Clinical significance of promoter hypermethylation of genes in ovarian cancer

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ABSTRACT

Ovarian cancer is the most lethal condition in gynecologic oncology. The known prognostic factors for ovarian cancer include tumor stage, histological grade, lymph node, and distant metastasis; however, handy and reliable molecular biomarkers for prevention, diagnosis, personalized treatment, and prognosis are scarce. Despite histological differences, the clinical treatment strategy is similar for ovarian cancers. The survival rate for ovarian cancer, however, remains relatively low. Accumulating evidence suggests that hypermethylated promoters of genes can be promising candidates as molecular biomarkers in ovarian cancer risk evaluation, early detection, personalized treatment, and prognosis. With advancements in immunology, hypermethylation of gene promoters was found to alter the tumor immune microenvironment, and gene methyltransferase inhibitors can contribute to ovarian cancer immunotherapy by boosting tumor immunogenicity and immune response and decreasing immunosuppression. Although DNMTis demonstrate high efficacy in hematologic malignancies, the application of DNMTis in solid tumors is just in its beginning. This article, drawing on both preclinical and clinical data, systematically reviews the common hypermethylated genes in ovarian cancer and their clinical applications, evaluating their usefulness in early diagnosis, personalized treatment, prognosis prediction, and the establishment of combined therapy of methyltransferase inhibitors and immunotherapy.

INTRODUCTION

Ovarian cancer (OC) is one of most lethal gynecological malignancies in women, with an estimated 238,700 new cases and more than 150,000 deaths in 2012 worldwide [1]. The high lethality of OC is mainly attributed to the lack of early detection strategies and to nonspecific symptoms that cause most patients to be diagnosed only at an advanced stage (International Federation of Gynecology and Obstetrics III–IV stage). The standard first-line treatment for OC is debulking surgery and platinum/taxane-based chemotherapy [2], which is curative in up to 90% of patients with early stage OC. The 5-year overall survival rate after the initial diagnosis in these patients is more than 70%. However, this rate sharply decreases to less than 30% for those with advanced-stage OC [3, 4]. The short of early screen biomarkers and the development of chemotherapeutic resistance are main obstacles to effective therapies. Therefore, early detection, and individualized treatment are imperative to improve the outcome for patients with advanced-stage OC.

The accumulation of genomic mutations has long been considered to be the core driver of tumorigenesis and progression [5]. With the developments in epigenetics, it has been confirmed that genetic mutation alone cannot account for the complexity of malignant tumors; epigenetic changes also play influential roles in the abnormal events of cancers, including OC [6–9].

The aberrant epigenetic alteration can lead to tumor formation and development, rendering the understanding and inhibition of these mechanisms essential. Unlike the nearly irreversible gene mutations, the epigenetic alterations are potentially reversible which make them attracting and promising biomarkers to prevent or treat OC [10]. In this systematic review, we discuss the common genes currently known to be silenced by hypermethylation in OC; investigate whether these methylated genes can serve as the biomarkers for early diagnosis, individualized treatment, therapeutic response, and prognosis evaluation; and explore the possibilities for combined cancer therapy comprising deoxyribonucleic acid (DNA) methyltransferase (DNMT) and immunotherapy.

DNA HYPERMETHYLATION

Conrad Waddington first proposed the concept of epigenetics in 1942 [11]. Epigenetic events lead to heritable modifications in gene expression other than the changes in DNA nucleotide sequences [12–14]. A relation between aberrant DNA methylation and human cancers was first reported in 1983 [15]. Since then, numerous studies have investigated its role in tumorigenesis, progression, and prognosis. Approximately 70% of human gene promoters feature cytosine guanine (CpG)-rich sequences (CpG loci) [16]. Compared with the bulk of DNA, the CpG loci within genes promoters are often methylation-free, which is a prerequisite for normal gene transcription [10, 17,18].

DNA hypermethylation is mediated by the DNMT enzymes, which depend on the methyl donor S-adenosyl methionine (SAM). The methyl group is transferred to the 5′ carbon of the cytosine ring within CpG dinucleotides [10, 19]. Generally, when cytosines are methylated from a CpG island (CpGI) of a gene, then the gene is silenced, and this CpGI is termed "hypermethylated" [20]. Normal cell differentiation requires appropriate DNA methylation/demethylation status, hypermethylation limits the capacity of cells to differentiate into cell-specific lineages and may ultimately induce a state of disease, such as tumorigenesis [21–23]. It is reported that DNA hypermethylation can silence the gene that is required for early stages of disease development; therefore, the evaluation of DNA hypermethylation may be valuable in the identification of potential biomarkers for detecting cancer early, monitoring progress, and facilitating personalized cancer therapy [24]. Moreover, the DNMT inhibitors (DNMTis) in cancer increase the immunogenicity, which inspired investigation of DNMTis in combined immunotherapy in cancer [25–27].

DNA HYPERMETHYLATION IN OC

The hypermethylated and silenced genes can induce events such as uncontrolled cell division, sustained angiogenesis, and avoided apoptosis, all of which are responsible for tumorigenesis and tumor progression [28, 29]. As in other cancers, DNA hypermethylation in CpGIs is common in OC [30]. To date, the identification of genes that are altered by DNA methylation is an area of intense research [10]. In OC, a large number of genes undergo hypermethylation. The most common seen or the most common used genes in OC are shown in Table 1.

BRCA1

The most extensively studied gene is *BRCA1* (breast cancer susceptibility gene 1) due to its well-known role in inherited forms of breast and ovarian cancers [10]. *BRCA1* was mapped by Mary-Claire King's group in 1990 [31] and subsequently cloned in 1994 [32]. *BRCA1* encodes a protein for DNA repair mechanism, and accordingly, its hypermethylation is linked to the reduced *BRCA1* expression and functions [10, 33], which eventually results in the formation and development of breast and ovarian cancers [34, 35]. The *BRCA1* hypermethylation rate in OC is reported to be 5%–89.9% (Table 1). The histological heterogeneity of OC as well as differences in sample collection and processing, assay design, and detection approach may account for the variation in DNA methylation frequencies [36]. *BRCA1* hypermethylation is significantly related to specific type of OC and high-grade OC [37, 38]. The frequency of *BRCA1* promoter hypermethylation was markedly higher in serous OC [37]. Compared with healthy controls and stage I OC, the methylation frequencies of *BRCA1* promoter were higher in stage II and III OCs [39]. Similarly, another study found that the promoter hypermethylation was in 31% of OCs and in none of the benign and borderline cases [40]. *BRCA1* promoter methylation has been elucidated as a useful tool to evaluate the prognosis of OC patients as well [41]. OC patients with *BRCA1* promoter hypermethylation showed a significantly shorter median disease-free survival (PFS, $P = 0.04$) and median overall survival (OS, $P = 0.02$) compared with those harboring mutated *BRCA1* [42]. Of note, *BRCA1* promoter hypermethylation is frequently detected in sporadic OC, whereas it has not been reported in the samples from germ-line *BRCA1* mutation cases [43, 44].

MLH1

As is well known, *MLH1* is an important tumor suppressor gene for DNA mismatch repair (MMR), which plays a vital role in reversing the replicative errors that escape the correction by DNA polymerases III [45]. In OC cells, chemotherapeutic agents such as carboplatin and cisplatin can damage DNA by forming intrastrand and interstrand adducts. Detection of these adducts by the MMR system leads to p53 protein phosphorylation, activation of the mitogen-activated protein kinase pathway, induction of the proapoptotic protein BAX, and finally

Table 1: Selected genes that undergo CpG island hypermethylation in epithelial ovarian cancer

Gene	Location	Function	Percentage Methylated	OC type	N	Ref
BRCA1	17q21.31	DNA damage repair	15%	sporadic OC	98	32
			10%	sporadic OC	88	42
			5%	sporadic ovarian adenocarcinoma	43	137
			89.9%	EOC	69	138
MLH1	3p22.2	DNA mismatch repair	9%	EOC	234	36
			30.4%	EOC	76	47
			56.3%	EOC	36	48
			37.5%	EOC	137	139
HOXA9	7p15.2	controlling cell growth, differentiation, proliferation	51%	EOC	52	59
			95%	HGSOC	92	61
RASSF1A	3p21.3	microtubulin stability	40%	OC	20	51
			26.4%	stage III/IV EOC	106	140
			67.8%	EOC	69	141
			58%	OC	119	142
APC	5q22.2	regulation of cell migration and adhesion, transcriptional activation and apoptosis	29%	OC	119	142
			22%	invasive OC	69	143
			47.2%	OC	140	144
HIC1	17p13.3	regulation of apoptosis, encoding transcriptional repressor	35%	primary sporadic OC	88	42
			17.3%	stage III/IV EOC	106	140
DAPK	9q21.33	regulation of apoptosis and metastasis	67%	OC	32	145
P15		cell cycle control	64.29%	epithelial serous ovarian	50	146
P16		cell cycle control	50%	epithelial serous ovarian	50	146
			43 %	EOC	198	147
OPCML	11q25	signaling and growth inhibition	78.4%	OC	217	148
			88.1%	EOC	46	149
CCBE1	18q21.32	inhibition matrix emodeling and migration	41%	OC	81	150
Wnt pathway						
SFRP5	10q24.2	inhibition epithelial-mesenchymal transition , Wnt antagonist	44.4%	OC	215	71
			64.6%: OCCA 13.3%: OSA	OCCA, OSA	78	151

FZD4, DVL1, NFATC3, ROCK1, LRP5, AXIN1, NKD1	-	control biological processes including cell proliferation, differentiation migration and tissue homeostatis	25% (average)	EOC	181	73
TGF-beta pathway						
FBXO32	8q24.13	regulates cell apoptosis	29.3%	advanced-stage OC	96	69

EOC, epithelial ovarian cancer; OC, ovarian cancer; OCCA, ovarian clear cell adenocarcinoma; OSA, ovarian serous adenocarcinoma.

apoptotic cell death [46, 47]. In the MMR-defective cells, both insertion/deletion loops and base–base mismatches are left uncorrected. Approximately 10% of OC is related to this molecular pathway [48, 49]. *MLH1* promoter hypermethylation has been identified in OC cases that acquired resistance to platinum-based chemotherapy [50–52]. Hypermethylated silencing of *MLH1* and the resultant reduced apoptotic response to platinum-DNA adducts are the contributing factor to platinum resistance in OC. Gifford *et al*. [53] investigated the prognosis of acquired resistance to chemotherapy by comparing *MLH1* hypermethylation status in epithelial OC (EOC) patients before and after carboplatin/taxoid chemotherapy. They discovered that hypermethylation of *MLH1* promoter during treatment was significantly linked to relapse and poor outcome. Another study confirmed that *MLH1* promoter hypermethylation showed a significant correlation with lymphatic metastasis ($P = 0.003$) and histological grade ($P = 0.028$) [50].

RASSF1A

Another gene found to be hypermethylated and silenced in OC is the gene encoding the RAS association domain family protein 1a (*RASSF1A*) [54, 55]. *RASSF1A* is a vital tumor suppressor gene which can regulate cell cycle, bind to tubulin and stabilize microtubules [56]. Studies have demonstrated that *RASSF1A* is among the most frequently hypermethylated genes in OC [10, 57]. To determine the potential functions of hypermethylated patterns of free-circulating DNA in patients' plasma, Liggett *et al*. [58] investigated the methylation patterns of cell-free plasma DNA in three cohorts (invasive EOC cases, benign ovarian cases and healthy controls). This study showed that the hypermethylation of three gene promoters (*RASSF1A*, *CALCA*, and *EP300*) differentiated between EOC vs. healthy control with a sensitivity of 90.0% and a specificity of 86.7%. Moreover, *RASSF1A* and *PGR-PROX* promoter hypermethylation can effectively distinguish the plasma of EOC patients from benign cases, with a sensitivity of 80.0% and a specificity of 73.3%. This proof-of-principle data further verify earlier findings that have demonstrated frequent

RASSF1A promoter hypermethylation in invasive ovarian cancer but rarely in normal cases [44, 59]. Hence, it can be concluded that the frequency of *RASSF1A* promoter hypermethylation in OC establishes a potential biomarker for the presence of OC.

HOXA9

Homeobox genes belong to the family of transcription factors. They are the key genes in regulating cell growth, differentiation, and proliferation during embryonic development [60]. Studies have suggested that OC is related to the methylomes of *HOX* genes [61]. Wu *et al*. [62] found that *HOXA9* was hypermethylated in 51% (26/51) of ovarian carcinomas which was higher among relatively early-stage carcinomas than those of later stages ($P = 0.002$). Widschwendter *et al*. [63] found that *HOXA9* hypermethylation was predictive of EOC formation, with an increase in *HOXA9* hypermethylated frequency correlating with an increase in risk of EOC. Another study demonstrated a similar result in high-grade serous OC (HGSOC) [64]. Furthermore, a combined methylation status of *HOXA9* and *EN1* could differentiate HGSOC from benign ovarian surface epithelium (OSE) with a sensitivity of 98.8% and a specificity of 91.7%, which increased to 100% sensitivity when pre-operative CA125 levels were also incorporated [64]. These results support *HOXA9* hypermethylation as a promising biomarker for detection of OC, likely in combination with other molecules and existing clinical methods [36].

TGF-beta pathway

The TGF-beta superfamily now contains around 40 secreted ligands [65], which play diverse and multifunctional roles in cell proliferation, differentiation, migration, immune response, angiogenesis and apoptosis [66]. It has been published that the hypermethylation of genes in TGF-beta signaling pathway were associated with many malignancies such as gastric [67], hepatocellular carcinoma [68], and OC [69]. In OC, the DNA hypermethylation can alter (ie, suppress) TGF-beta signaling expression [69, 70]. Kang *et al*. [69] found that

TGFBI hypermethylation was detected in two OC cell lines (ES-2 and OVCAR-3) and was present in 60.5% of OC patients, 27.8% of borderline ovarian diseases, and none of the normal cases. Furthermore, Matsumura *et al*. [70] conducted a microarray analysis of 39 OC cell lines and identified numerous candidate methylated genes, many relevant to TGF-beta signaling. Chou *et al*. [71] reported that the unmethylation of *FBXO32* (TGF-beta/SMAD4 target gene and regulator of apoptosis) [72] was observed in normal OSE, but not in OC cell lines. *In vitro* studies have shown that the expression of *FBXO32* restored sensitivity to cisplatin treatment, suggesting that *FBXO32* hypermethylation may act as a prognostic biomarker for EOC as well [72].

Wnt pathway

The Wnt pathway is one of the classical pathways in the process of cell signal transduction which participates in embryogenesis and cell homeostasis [73]. Several genes regulating the Wnt pathway are hypermethylated in EOC. *SFRP5*, a Wnt blocker, was detected methylated in 1.3% of benign ovarian cases, 21.4% borderline cases and 44.4% of EOC ($P < 0.001$) [74]. Moreover, the epigenetic silence of *SFRP5* is related to malignant phenotype and chemoresistance of OC and the unmethylation of *SFRP5* sensitizes OC to taxolin and cisplatin *in vitro* [75]. Dai *et al*. [76] evaluated the methylated status of 302 loci at 137 genes in Wnt signaling pathway in OC. They revealed that the increased methylation at 7 of these loci, at *FZD4, DVL1, NFATC3, ROCK1, LRP5, AXIN1*, and *NKD1* genes was associated with increased hazard of disease progression. They also analyzed the relation between DNA methylation and patient response to platinum and found that the hypermethylated alteration of *NFATC3* and *DVL1* showed significant correlation with poor response which could be independent predictors of PFS [76].

CLINICAL HYPERMETHYLATED MARKERS

As a means to detect, classify, and evaluate OC, DNA promoter hypermethylation has several advantages than other means of detection. First, the hypermethylation analysis utilizes DNA, which is more chemically stable [10]. Second, aberrant DNA methylation has a high sensitivity: it can be detected by signal amplification by sensitive polymerase chain reaction (PCR) at a low concentration in the background of excess normal DNA molecules [56]. Such detection methods include methylation-specific PCR (MSP) [77] and quantitative MSP [78]. The MethyLight and Headloop PCR are high-throughput quantitative MSP methods which are able to detect methylated alleles in the presence of up to a 100,000-fold excess of unmethylated alleles [79, 80]. These methods seem promising for clinical use because of their throughput capacity and high signal-to-noise ratio [81–83]. Third, the detection of methylated biomarkers is non-invasive because they mainly exist in bodily fluids draining, serum/plasma and near a tumor [84]. Publications have demonstrated that it was feasible to detect the methylated alterations in patient's circulating DNA in a variety of tumor cells, including OC [85–87]. The clinical applications of the hypermethylated genes include early diagnosis, treatment (chemotherapy, epigenetic therapy, immunotherapy, and combined immunotherapy), and prognosis evaluation. Since we have already discussed the functions of several common genes in early diagnosis, chemotherapeutic resistance, therapeutic responsiveness, and prognosis, we will only briefly mention these similar functions and focus on epigenetic therapy, immunotherapy, and combined immunotherapy.

Early diagnostic markers

The 5-year OS rate of advanced-stage OC sharply decreases to less than 30% [4], so early diagnosis is vital for effective treatment and better prognosis. Aberrant hypermethylation is considered to be a useful biomarker for early diagnosis of OC or potentially premalignant disease because of its early appearance during tumorigenesis [22]. However, compared with the genes panel, hypermethylation of a single gene has limited practical value. Detection of multiple genes (a gene panel) simultaneously is much more sensitive and specific which can provide more information for OC diagnosis. One study reported that promoter hypermethylation was common in OC, including stage I disease, and could be readily detected using the *BRCA1, RASSF1A, APC, p14ARF, p16INK4a*, and *DAPK* genes [59]. Another study reported that the multiplex MSP assay for 7 candidate genes (*APC, RASSF1A, CDH1, RUNX3, TFPI2, SFRP5* and *OPCML*) produced a sensitivity of 85.3% and a specificity of 90.5% in stage I OC, strikingly higher rates than that using only CA125, which produced a sensitivity of 56.1% and a specificity of 64.15% ($P = 0.0036$) [88]. Therefore, hypermethylated biomarkers, especially gene panels, may prove more valuable for early diagnosis for OC.

TREATMENT

Chemoresistance and therapeutic responsiveness

After an initial response to first-line chemotherapy, a majority of patients with OC relapse and progress within 16–18 months due to the development of resistance to chemotherapy [2]. Apart from gene mutation, aberrant DNA hypermethylation has been recognized as a common molecular event in cancer chemoresistance [89]. Hypermethylated genes implicated in drug resistance are usually those involved in processes that affect chemosensitivity, such as DNA damage and repair

pathways, cell cycle control, and apoptosis [10, 90]. As aforementioned, the hypermethylation of *SFRP5*, a Wnt antagonist, was associated with platinum resistance in OC [75]. Similarly, the hypermethylation of other genes such as *MLH1* [91], *MCJ* [92], *HSulf-1* [93], *ASS1* [94], and *DAPK* [95] is also involved in platinum resistance. Therefore, assessing the methylation of genes is helpful in evaluating chemotherapeutic efficiency and prognosis in OC patients [96]. Moreover, combining conventional chemotherapeutic drugs with epigenetic-based therapies (inhibition the formation of hypermethylation) may provide a means to resensitize OC [96].

Epigenetic therapy

As aberrant hypermethylation is frequently observed in drug resistance, re-expression of silenced genes might allow for resensitization of drug-resistant OCs [56]. Unlike cancer-associated gene mutations, DNA hypermethylation is potentially reversible, which makes epigenetic agents that reverse methylated alterations attractive for cancer prevention and resensitization to chemotherapy. There exist 2 kinds of methylation inhibitors that have been used to re-express hypermethylation-silenced genes: nucleoside and non-nucleoside analogs. The nucleoside analogs decrease DNA methylation by incorporating themselves into DNA strands and forming covalent compound with DNMTs [97]. Non-nucleoside, the small molecule inhibitors of DNMTs, can directly incorporate into the catalytic domain of DNMTs and inhibit gene translation [98]. The first 2 DNMTis approved to treat myelodysplastic syndromes (MDSs) are azacytidine (AZA) and decitabine (2′-deoxy-5-azacytidine, DAC) [99, 100]. AZA is a ribonucleoside that is incorporated into RNA and DNA (after conversion to the deoxyribose form) and combines to and inhibits DNMTs [101]. Treatment of chemotherapy resistant A2780 OC cell lines with 5-azacytidine resulted in re-expression of MLH1 and increased OC cells sensitivity to cisplatin [91]. DAC is another potent demethylation agent which can reverse the aberrant silencing of numerous genes in ovarian cells [102]. DAC also sensitized cisplatin-resistant OC xenografts which were *MLH1* silent because of gene promoter hypermethylation [103]. Two clinical trials have found that DNMTis increased the efficacy of chemotherapy for OC patients [104, 105]. Moreover, a clinical study showed that the combined application of DNMTi with carboplatin produced efficiency in platinum-resistant or platinum-refractory EOC [104].

Prognostic markers

The epigenetic modifications, especially hypermethylation of genes, act as biomarkers to evaluate the prognosis for cancer patients, and several studies have assessed this for OC. For example, promoter methylation of *IGFBP-3*, which regulates the mitosis

and antiapoptosis of insulin-like growth factors, was related to the disease progression and death of OC. The association was more evident in patients with early-stage disease [106]. A clinical phase III trial demonstrated that silencing of *MLH1* by hypermethylation of its promoter CpGI [107, 108] induced the relapse in EOC, with 25% of relapsed OC patients showing hypermethylated alteration of *MLH1*. The methylation of *MLH1* at OC relapse is associated with drug resistance and predicts poor OS [53]. Wei *et al*. [109] found that OC patients with a short PFS (with hypermethylation) showed less sensitive to chemotherapies than those with a longer PFS (with a low methylation), indicating that higher degree of CpGI methylation facilitates chemoresistance more readily and is associated with early recurrence after chemotherapy. Therefore, the identification of a gene panel to specifically predicate and evaluate the prognosis of OC awaits validation but looks encouraging.

PROMOTER HYPERMETHYLATION AND CANCER IMMUNITY

DNMTis boost tumor immunogenicity and immune response

DNMTis boost tumor major histocompatibility complex and tumor-related antigens

As mentioned earlier, researches on hypermethylation have been increasing; however, its roles and exact mechanisms in cancer immunity are poorly understood. Immune evasion is a hallmark of tumorigenesis and development [5]. One of the most potent evasion tactics adopted by cancers is the impairment the process of antigen presentation, such as the downregulation of major histocompatibility complex (MHC) by reversibly inactivating methylation patterns [25, 110]. DNMTi can upregulate MHC I/II and yield promising results in several cancer types such as chronic lymphocytic leukemia (CLL) [111] and acute myeloid leukemia (AML) [112]. DNMTi also upregulates immunogenicity by inducing the expression of various antigens such as cancer/testis antigens, including NY-ESO-1 [113] and SSX2 [112] and by increasing baseline expression of other molecules, such as the melanoma-associated antigens [114]. Apart from MDS and leukemia, DMNTi treatment can also increase the immunogenicity in solid tumors, especially in melanoma [115] and OC [26, 116]. It was discovered that DAC enhanced the MHC I molecules, as well as 11 cancer/testis antigen genes tested, including MAGE-A1, NY-ESO-1 and TAG-1, in OC cell lines [116].

DNMTi boosts tumor immune checkpoint marker

A study found that DNMTi reversed the repression of Th1-type chemokines CXCL9 and CXCL10, increased effector T cells infiltration in tumor site, and, thus,

improved the therapeutic efficacy of adoptive T-cell transfusion and programmed death ligand 1 (PD-L1) inhibitor in mice bearing OC [27]. This study also reported that mice treated with PD-L1 checkpoint blockade or DZNep (an inhibitor of all SAM-dependent enzymes) plus DAC exerted a decreased tumor size, increased CD8+ T lymphocytes and Th1-type chemokine expression [27]. Therefore, the inhibition of DNA hypermethylation synergistically increases the therapeutic efficacy of anti-PD-L1 therapy [117]. To evaluate the correlation of immune checkpoints (PD-L1, PD-L2, PD-1 and CTLA-4) with DNMTi in patients with myeloid malignancies, a study evaluated the effect of the treatment of leukemia cells with DAC and revealed that DAC leads to a dose-dependent upregulation of these 4 genes [118]. During first course of DNMTi therapy, PD-L1, PD-L2, PD-1, and CTLA-4 expressions were upregulated (≥2 fold) in 57%, 57%, 58% and 66%, respectively, of patients with MDS. Moreover, when MDS patients treated with 5-AZA and vorinostat (a histone deacetylases inhibitor), there is a trend toward enhanced expression of these 4 genes in epigenetic treatment–resistant patients compared with treatment–sensitive patients [118]. This phenomenon has strong implications for the development and application of combination strategies of DNMTi with immune checkpoint inhibitors in OC [118].

DNMTis stimulate natural killer cell– and CD8+ T-cell–mediated cytotoxicity

Apart from rendering tumor cells more recognizable to T lymphocytes in an antigen-specific manner, the epigenetic modifiers enhance cytotoxic natural killer (NK) and CD8+ T-cell function [110]. A study found that valproic acid and DAC increased the level of MICA molecule (expressed by NK cells) and activated CD8+ T cells [119]. Moreover, DNMTi-mediated demethylation induces expression of genes involved in CTL reactivity, most notably antitumor cytokines such as interleukin-2 (IL-2). For instance, the transcription of IL-2 gene in T cells is associated with the demethylation of the promoter-enhancer domain of IL-2 upon activation [110, 120]. This demethylation status was found early after CD8+ T-cell antigen exposure and is maintained throughout CD8+ T-cell memory development [121]. This indicates that IL-2 levels may be increased and maintained by DNMTi-mediated hypomethylation [110]. Similarly, culturing primary mouse CD8+ T cells with the treatment of 5-AZA increased interferon-gamma expression up to 25-fold and IL-3 (related to T-cell growth, differentiation, and myeloid proliferation) production up to 14-fold [122]. Another study found that low-dose DAC increased the expression of antitumor chemokines released by NK cells and CD8+ T cells in the ascites of OC mice [26]. Taken together, these findings indicate the promising applications of DNMTi to increase NK and CD8+ T lymphocytes,

especially via the induction or enhancement of critical immunostimulatory cytokines.

DNMTi decreases immunosuppression by regulating immune cells

Clinical outcomes reveal that the inhibition of immunosuppressive cells is equally important with activating cytotoxic NK/T cells–mediated antitumor immunity [123]. Apart from improving cancer immunogenicity and cytotoxic cells functions, DNMTi decreases natural (innate) and tumor-induced (adaptive) immunosuppression. Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) are vital immunosuppressive components in the immune system. In melanoma-bearing mice, the macrophage effector and dendritic cell activation increased with the low-dose of DAC treatment, while the number of MDSCs decreased [124]. Similarly, DAC decreased the percentage of MDSCs in their peritoneal cavity of murine OC model [26]. A clinical study found that the number of Tregs of 68 patients with MDS was significantly reduced following treatment with DNA methyltransferase inhibitor [110, 125].

Rationale for combination of DNMTi with immunotherapy

Based on the aforementioned evidence, DNMTi's great ability to prime the antitumor immunoresponse makes it a promising candidate for combinations with immunotherapies [110]. Application DNMTi alone or in combination with a histone deacetylase inhibitor to patients with MDS or AML upregulated the expression of immune checkpoint markers (CTLA-4, PD-1, PD-L1, and PD-L2), which is the prerequisite for blocking the tumor-related CTLA-4 and PD-1 pathways [120, 110]. Additionally, PD-1 [126] and CTLA-4 [127] blockades as well as DNMTis [26, 115] can elevate interferon-gamma production which further support the possibility of combined application of immune checkpoint blockades and DNMTi. More than 80% of the 4T1 tumor-bearing mice were cured with the cotreatment of epigenetic-modulating drugs and checkpoint inhibitors [128]. A similar result was found in a syngeneic murine OC model on the synergistic antitumor roles of DAC with anti-CTLA-4 [26]. This combination induces the differentiation of naïve T cells into effector T cells and prolongs CTL responses. Based on this encouraging evidence, multiple clinical trials applying DNMTi in combination with various immune-based therapies have been carried out [110]. A clinical trial (NCT01928576) combined 5-AZA (with or without histone deacetylase inhibitor entinostat) and checkpoint blockade (nivolumab, anti-PD-1 drug) to treat patients with non-small cell lung cancer (NSCLC). Considering the phenomenon that AZA can upregulate *PD-L1* gene transcripts and PD-L1 protein expression

[129], the outlook for this clinical trial seems to be promising. The combined therapy may warrant further study and consideration for the treatment of drug-resistant OC. However, we should take the potential variables that could influence the outcome of such combinatorial therapy into account, such as the sequences of the treatment [129], the dose of DNMTis and checkpoint blockade should be used [130], the stage of tumor [131] and so on.

Toxicity issue associated with DNMTis

AZA and DAC have evidently demonstrated efficacy in hematologic malignancies such as MDS, AML and CLL, however, the use of DNMTis in solid tumors seems limited because of the low specificity, substantial toxicity, and poor bioavailability [132]. Moreover, the solid tumors patients also have DNMTis associated toxicity issue. As nucleoside analogs, AZA and DAC can be incorporated into DNA strands and act as covalent inhibitors, with low specificity and common toxic side effects, such as nausea, vomiting, diarrhea, myelosuppression, neurotoxicity, mutagenic lesions and even death [131, 133–136]. This is probably the main reason why the progress in the FDA approval of DNMTi in solid cancer is so slow. To overcome the toxicity, the non-nucleoside compounds have attracted people's attention, such as DC_517 [137], SGI-1027 [138] and RG108 [139]. In comparison with oligonucleotide derivatives, non-nucleoside molecules serve as a comparatively safer option to regulate DNMTs methylation [137–139]. Besides, clinical studies suggested that low-dose DNMTi could be a regimen for cases with refractory-advanced solid tumors [130, 131].

CONCLUSIONS

DNA hypermethylation is a promising and rapidly evolving area of research. The profiling of DNA hypermethylated patterns can provide new insights into their use in OC risk evaluation, early detection, personalized treatment, and prognosis. Because of its stability, sensitivity, specificity, and restriction to limited regions of DNA (ie, CpGIs) in comparison with proteins or DNA mutations, the hypermethylation status as a biomarker holds potential. However, several problems must be considered before translating OC hypermethylation into clinical practice. EOC is a heterogenous tumor; the frequencies of DNA methylation vary greatly for different genes and tumor types. Moreover, rather than an individual gene, a panel of gene methylations, which can increase the test's sensitivity and specificity, should be explored in the future. Furthermore, the side effects of DNMTis such as low specificity, substantial toxicity, and poor bioavailability should be taken into consideration. Although the epigenetics of OC is still in its infancy, the developments in oncologic epigenetics and immunology will move the applications of gene hypermethylation into a new territory.

Author contributions

Xinxin Zhu contributed to data collection, discussing content and writing. Jinghe Lang designed and reviewed the article.

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CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article is reported.

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