Targeting DNA methylation for treating triple-negative breast cancer

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Triple-negative breast cancer (TNBC) accounts for 15–20% of all invasive breast cancers and tends to have aggressive histological features and poor clinical outcomes. Unlike, estrogen receptor- or HER2-positive diseases, TNBC patients currently lack the US FDA-approved targeted therapies. DNA methylation is a critical mechanism of epigenetic modification. It is well known that aberrant DNA methylation contributes to the malignant transformation of cells by silencing critical tumor suppressor genes. DNA methyltransferase inhibitors reactivate silenced tumor suppressor genes and result in tumor growth arrest, with therapeutic effects observed in patients with hematologic malignancies. The antitumor effect of these DNA methyltransferase inhibitors has also been explored in solid tumors, especially in TNBC that currently lacks targeted therapies.

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DNA methylation is one of the best described epigenetic events that regulate chromatin compaction and gene expression. Cancer cells utilize DNA methylation as a strategy to abnormally silence a variety of genes, including tumor suppressors and genes controlling the drug sensitivity [6]. The inhibition of DNA methylation with cytidine analogs such as 5-azacytidine (Vidaza®) or its deoxy derivative decitabine (5-aza-2′-deoxycytidine, Dacogen®) reactivates the expression of genes silenced by hypermethylation, reduces the malignant cell burden and improves patient survival [7,8]. This led to the US FDA approval of the DNA methyltransferase (DNMT) inhibitors for the treatment of hematological malignancies, and has become the standard of care for patients with high-risk myelodysplastic syndrome (MDS) [9,10].

DNA methylation in TNBC

DNA methylation is a reversible process of adding a methyl group to the fifth position of the pyrimidine ring of cytosine residues to form 5-methylcytosine (5-mC). This commonly occurs at CpG sites within the genome [11,12]. DNA methylation is a heritable epigenetic process regulating gene function in normal mammalian development [13,14]. However, aberrant methylation of CpG islands in the promoter regions of genes critical for cell growth is a hallmark of many cancers [15,16]. The phenomenon of regional DNA hypermethylation and silencing of tumor suppressor
genes in cancer has drawn the attention of researchers and clinicians for the last decade. Recent whole-genome approaches have identified breast cancer-specific DNA methylation signatures that were correlated with cancer stage and this finding will extend our ability to classify breast cancer to better predict clinical outcome beyond what is currently possible [17–19].

The Cancer Genome Atlas Network and several other groups have demonstrated that TNBC tumors are characterized by the most extensive hypomethylation whereas ER-positive breast cancers show the highest degree of hypermethylation [20–23]. Moreover, TNBC have a distinct methylation pattern. A recent comprehensive TNBC methylome analysis by Stirzaker et al. found that TNBC patients can be stratified into three methylation clusters. TNBC patients with a hypomethylation profile were associated with a better 5-year survival compared with clusters with heavier methylation. They also identified a series of differentially methylated regions that can stratify TNBC patients into a better or worse prognosis [24]. By comparing the methylation pattern in human embryonic stem cells, cancer cell lines, as well as human primary tissue, studies showed that the CpG sites in TNBC tumors mostly were not hypermethylated, similar to human embryonic stem cells and normal breast tissues. However, in the rest of the breast cancer subtypes, these sites were hypermethylated [25].

Interestingly, a hypermethylation phenotype of specific genes has also been described for TNBC despite global hypomethylation [26]. Methylation of genes involved in DNA damage response, such as BRCA1 [27], or 14–3–3σ (also known as HME1) [28], has been described in TNBC. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA expression [27]. While CpG island methylation of 14–3–3σ gene caused the gene expression reduction and this mechanism was demonstrated both in cell line-based and tissue-based studies [29]. Cell-to-cell adhesion molecules such as E-cadherin, whose silencing might promote metastasis were also found to be methylated in TNBC [30]. In addition, DNA methylation in genes involving in stem cell properties changed in TNBC primary breast cancer samples and was found to be correlated with TNBC subtype and clinically aggressive phenotype [31,32]. Recently, Coyle et al. discovered over 1400 CpG sites with differential DNA methylation status between all-trans retinoic acid-sensitive and -resistant TNBC cell lines. And they further found that these sites methylation status can predict TNBC patient-derived xenograft (PDX) response to all-trans retinoic acid [33].

**DNA methyltransferases normal function**

In human cells, DNA methylation is mainly catalyzed by the DNA cytosine methyltransferases family members including DNMT1, DNMT3A and DNMT3B [34]. DNMT1 is the most abundant form of DNA methyltransferase and is responsible for the maintenance of methylation pattern during DNA replication [35,36]. DNMT1 methylates newly synthesized DNA strands to effectively reinstate the methylation patterns that originated in the parental strands. DNMT3A and DNMT3B are the de novo DNA methyltransferases, which are responsible for de novo DNA methylation during early development and gametogenesis [37]. DNMT3L is another member of the DNMT3 family proteins and encodes a catalytically inactive protein. DNMT3L does not bind to DNA, but instead binds to DNMT3A/B and strikingly increases their catalytic activity [38]. The maintenance versus de novo function of these enzymes is not mutually exclusive. For example, DNMT1 can function as a de novo DNMT, and overexpression of DNMT1 leads to de novo methylation of CpG islands [39], and human cancer cells lacking DNMT1 have shown only a 20% reduction in methylation of CpG sties [40]. Similarly, DNMT3A or DNMT3B can function as maintenance DNMTs [40].

**DNA methyltransferases in TNBC**

**DNMT1**

DNMT1 is overexpressed in many types of cancers, including breast cancer, and the expression of DNMT1 was found to be higher in TNBC and lower in luminal samples [41]. Overexpression of DNMT1 is associated with cellular transformation while reduced DNMT1 expression seems to be associated with a protective effect [42,43]. DNMT1 was also observed to be upregulated in most cancer-associated fibroblasts relative to their adjacent normal fibroblasts and enhanced cancer-associated fibroblasts tumor-promoting properties [44].

**DNMT3A/B**

DNMT3A and DNMT3B are also reported to be overexpressed in cancer tissues [35]. DNMT3B overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines [45]. DNMT3B overexpression is associated with increased DNMT activity and corresponds to high rates of methylation-dependent gene silencing compared with low-frequency methylator cells. DNMT3B overexpression is found associated with TNBC and
displayed increased proliferation and poor patient prognosis [46]. These findings are in agreement with our recent study revealing that DNMT inhibitors might be more effective in treating TNBCs overexpressing DNMT3B [47]. Additionally, transcriptional induction of DNMTs, including DNMT3A, has also been reported in cancer [48,49]. Mutations in DNMT3a are also associated with poor overall survival in acute myeloid leukemia (AML), leading to potentiation of aberrant stemness genes linked to AML development [50].

**DNA methyltransferase inhibitors**

With the understanding of how DNA methylation can drive cancer, there is an increasing focus on developing pharmacological interventions for treatment of cancer. Currently, two DNMT inhibitors (DNMTi) have been approved by the FDA: azacitidine (Vidaza; Celgene) and decitabine (5 aza 2′ deoxycytidine; Dacogen; SuperGen) for the treatment of patients with AML and MDS, respectively [9]. These nucleoside analogs were originally designed as cytotoxic agents with high dose. However, patients experienced severe adverse side effects. With improved understanding of their mechanism of action, low dose DNMTi (nanomolar range) is being used to achieve effective inhibition of DNA methylation in patients while also improving the tolerability [51]. Another demethylating agent in the family of nucleoside analogs is zebularine, which has been characterized as a potent, chemically stable and better bioavailable oral compound. It has promising in vitro results, encouraging zebularine use for future clinical trials [52,53].

The next-generation DNMTi includes guadecitabine (SGI-110), a produrg whose active metabolite is decitabine. This compound has a novel molecular structure, which makes it resistant to degradation by the enzyme, cytidine deaminase and thereby prolongs in vivo exposure, increasing its efficacy [54]. The Phase II clinical trials for the treatment of AML cohorts showed promising activity [55]. Recent Phase II trial for treating MDS has indicated that guadecitabine may improve the responses and overall survival in patients who progressed on previous demethylating agents, offering a new therapeutic option for patients who might fail on currently available demethylating agents [56]. Additional demethylating agents have been explored and reviewed previously [57].

Early Phase I and II trials investigating the role of demethylating agents in solid tumors, including breast cancer, yielded little success [58,59]. TNBC tumors do not express ER or HER2, and they are not amenable to the conventional targeted therapies. DNMTi can induce the re-expression of endogenous ERα and PRs in TNBC cells [60,61]. Therefore, a Phase II study was conducted to evaluate the efficacy of hormonal therapy following epigenetic therapy in TNBCs [62]. Although the results from using DNMTi alone in treating cancer are modest, DNMTi in combination with histone deacetylase (HDAC) inhibitors have shown robust efficacy in clinical trials [63,64].

**Conclusion**

Despite the extensive studies regarding the role of DNA methylation in TNBC, significant challenges remain in terms of successful application of demethylating agents in the clinic. With the recent advances in ‘omics’, future identification of effective biomarkers will enhance our ability to use these agents in a most appropriate way for any individual patient. With the success of developing TNBC PDX models [65,66], patient-derived organoid models [67,68] and 3D high-throughput screening platforms [69], preclinical studies can be conducted to test related hypotheses and validate in vitro findings. Robust preclinical and translational studies are essential to support the development of new regimes and to maximize the chance of success in treating TNBCs.

**Future perspective**

Alterations in DNA methylation are frequently detected in TNBCs, hence agents that target these alterations are of great interest and are under investigation. Besides developing new DNMTis with higher potency, new strategies of combination therapies, targeting specific processes involved in tumorigenesis, or identifying new biomarkers predicting treatment response are required to allow achieving optimal therapeutic effect of epigenetic therapies in TNBC.

Abrupt promoter methylation of key genes may cause gene expression silencing and contribute to tumor cell spread and metastasis, thus, DNMTi can reverse the process and inhibit cancer metastasis [70]. Su et al. also found that TNBC was sensitive to DNMTi treatment and that DNMTi exerts antitumor activity, in part, by epigenetically reprogramming epithelial-to-mesenchymal transition [71]. DNMTi may provide extended value in inhibiting epithelial-to-mesenchymal transition and cancer metastasis.
Cancer stem cells (CSCs) are found within tumors and they possess self-renewal and tumorigenic characteristics. CSCs can survive chemotherapies and cause cancer relapse. Pathania et al. found that DNMT1 was required for the maintenance of cancer stem cell in breast cancer, and inhibition of DNMT activity in breast cancer reduces cell growth, migration and cancer stem cell formation [72], suggesting that DNMTIs may target the CSCs.

A recent study identified that major histocompatibility class-I genes were methylated in human breast cancers and their expression were suppressed. The DNMT inhibitor, guadecitabine, could upregulate major histocompatibility class-I in breast cancer cells, which promoted recruitment of CD8 T cells to the microenvironment [73]. This immunomodulatory effect of demethylating agents may be useful to potentiate antitumor immunity and responses to checkpoint inhibition in immune-refractory breast cancers.

Although DNMTIs are widely used in treating AML and MDS, the response rate in patients is around 50% [7]. Given the heterogeneity of TNBC, identification of predictive biomarkers is very crucial for successful application of these drugs in clinic. DNMT3A has been associated with CpGs hypermethylation in AML [74]. Moreover, the expression levels of DNMT3A and DNMT3B are also shown to be associated with decitabine sensitivity in embryonic cells [75]. Furthermore, DNMT3A/DNMT3B double-null embryonic stem cells are more resistant to decitabine treatment than DNMT1 null cells, suggesting that decitabine may be more effective for selected types of cancer cells in which DNMT3 expression is upregulated. We also found that DNMT protein levels, especially DNMT3A and DNMT3B, can be predictive of decitabine treatment response in TNBC cells and PDX models [47]. This accumulation of evidence suggests that DNMT3A/3B levels might be the biomarkers predictive of decitabine response. We also found that decitabine could induce ubiquitin-dependent degradation of DNMTs, raising a possibility of using DNMT as pharmacodynamics biomarkers for decitabine response [47].

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