OncoTargets and Therapy

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REVIEW

The Role of DNA Methylation in Osteosarcoma Pathogenesis and Therapy

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Abstract: Osteosarcoma (OS) is a highly malignant bone tumor primarily affecting children and adolescents. Clinical treatment has consistently encountered challenges, including chemotherapy resistance, high recurrence rates, and metastasis. Research has demonstrated that epigenetic regulation, particularly DNA methylation, can stably modify the DNA sequence without altering it, playing a key role in the development and progression of OS. Compared with normal tissue, OS exhibits distinctive alterations in DNA methylation, characterized by genome-wide hypomethylation and hypermethylation of specific gene promoter regions. This "dual pattern" not only promotes tumor proliferation, invasion, and metastasis but also maintains cancer stem cell characteristics and modulates the tumor immune microenvironment (TIME). Molecular classification based on DNA methylation profiles offers a new tool for the diagnosis and prognosis of OS. Drugs targeting DNA methylation, such as decitabine, have shown promising results for reversing gene silencing and suppressing tumor progression. This article systematically reviews the core mechanisms by which DNA methylation contributes to OS development, progression, and metastasis, and examines its potential for clinical translation.

Keywords: biomarkers, DNA methylation, epigenetics, osteosarcoma, targeted therapy, tumor microenvironment

Introduction

OS is an ordinary malignant bone tumor that mainly occurs among children and adolescents.[1] Owing to its fast growth rate, strong metastatic potential, and intricate tumor microenvironment (TME), the tumor is highly malignant.[2] According to an epidemiological survey conducted in the year 2009, the incidence rates observed among males younger than 24 years old were 4.3 among each million and slightly lower among females.[3] Currently, the mainstay of treatment involves neoadjuvant chemotherapy followed by surgical resection and adjuvant chemotherapy. Surgery and several chemotherapy regimens are the standard clinical management strategies. For far-distant metastatic patients, the survival rate is around 5 years about 20%.[4,5] Despite early diagnosis, the five-year survival rate is merely around 60%, and the patients tend to encounter invasive therapies, including amputation.[6,7] Though the therapies increased the survival rates through these therapies, the complications like chemotherapy resistance and elevated recurrences remain and signify the necessity to boost the effectiveness and quality of life among the patients.[8,9] Various studies are now working on the immunotherapies treating the OS, including the cGAS-STING signaling pathway. However, some underlying mechanisms remain undefined and the translation to clinic still poses difficulties. Early, accurate, and effective therapeutic remedies against the OS are therefore required on an emergency basis.[10]

With the explosion of epigenetics, interest has intensified on interactions between epigenetic mechanisms and variations of the DNA nucleotide sequence and also on consequences thereof on biology, disease and evolution. Epigenetics is alterations to the molecules of the DNA to control the activity of the genes without modifying the nucleotide sequence.[11]

Epigenetic changes have become an overriding mechanism leading to the development of numerous diseases.[12] They control gene expression by varying the local and global accessibility of the epigenetic codes within the chromatin and

https://doi.org/10.2147/OTT.S581744
Received: 15 November 2025
Accepted: 19 February 2026
Published: 23 February 2026

therefore affect various physiological and pathological processes.[11,13,14] In the progression of cancer, particularly the earlier phases, aberrant metabolic processes and environmental exposures commonly cause epigenetic changes. Indeed, modification such as DNA methylation, histone modification, microRNA expression, and nucleosome remodeling are specifically critical. They cause the disruption of fine regulation within the genome within cells and result in carcinogenic processes involving the unassociated division of cells, unassociated differentiation, and evasion from apoptosis.[15] Therefore, epigenetic changes represent the hallmarks of cancer.[16–19]

DNA methylation is an extremelystable epigenetic mark that greatly affects gene expression and controls the phenotype and working of cells by quelling genes without the altering of the DNA nucleotide sequences.[20] DNA methylation comprises various sorts, such as 5mC, 6mA, and 4mC,[21,22] where among them 5mC is predominant incellular organisms.[23] This biochemical process involves the covalent attachment of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon atom of cytosine, forming 5-methylcytosine (5mC), catalyzed by enzymes known as DNA methyltransferases (DNMTs). This reaction predominantly occurs at cytosines within CpG dinucleotides, where cytosine is paired with guanine.[24,25] The DNMT enzyme family includes Dnmt1, Dnmt3a, and Dnmt3b. Another member, Dnmt3L, lacks a catalytic domain and thus has no enzymatic activity.[26,27] DNMT3a and DNMT3b are de novo methyltransferases, which establish new methylation patterns at previously unmethylated CpG sites during embryonic development and cell differentiation, thereby setting initial gene expression states.[28] Although Dnmt3L has no catalytic function, it can interact with DNMT3a and DNMT3b to enhance their methylation activity.[29] DNMT1 is a maintenance methyltransferase that specifically recognizes and binds to hemimethylated DNA after replication, methylating corresponding cytosines in daughter strands.[30] This ensures faithful inheritance of epigenetic information during cell division and preserves cellular identity[24,31] (Figure 1).

DNA methylation distribution across the genome is not uniform, exhibiting significant regional specificity associated with various biological functions. CpG dinucleotide distribution is highly uneven: while most mammalian DNA lacks CpG dinucleotides,[32] specific genomic regions, known as CpG islands (CGIs), have high G+C content and near-expected CpG frequencies.[33] Bioinformatically, CGIs are typically defined as DNA segments longer than 200 bp, with G+C content exceeding 50%, and an observed-to-expected CpG ratio of at least 0.6.[33] Most CpG sites outside CGIs are methylated, whereas gene promoters typically contain unmethylated CGIs, enabling transcription. Approximately 50,200 CGIs exist within the human genome.[34] Increased CpG sites are often linked to hypomethylation, as these sites are protected from methyltransferases by DNA-binding proteins like Sp1.[35,36] Promoter hypermethylation is a major dysregulation mechanism in cancer, silencing about 70% of genes with CpG islands.[37,38] Hypermethylation physically prevents transcription factor binding and recruits methyl-CpG-binding proteins (MBDs) and histone-modifying enzymes (eg, HDACs), leading to chromatin condensation and transcriptional silencing.[39] In contrast to promoters, gene bodies often exhibit moderate methylation, positively correlated with active gene expression. Although it does not impede transcriptional elongation, gene body methylation may influence RNA splicing.[40,41] Furthermore, methylation of repetitive sequences and transposable elements is essential for transcriptional repression and genomic stability.[42]

Epigenetic variation is common in cancer.[43,44] Dysregulated expression of oncogenes and tumor suppressor genes plays a central role in cancer, driven significantly by epigenetic abnormalities.[19] DNA methylation changes are particularly prominent and occur more frequently than genetic mutations. Therefore, epigenetic changes are promising therapeutic targets and consistent clinical diagnosis markers. Epigenetic changes can be involved in the progression of OS and can serve potential indicators of prediction and therapy.[45] This review demystifies the distinct roles played by DNA methylation within OS, highlighting key signaling pathways and corresponding genes and discusses its translational clinical potential and future research direction.

DNA Methylation in OS Pathogenesis

Global Methylation Patterns

DNA methylation, an important epigenetic modification, has a distinct "dual pattern" expression during the development and progression of OS: genome-wide hypomethylation and localized hypermethylation. Compared to normal tissue, OS has more differentially methylated sites.[46] Another study detected 2845 differentially methylated sites within the OS

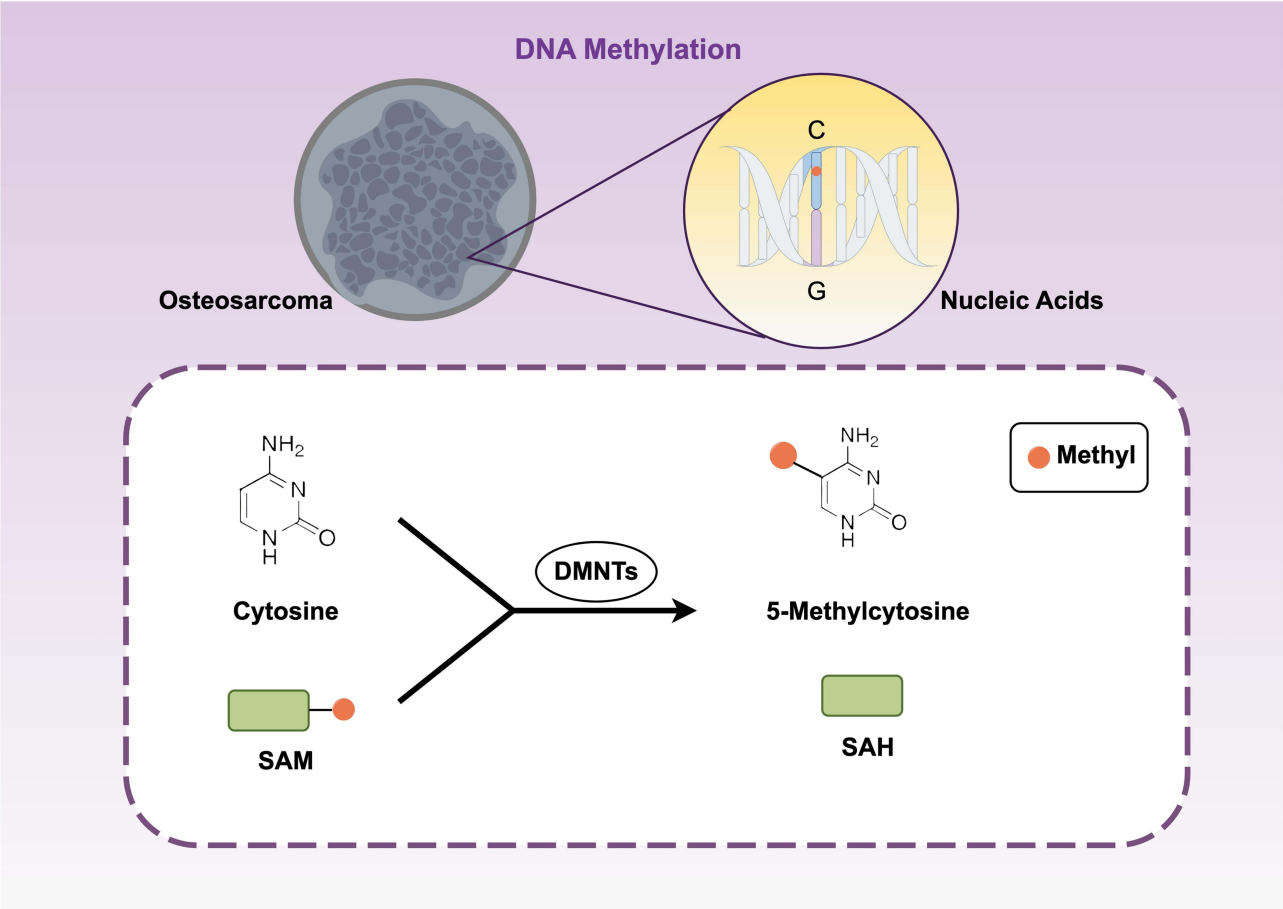

Figure 1 Under DNMT catalysis, cytosine bases are converted into 5mC. A methyl group from the donor SAM is covalently attached to the fifth carbon atom of cytosine, primarily at CpG dinucleotide sites, generating SAH as a byproduct.

specimens, of which 1379 were hypermethylated and 169 hypomethylated promoter sites. Such two apparently opposite processes work together to promote tumor progression.[47–49] Genome-wide hypomethylation primarily happens within non-coding areas. This ubiquitous hypomethylation directly destroys genome stability because the hypermethylation of repeat sequences usually keeps repeat sequences and chromosomes transcriptionally silenced. Removing the methylation activates the genomic "dark matter" to cause chromosomal translocations, gene fusions and aneuploidy. Besides, hypomethylation can indirectly activate proto-oncogenes. Demethylation within the promoters of the oncogenes eliminates the actedness of transcription repression and thus stimulates the expression of the genes to cause uncontrolled proliferation of the cells.

In contrast, hypermethylation predominantly occurs in CpG islands, especially in gene promoter regions.[47] CpG islands are normally unmethylated and allow gene transcription. However, aberrant hypermethylation attracts the binding of methylation-binding proteins and histone deacetylases and results in the formation of bulky heterochromatin complexes. This physically impedes the binding of transcription factors, leading to permanent gene silencing. Large-scale epigenomic data confirm the observation. For example, about 3.8% and 2.2% of the CpG sites are respectively hypermethylated and hypomethylated within OS tissues. This aberrant pattern is more intensified within recurrent or metastatic specimens and is highly correlated with the degree of disease aggressiveness and adverse outcome.[50] Promoter CpG island hypermethylation is the prevailing mechanism responsible for tumor suppressor gene silencing commonly illustrated within the malignancies of human beings, including OS.[51] Model studies involving the OS cell lines U2OS and MG63 experimentally confirmed the pattern of this methylation. Deep high-throughput sequencing identified hundreds of gene promoters with aberrant hypermethylation and hypomethylation. They included genes related to cell

https://doi.org/10.2147/OTT.S581744

cycle regulation, DNA repair, apoptosis, signal transduction pathways, differentiation, and invasion. For example, cyclin-dependent kinase inhibitor gene (CDKN2A) promoter hypermethylation abolishes cell cycle check-points, and MGMT gene silencing impairs chemosensitivity against chemotherapy agents.[48]

Importantly, these "dual patterns" mutually interact extensively. Genome-wide hypomethylation-induced relaxed chromatin structure is postulated to facilitate an favored "epigenetic landscape" for localized hypermethylation to induce malignant transformation. Consequently, the simultaneous existence of worldwide hypomethylation and localized hypermethylation is the defining epigenetic characteristic of OS. It accounts for primary tumor behaviors, such as unlimited proliferation, escape from growth inhibition, and anti-apoptosis. This opens to clinicians new avenues: methylation profiles can be used as biomarkers to diagnose and categorize diseases and predict progression. Furthermore, because DNA methylation is reversible, demethylating agents are promising therapeutic interventions to restore tumor suppressor gene expression, break through chemotherapy resistance, and suppress metastasis.

Methylation of Genes in Key Signaling Pathways

In OS, DNA methylation precisely controls key signaling pathways responsible for its malignant phenotype. Hypermethylation of gene promoters can inactivate crucial tumor suppressor pathways, supporting tumor proliferation, survival, invasion, and metastasis. It is important to note that several well-characterized mechanisms of methylation-mediated silencing, identified primarily in other cancers, provide valuable references for understanding epigenetic dysregulation in OS. For instance, studies across various solid tumors have shown that hypermethylation of the tissue inhibitor of metalloproteinases 3 (TIMP3) promoter silences its expression, leading to impaired natural inhibition of matrix metalloproteinases (MMPs) and disruption of angiogenic balance. This mechanism may similarly contribute to promoting angiogenesis, local invasion, and distant metastasis in OS. Likewise, research in other cancer models indicates that hypermethylation of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) reduces cellular capacity to repair DNA damage, thereby accelerating genomic mutation accumulation and tumor evolution. The potential role of this pathway in OS warrants investigation. Furthermore, studies have demonstrated that expression of death-associated protein kinase (DAPK) can be suppressed by hypermethylation, resulting in apoptosis evasion and chemotherapy resistance. This offers clues for exploring therapeutic resistance mechanisms in OS. These findings from other tumor models illustrate the general role of methylation-mediated inactivation of tumor suppressor genes in driving malignant transformation, providing significant insights for osteosarcoma research.[52–58]

Besides classical tumor suppressors, OS progression also depends on abnormal activation of major signaling pathways. The Wnt/β-catenin pathway regulates tumor immunity, bone remodeling, angiogenesis, hypoxia response, and epithelial-mesenchymal transition (EMT).[59] Aberrant activation involves precise DNA methylation regulation. For example, the Wnt pathway inhibitor gene APCDD1 undergoes hypermethylation mediated by DNMT3a, leading to pathway activation, increased invasion, and EMT.[60]

Research has expanded from protein-coding genes to non-coding RNAs (ncRNAs), whose dysregulation is closely linked to tumor progression.[61] Their regulatory mechanisms often involve epigenetic modifications. Some long non-coding RNAs (lncRNAs) recruit DNMTs to specific gene promoters, modifying methylation levels and downstream gene expression.[62] In OS, lncRNA THAP9-AS1 recruits DNMTs to silence suppressor of cytokine signaling 3 (SOCS3), a negative regulator of the JAK2/STAT3 pathway, enhancing tumorigenesis, metastasis, and oxidative stress resistance.[63] Similarly, DNA methylation regulates microRNAs (miRNAs), which modulate signaling pathways and invasive behaviors, representing therapeutic targets[64–66] (Figure 2).

Methylation and OS Stem Cells (OSCs)

Progresses in the epigenetic evaluation technology have further elaborated the function of DNA methylation involvement in the maintenance and differentiation of stem cells.[67] Epigenetic processes manage the expression of genes during the differentiation and self-renewal of stem cells by adjusting the structure of chromatin. Among these processes, the study on DNA methylation is highly intensive.[68] Clinical challenges in OS, such as chemotherapy resistance, relapse, and heterogeneity of tumors, are all related to OSCs. They are thought to be responsible for tumor initiation, maintenance, progression, and chemotherapy failure. OSCs' self-renewal, tumorigenicity, and chemotherapy resistance are controlled

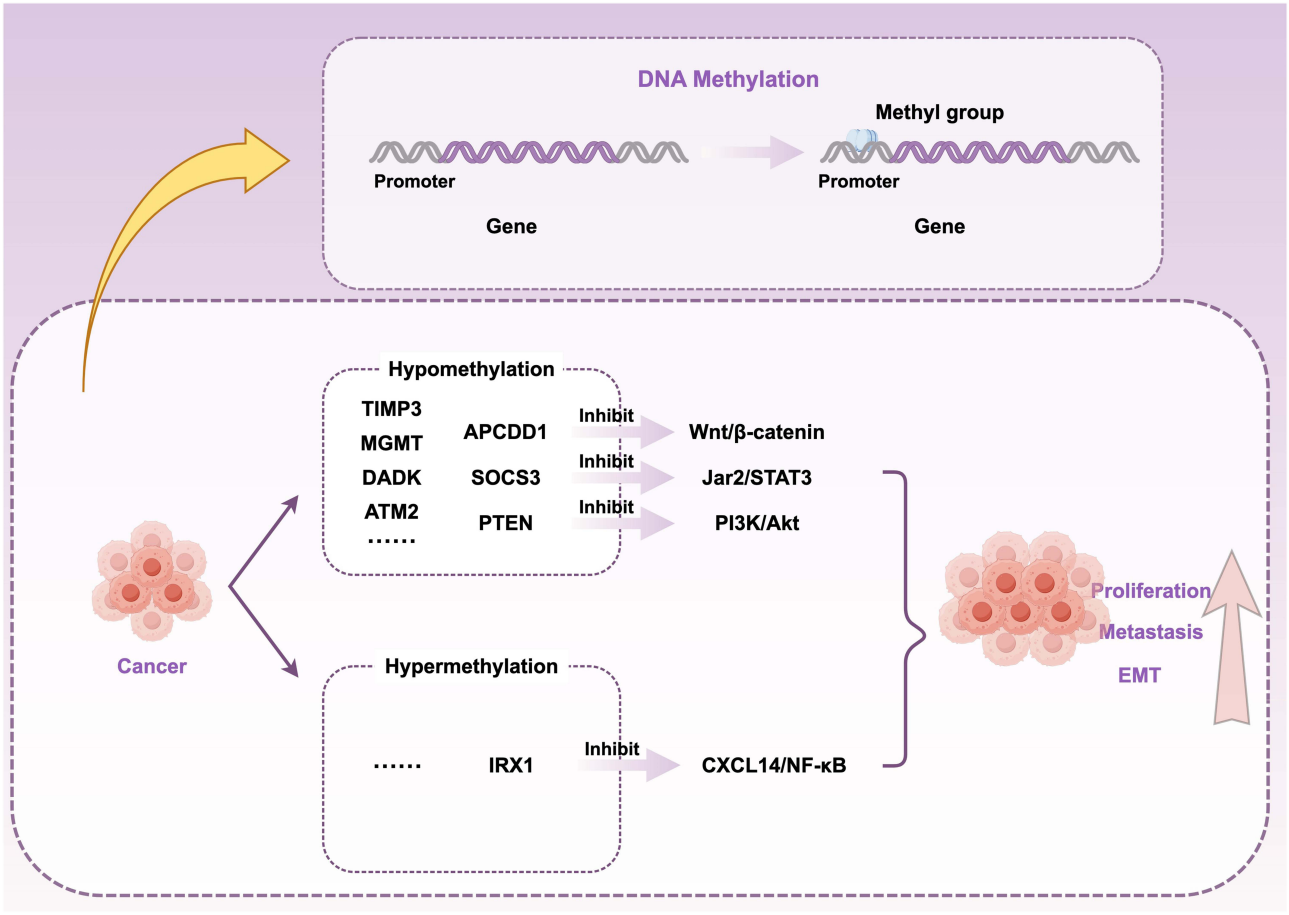

Figure 2 Cancer genomes exhibit a "dual pattern" of DNA methylation, characterized by genome-wide hypomethylation coexisting with hypermethylation at specific gene promoters. This pattern drives tumor proliferation, invasion, and metastasis by regulating key genes and reshaping the TIME. Genes such as APCDD1, and TIMP3 are silenced by promoter hypermethylation, affecting signaling pathways including Wnt/β-catenin, JAK/STAT, and PI3K/Akt, thereby promoting tumor progression. Conversely, genes like IRX1 are activated by promoter hypomethylation, further contributing to tumor progression. These mechanisms collectively form the core epigenetic regulatory network in cancer.

not just by genetic mutations but also by epigenetics, particularly by DNA methylation. DNA methylation downregulates tumor suppressor genes and key pathways involving differentiation control and cell cycle check points and apoptosis and thus widens the stemness of OSCs. This sheds new evidence on OS progression and chemotherapy resistance.

Recent research has elucidated the roles of distinct molecular pathways. For example, the aberrant DNMT1 activation sustains OSC stemness by facilitating the hypermethylation of the miR-34a promoter and thereby silencing its expression. miR-34a is an established tumor suppressor protein inhibiting self-renewal and proliferation through the targets stemness-associated proto-oncogenes Notch and Bcl-2. Consequently, the silencing of miR-34a is therefore augmented OSC stemness by freeing its inhibition on downstream signaling pathways. This finding explains the key involvement of the DNMT1/miR-34a axis in OSC biology and lends themselves to the exploitation in the generation of new therapeutic interventions aimed at OSCs.[69]

Exogenous molecules also affect OSC characteristics by altering DNA methylation profiles. For instance, the cardiac glycoside ouabain suppresses Na^+/K^+-ATPase and activates intracellular signaling pathways in OS cells. Ouabain increases intracellular sodium levels, subsequently raising intracellular calcium (Ca^{2+}) by the sodium-calcium exchanger (NCX). Calcium signaling thus generated alters calcium-dependent enzymes and causes massive DNA methylation alterations at stemness transcription factor promoter areas including Oct4, Sox2, and Nanog. Epigenetic reprogramming by these changes downregulates these genes and decreases stemness and chemotherapy resistance. Pharmacological intervention therefore on DNA methylation assumes significance as an attractive strategy to suppress OS progression and metastasis.[70]

https://doi.org/10.2147/OTT.S581744

Continued research on the involvement of DNA methylation in cell fate determination will clarify the differentiation barrier of OS. DNMT3B controls the fate of mesenchymal stem cells through the hypermethylation of the tumor suppressor gene PTEN's promoter. PTEN silence stimulates the PI3K/Akt pro-survival signaling pathway and suppresses osteoblastic differentiation to preserve cells within an undifferentiated and proliferative state. Such an epigenetic mechanism is potential bait to be pursued in bone regeneration studies and clarifies PTEN silencing commonly seen within OS. PTEN promoter hypermethylation is the leading cause of malignant proliferation, dysfunctional differentiation, and stem-like characteristics' acquirement within OS cells.[71]

In summary, DNA methylation controls the non-coding RNAs, key stemness transcription factors, and essential signaling pathways. Such an intricate control system accurately regulates OSC stemness, self-renewal, differentiation inhibition, and therapeutically induced resistance.

DNA Methylation and OS Progression and Metastasis

Methylation of Genes Related to Invasion and Metastasis

Though chemotherapy and surgery have improved, the five-year survival rate is low for metastatic OS. So there is an imperative to identify biomarkers to allow early diagnosis and to introduce new therapeutic strategies. IRX1 (Iroquois homeobox 1) is an OS pro-metastatic gene induced by DNA hypomethylation. Hypomethylation results in the increased expression of IRX1 and highly elevated the migration and invasion characteristics of tumor cells and anti-apoptosis by the activation of the CXCL14/NF-κB signaling pathway and thereby induced lung metastasis. Hypomethylation of IRX1 can be used as an indication marker to predict metastasis and by restoring its methylation status new anti-metastasis therapies can be introduced.[72,73]

It is important to note that the aforementioned methylation alterations in these genes do not occur in isolation; environmental factors play a significant driving role. Exposure to environmental factors such as carcinogens, radiation, or specific nutritional factors can induce aberrant DNA methylation patterns either genome-wide or at specific gene loci.[74–76] This occurs by interfering with DNA methyltransferase activity or affecting the availability of methyl donors. Environmental carcinogens may directly cause DNA damage and trigger abnormal localized methylation reprogramming, while radiation could indirectly affect the epigenetic regulatory network through pathways like oxidative stress. These environmentally induced methylation changes may act as early events, synergizing with genetic variations to collectively shape the epigenomic landscape of osteosarcoma, thereby promoting tumor initiation, invasion, and metastasis. Therefore, incorporating consideration of environmental factors is essential for understanding the etiology behind methylation changes in osteosarcoma, providing a broader perspective for disease prevention and risk intervention.

Methylation and TME and Extracellular Matrix (ECM)

DNA methylation has an important determinant role to play in the development and progression of cancers. Its involvement is not limited to tumor cells but is also fundamentally important to the shape the TME takes, specifically by shaping localized immune responses.[77–79] DNA methylation controls the expression of genes related to immunity precisely. Modulations within the methylation state at gene promoters/regulator elements can cause altered gene expression to produce specific DNA methylation patterns (IMPs). According to these patterns, OS can be stratified by immune subtypes: an immunosuppressive TME and an immune-activated TME.

In the immune-activated TME, specific DNA methylation patterns promote the upregulation of genes beneficial to anti-tumor immunity. For example, the promoters of chemokines (CXCL9, CXCL10) and antigen-presenting molecules (HLA family genes) exhibit hypomethylation. This increases their transcription, enhancing recruitment, infiltration, and activation of $CD8^+$ T cells and NK cells at the tumor site, thus establishing an effective anti-tumor immune response. Clinical studies show that this immune-activated TME significantly correlates with improved treatment response and prognosis in OS. In contrast, the immunosuppressive TME features abnormal DNA methylation, causing dysregulated expression of immunosuppressive genes. Specifically, DNA methylation promotes tumor immune escape through the regulation of multiple key mechanisms: Promoters of immune checkpoint molecules and anti-inflammatory cytokines may become demethylated and upregulated, while pro-inflammatory genes may be silenced by hypermethylation. This

epigenetic imbalance, particularly the abnormal demethylation and upregulated expression of immune checkpoint molecules such as PD-L1 and CTLA-4, directly weakens the immune system's ability to attack tumor cells. Simultaneously, it recruits and activates immunosuppressive cells, including γδ T cells, naïve CD4+ T cells, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs). These cells inhibit cytotoxic T and NK cell functions through cytokine secretion or direct cell interactions, creating a highly immunosuppressive microenvironment. This TME protects tumor cells, promotes tumor growth, angiogenesis, invasion, and metastasis, and closely associates with poor patient prognosis.[80] Therefore, DNA methylation plays a pivotal role in shaping the TME by precisely regulating the expression of immune checkpoints, immunosuppressive factors, and related cellular functions. It determines the intensity of the immune response and mediates interactions between tumor cells and stromal immune cells. Together, these mechanisms constitute critical pathways of immune escape in osteosarcoma.

Beyond the immune compartment, DNA methylation also critically regulates the extracellular matrix, a key non-cellular component of the TME that provides structural support and biochemical cues essential for tumor progression.[81] Aberrant DNA methylation can directly influence the expression of genes encoding ECM components and ECM-remodeling enzymes, such as matrix metalloproteinases (MMPs) and lysyl oxidases (LOXs).[82] Promoter hypomethylation of specific genes can lead to their overexpression, enhancing ECM degradation and facilitating local invasion and metastasis of osteosarcoma cells.[83] This epigenetic reprogramming of the ECM creates a physical path for tumor spread and releases growth factors and alters mechano-signaling, further driving proliferation and survival. Therefore, the interplay between DNA methylation and ECM dynamics is a crucial layer of regulation in osteosarcoma invasion and metastasis, warranting further investigation as a potential therapeutic target.

Clinicopathological Correlations

For many years, tissue morphology has served as the primary tool in anatomical pathology for cancer diagnosis, particularly in bone tumors.[84] However, some tumors share similar morphologies and are difficult to distinguish. DNA methylation patterns simultaneously reflect the cell of origin and tumorigenesis-related changes, enabling precise identification of histological subtypes.[85,86] Genome-wide methylation profiling combined with machine learning methods has facilitated the creation of various clinicopathological classifiers, increasingly employed as diagnostic tools for diverse tumors.[87] Researchers developed a sarcoma classifier based on methylation profiles, including OS and normal tissues, detecting CpG methylation levels for accurate pathological classification and prognosis prediction.[88–90] This classifier demonstrated high accuracy in pediatric sarcomas.[91] For example, methylation profiles distinguish undifferentiated round cell sarcomas (URCS) harboring the EWSR1–NFATc2 fusion from Ewing sarcoma (EwS) with the classic TET–ETS fusion.[92] Integration of methylation profiles with WHO classification identified four correspondence types: complete matches with WHO entities, intra-entity heterogeneity, merging similar biological entities, and identifying new disease types. This molecular classification has strong correlations to clinical behaviors in OS, individual subtypes of methylation predict various tumor grades and malignancy. High-risk patterns of methylation are also often observed in advanced and metastatic examples. Also, categories of methylation can be an independent category used to predict prognosis. For example, TLR4 promoter status of the methylation highly correlates to the five-year event-free survival and is therefore an important marker to predict recurrence50. Despite the addition of data on the methylation along with CNV analysis to better predict chemotherapy resistance and metastatic potential and overall survival is also improved by adding CNV analysis to the data on the methylation.[88] Consequently, DNA methylation Profiling is an effective instrument that not only aide accurate diagnosis and classification of the OS but also is an important differentiator to be used to make clinical decisions on risk stratification and individualized therapy.

Clinical Translational Applications

Prognostic and Predictive Markers

DNA methylation is an important epigenetic mechanism with immense clinical translational potential in OS, acting as an epigenetic biomarker to predict prognosis and monitor therapeutic efficacy. One study involving database integration identified three critical methylation-driven genes: COL13A1, MXI1, and TBRG1.[93] Type XIII collagen is encoded by

COL13A1 and is a hypermembrane protein whose reduced expression is caused by its hypermethylation to facilitate tumor advancement through the enhancement of cell invasion. MXI1 is a tumor suppressor that regulates growth and differentiation through competitive inhibition to form the MYC-MAX complex and has its hypermethylation related to adverse prognosis.[94–96] TBRG1 is a growth inhibitor and has tumor-suppressive action through the induction of the p53 action and chromosomal stabilization but has its action inhibited by its hypermethylation.[97] According to mRNA expression of these genes, an mRNA expression-derived risk score model with strong predictive power was established. Characterization of these methylation-promoted biomarkers sheds light on OS epigenetic mechanisms and initiates an innovative clinical risk prediction tool. Another study generated an assay to generate a risk prediction model from the basis of the data on the methylation data through nine genes (ARHGAP9, CADM1, CPE, DUSP3, FGFR1, GALNT3, IGF2BP3, KIF26A, and ZFP3) that showed high prognostic ability.[98] Likewise, four DNA methylation loci-based prognostic models were generated by Zhang et al.[99] Successful predictive capability by these models suggests the potential to monitor the gene-specific methylation status to allow future individualized therapeutic efficacy monitoring and tailoring.[93] Looking ahead, the integration of emerging technologies such as single-cell sequencing and spatial transcriptomics holds great promise for advancing DNA methylation research in osteosarcoma. These cutting-edge approaches enable the precise mapping of methylation changes at the single-cell resolution and within specific tissue architectures, thereby uncovering spatial and cellular heterogeneity in the tumor microenvironment. By correlating methylation profiles with transcriptional activity and cellular phenotypes in situ, these tools may refine prognostic models, identify novel methylation-driven subpopulations, and ultimately guide more personalized therapeutic strategies.

Targeting DNA Methylation

Decitabine, a DNA methylation inhibitor, reverses hypermethylation of gene promoters in OS, thus inhibiting tumor proliferation and metastasis. Decitabine also reverses methylation of the GADD45A gene's 5′ CpG island, activating its expression and re-establishing the GADD45A-mediated apoptotic pathway, specifically inducing OS cell apoptosis.[100] Additionally, decitabine restores expression of the estrogen receptor α (ERα) by reversing promoter hypermethylation, thus activating osteogenic differentiation and reducing proliferation and metastasis markers.[101] Furthermore, decitabine upregulates expression of the NKG2D ligand genes MICB and ULBP1, enhancing γδ T-cell-mediated immune recognition and killing of OS cells via the NKG2D–NKG2DL axis.[102] Azacitidine, another DNA methylation inhibitor, similarly reverses or blocks aberrant methylation in cancer cells.[103] In chemotherapy-resistant OS, azacitidine combined with methionine restriction therapy (o-rMETase) effectively inhibited tumor growth by reducing the methylation capacity essential for cancer cell survival.[104] Thus, DNA methylation inhibitors like decitabine and azacitidine provide comprehensive anti-OS effects, ranging from proliferation inhibition and induction of apoptosis and differentiation to immune activation[105] (Table 1).

However, demethylating drugs alone may be insufficient for permanently reversing highly stable epigenetic states. Stable DNA methylation states depend on complex regulatory circuits. Studies indicate that imprinting control regions exhibit "epigenetic bistability", where methylation states are maintained by positive feedback involving DNA-binding factors and chromatin modification complexes.[106–108] Future strategies could target specific components of these stable complexes, enabling more precise and lasting desilencing of tumor suppressor genes, and providing new therapeutic approaches for OS treatment. Although preclinical studies demonstrate the potential of DNA methylation-targeting drugs,

Table 1 Mechanisms of DNA Methylation-Targeting Agents in OS via Gene Regulation

	Gene loci	Influence	References
Decitabine	GADD45A gene	Restoring the apoptosis pathway to specifically induce apoptosis in OS cells	[100]
	Estrogen receptor α gene	Activate osteogenic differentiation pathways, inhibit proliferation and metastasis-related markers, and thus inhibit tumor progression	[101]
	NKG2D ligand genes MICB and ULBP1	Enhances the recognition and killing ability of γδ T cells via the NKG2D–NKG2DL axis, inhibiting OS growth	[102]
Azacitidine	Methionine	Inhibit tumor growth, promote tumor necrosis and degenerative changes	[104]

their clinical translation in osteosarcoma faces three major challenges. Current agents are broad-spectrum demethylating agents that may non-specifically activate proto-oncogenes and cause off-target effects. These drugs often induce side effects such as myelosuppression and gastrointestinal reactions, limiting dosage and duration, with increased risks when combined with chemotherapy. Tumor cells may develop resistance through upregulation of drug-metabolizing enzymes, altered drug transport, or activation of compensatory epigenetic pathways. Therefore, future research should focus on improving target specificity, optimizing dosing regimens to balance efficacy and toxicity, and exploring combination strategies to overcome resistance, thereby advancing the safe and effective application of such epigenetic therapies in osteosarcoma.

Conclusions

In summary, DNA methylation, as a crucial epigenetic regulatory mechanism, plays a multifaceted and central role in the initiation and progression of osteosarcoma. It directly regulates the expression of numerous key genes, including tumor suppressor genes, DNA repair genes, and metastasis-related genes. This regulation occurs through promoter hypermethylation leading to gene silencing or hypomethylation activating proto-oncogenes, thereby driving tumor proliferation, invasion, metastasis, and therapy resistance. DNA methylation profiling demonstrates significant advantages in OS research, providing powerful tools for pathological classification, subtype identification, prognostic prediction, and treatment response evaluation.[109–111] Studies indicate that methylation profiling offers independent and more robust chemotherapy-response predictions than other molecular markers, such as miRNAs. Methylation profiles effectively complement other molecular patterns and can jointly identify clinically relevant tumor subtypes.[112] However, clinical translation faces several challenges. The primary issue is that current risk-prediction models based on multi-gene methylation markers mainly rely on small cohorts, lacking validation through large-scale, multicenter, prospective studies, thus limiting their reliability and universality.[112,113] Additionally, many studies remain at the bioinformatics analysis stage without subsequent functional validation experiments, making the clinical significance of these findings uncertain.[49] Future research must expand sample sizes and conduct multicenter validations to enhance the predictive efficacy of methylation biomarkers. Moreover, integrating functional experiments to clarify the mechanisms by which key methylation events drive OS progression is essential for converting biomarkers into therapeutic targets. Ultimately, combining DNA methylation data with other omics data to establish comprehensive molecular classification systems will facilitate precision diagnosis and treatment of OS.

Disclosure

The authors report no conflicts of interest in this work.

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