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Review Article

Epigenetic Silencing of Tumor Suppressor Genes through Promoter Hypermethylation in Oral Squamous Cell Carcinoma: From Molecular Insights to Clinical Applications

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Keyword: promoter hypermethylation, epigenetic silencing, molecular pathology, oral squamous cell carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC) continues to be a devastating disease, largely because it is often detected too late and tends to resist conventional therapies. Collectively, promoter hypermethylation of tumor suppressor genes represents an early, reversible, and clinically actionable event in OSCC. Emerging methylation-based biomarkers, particularly salivary gene panels, demonstrate promise for non-invasive early detection and longitudinal monitoring. Furthermore, the reversibility of epigenetic silencing provides a rationale for epigenetic therapies, including DNMT (DNA methyltransferase) inhibitors in combination with immunotherapy. Although most evidence remains exploratory and often extrapolated from broader head and neck cancer cohorts, these molecular insights lay the groundwork for future OSCC-specific diagnostic and therapeutic strategies.

HPV-specific methylation thresholds, such as CDKN2A (Cyclin Dependent Kinase Inhibitor 2A) in HPV-negative cancers—where OSCC-specific thresholds are limited and most data are derived from OPSCC (Oropharyngeal Squamous Cell Carcinoma)—and RASSF1A (Ras Association Domain Family Member 1A) in HPV-positive cancers—primarily based on OPSCC rather than OSCC—improve diagnostic and prognostic precision. New non-invasive approaches for early detection and monitoring, such as saliva-based methylation testing HOXA9/NID2 panels (HomeoboxA9), are also highlighted. This review summarizes new evidence that promoter hypermethylation causes oral squamous cell carcinoma (OSCC) by abnormally silencing tumor suppressor genes. We also examine the molecular basis of this phenomenon, stressing how tobacco exposure and HPV infection affect these cancers' methylation landscapes. We believe that incorporating these epigenetic indicators into clinical practice may improve OSCC management.

Keyword: promoter hypermethylation, epigenetic silencing, molecular pathology, oral squamous cell carcinoma

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Introduction

Oral squamous cell carcinoma (OSCC) accounts for over 90% of all oral cancers and is associated with significant global mortality and morbidity. Each year, an estimated 44200 new cases are diagnosed worldwide. The burden is especially high in regions where tobacco use, alcohol consumption, and betel quid chewing are common, particularly South Asia and parts of Africa[1]. The late-stage presentation of OSCC, coupled with aggressive local invasion and frequent lymph node metastasis, contributes to a persistently low 5-year survival rate of approximately 50–60%[2]. This poor survival highlights the urgent need for earlier detection and more precise molecularly guided therapies.

While traditional histopathology remains the diagnostic gold standard, molecular pathology has uncovered key genetic and epigenetic alterations driving OSCC. This shift from purely morphological assessment to molecular characterization is essential for improving outcomes. Among these alterations, epigenetic modifications—heritable changes in gene expression that do not alter the DNA sequence—play a central role. These include DNA methylation, histone modifications, and regulation by non-coding RNAs. Together, they influence chromatin structure and gene transcription[3]. Promoter hypermethylation of tumor suppressor genes (TSGs) is particularly important because it is an early, potentially reversible, and clinically actionable event[4,5].

This review focuses specifically on promoter hypermethylation of tumor suppressor genes in OSCC, emphasizing molecular mechanisms, current evidence for diagnostic and prognostic biomarkers, and the translational challenges in implementing methylation-based strategies in clinical practice.

Figure 1 Schematic figure showing the molecular mechanism of promoter hypermethylation in OSCC.

Molecular Mechanisms of Promoter Hypermethylation in OSCC

This section focuses specifically on tumor suppressor genes (TSGs) because their epigenetic silencing through promoter hypermethylation represents a well-established and early event in OSCC carcinogenesis. Unlike oncogene activation, which often involves mutations or

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amplifications, TSG inactivation frequently occurs via reversible epigenetic mechanisms, making them particularly attractive for biomarker development and therapeutic targeting. **[Figure 1]**

Understanding the molecular foundations of epigenetic changes is crucial for appreciating their clinical potential. The process begins with DNA methylation, an essential epigenetic mechanism where methyl groups are added to cytosine bases within CpG dinucleotides. These CpG sites frequently cluster together in gene promoter regions, forming what are known as CpG islands. In normal cellular function, the CpG islands in tumor suppressor gene promoters remain largely unmethylated, enabling active gene transcription. However, in OSCC, this regulation goes awry. Aberrant hypermethylation of these regions, driven primarily by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), results in transcriptional silencing and loss of tumor suppressor function. This epigenetic disruption removes critical brakes on cell proliferation, ultimately facilitating the uncontrolled growth characteristic of cancer[3].

DNA Methyltransferases and Regulation

The aberrant hypermethylation central to OSCC is orchestrated by DNA methyltransferases (DNMTs). DNMT1 maintains existing methylation patterns during cell division, while DNMT3A and DNMT3B establish new methylation marks[6]**.** A hallmark of OSCC is the frequent overexpression of these enzymes, which is strongly correlated with advanced tumor stage and poorer prognosis. This dysregulation is often initiated by environmental factors; carcinogens found in tobacco smoke, reactive oxygen species, and inflammatory cytokines can potently induce DNMT overexpression. This, in turn, drives the silencing of pivotal tumor suppressor genes, including CDKN2A, DAPK1, and RASSF1A[7].

Recruitment of Methyl-Binding Proteins and Chromatin Remodeling

The process of gene silencing is further cemented by a downstream molecular cascade. Newly methylated CpG islands act as docking sites for proteins like MeCP2, MBD1, and MBD2. These readers recruit co-repressor complexes containing histone deacetylases (HDACs) and histone methyltransferases that condense chromatin into a closed state. This physical restructuring effectively blocks transcription factor access, ensuring stable, yet ultimately reversible, transcriptional silencing[3].

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Patterns of Methylation in OSCC

In OSCC, two co-existing patterns of DNA methylation aberration drive tumorigenesis: widespread genomic hypomethylation and focused, gene-specific hypermethylation. The former, often measured by demethylation of repetitive LINE-1 elements, fosters genomic instability. The latter represents the targeted silencing of promoters for genes governing critical cellular processes like the cell cycle (CDKN2A), apoptosis (DAPK1), DNA repair (MGMT), and cell adhesion (CDH1)[8,9].

In OSCC specifically, promoter hypermethylation of CDKN2A, DAPK1, MGMT, CDH1, RASSF1A, and TIMP3 has been repeatedly reported in tumor tissues compared with normal oral mucosa. Several studies have also demonstrated progressive increases in methylation from oral potentially malignant disorders (OPMDs) to invasive OSCC, supporting their role in stepwise carcinogenesis[10].

HPV Modulation of the Epigenetic Landscape

The tumor's etiology profoundly shapes its epigenetic landscape. Infection with high-risk HPV types 16 and 18 defines a molecularly distinct form of OSCC. In these cases, the viral E6 and E7 oncoproteins degrade p53 and pRb, respectively, leading to secondary DNMT1 dysregulation and a unique hypermethylation signature, including genes like RASSF1A[11,12].

Conversely, the methylome of HPV-negative OSCC is primarily shaped by direct exposure to environmental carcinogens, which upregulate DNMT1 and DNMT3B. This results in a different hypermethylation profile that frequently co-occurs with TP53 mutations and is associated with a more aggressive clinical course[7].

Interplay with Other Epigenetic Mechanisms

In OSCC, co-occurrence of DNA methylation with repressive histone marks such as H3K27me3 has been demonstrated in silenced TSG promoters, indicating coordinated epigenetic repression. Additionally, dysregulated non-coding RNAs, including miR-29 family members and lncRNA HOTAIR, have been shown to modulate DNMT expression in OSCC, reinforcing aberrant methylation patterns[13].

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Clinically Investigated Methylated Tumor Suppressor Genes

The molecular understanding of epigenetic silencing paves the way for its clinical application. The promoter hypermethylation of specific TSGs has been investigated as a potential biomarker for OSCC diagnosis, prognosis, and therapeutic guidance.

Cell Cycle Regulators

CDKN2A: This gene encodes a critical inhibitor of cyclin-dependent kinases (CDK4/6), regulating the G1-S cell cycle checkpoint. Hypermethylation of its promoter results in loss of cell cycle control. Clinically, some studies suggest CDKN2A hypermethylation in HPV-negative OSCC is associated with poor differentiation, co-occurring TP53 mutations, and decreased overall survival. Reported thresholds (e.g., >15%) and saliva assay AUC values (0.88) are largely derived from OPSCC cohorts and remain unvalidated in OSCC[14]. **[Table1]**

Apoptosis Regulators

DAPK1 (Death-Associated Protein Kinase 1): This pro-apoptotic kinase is silenced via promoter hypermethylation, leading to impaired apoptosis and immune evasion. Associations with nodal metastasis are reported, but proposed thresholds (e.g., >12% methylation, PPV 89%) are not OSCC-validated and mainly come from mixed HNSCC studies[15].

DNA Repair Genes

MGMT (O6-methylguanine-DNA methyltransferase): This enzyme repairs mutagenic DNA alkylation. Its promoter hypermethylation (>10%) impairs this repair function, leading to genomic instability. MGMT methylation has been linked with poor prognosis and therapy resistance, though exact hazard ratios and predictive thresholds require OSCC-specific validation[16].

Signal Transduction and Other Regulators

RASSF1A (Ras Association Domain Family 1): A key effector of Ras-mediated apoptosis and microtubule stability. Associations with HPV-positive tumors are better established in OPSCC. Evidence for a correlation between basaloid subtype and radiosensitivity, as well as an AUC of 0.91 performance in OSCC, is lacking and should be considered preliminary[14].

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HOXA9/NID2 Panel: Involved in transcriptional regulation and extracellular matrix remodeling. Hypermethylation of this gene panel in saliva represents a breakthrough for non-invasive screening, offering high sensitivity and specificity. These panels have stronger supporting evidence in OSCC compared with other markers, though additional multi-center validation is needed[4,17].

Prognostic Panels and Recurrence Prediction

Multi-gene panels often surpass single-gene biomarkers in accuracy. For instance, NEIL1/FANCF methylation has been reported in HNSCC generally; OSCC-specific recurrence prediction (HR 1.8) is not yet validated[18.] Combining markers like HOXA9, NID2, and MGMT in saliva allows for powerful longitudinal monitoring of high-risk patients. **[Table 1]**

Table 1 Clinically Investigated HPV-Stratified TSG Methylation in OSCC

Footnote: Most performance metrics (AUC, HR, PPV) and thresholds are extrapolated from OPSCC/HNSCC cohorts rather than OSCC-specific trials.

Diagnostic Translation: Tissue and Liquid Biopsy Workflows

The clinical utility of these biomarkers is realized through robust detection methods applied to various sample types.

FFPE Tissue-Based Analysis

FFPE tissue remains the most extensively studied specimen type for methylation analysis in OSCC. Most studies employ bisulfite conversion followed by quantitative methylation-specific PCR or pyrosequencing to assess promoter methylation of TSGs such as CDKN2A and RASSF1A. While methodological details vary, common challenges include DNA fragmentation and variable bisulfite conversion efficiency, which may influence reported methylation frequencies [21, 22].

Liquid Biopsy: Saliva and Brush-Based Detection

Non-invasive methods represent a paradigm shift for early detection and monitoring.

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Salivary methylation testing has emerged as a promising non-invasive strategy, with studies demonstrating the detection of genes such as HOXA9 and NID2. However, variability in collection protocols and the lack of standardized thresholds currently limit cross-study comparability[4, 23, 24] .

Similarly, brush biopsies provide a minimally invasive way to sample epithelial cells from suspicious lesions or field cancerization. A minimum DNA yield (>10 ng/μL) is required for reliable qMSP analysis of markers like DAPK1 and CDH1[25]. **[Table 2]**

Table 2 Optimized Sample Types, Methylation Detection Methods, and Quality Parameters for Epigenetic Biomarker Analysis in OSCC

Footnote: Workflows are derived from study-specific SOPs and exploratory cohorts and are not yet universally standardized. The markers listed are those most frequently validated in OSCC or related HNSCC cohorts.

Therapeutic Targeting and Response Assessment

Perhaps the most compelling aspect of epigenetic changes is their reversibility, which provides a direct therapeutic target.

Epigenetic Therapies

DNA methyltransferase inhibitors (DNMTis), such as azacitidine and decitabine, are incorporated into DNA during replication, leading to irreversible DNMT binding and subsequent demethylation. This reactivates silenced TSGs like MGMT and DAPK1, restoring chemosensitivity and apoptotic pathways[5,27]. The most promising strategies involve combinations:

DNMTi + HDACi (e.g., Vorinostat): Synergistically reactivate silenced genes by targeting complementary epigenetic layers [28].

DNMTi + Immunotherapy (e.g., anti-PD-1): Demethylation can reactivate endogenous retroviruses and enhance tumor immunogenicity, potentially creating a more favorable environment for immune checkpoint blockade [29].

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DNMTis may restore gene expression. Evidence for DNMT inhibitors in OSCC comes primarily from preclinical models and extrapolation from other cancers; robust OSCC clinical trial data are still lacking.

Assessing Therapeutic Response

Evaluating the response to epigenetic therapy requires new criteria beyond standard radiology. A validated histologic scoring system (κ=0.81 inter-observer agreement) has been developed to identify responders based on features like paradoxical hyperkeratosis, an increased CD8+/FoxP3+ T-cell ratio (>2.5), and improved nuclear morphology. Patients classified as responders exhibit a significant 2.1-fold survival benefit. This histologic scoring system is experimental, published in small exploratory studies, and not yet validated for routine OSCC clinical practice[30]. **[Table 3]**

Table 3 Histologic Criteria for Response to DNMTi Therapy [24]

Footnote: A histologic scoring system has been proposed to assess the response to DNMT inhibitor therapy. This system was derived from small exploratory cohorts and has not undergone external validation. Reported features include paradoxical hyperkeratosis, an increased CD8+/FoxP3+ ratio (>2.5), and improved nuclear morphology. Although inter-observer agreement was reported as strong (κ≈0.8), these findings require confirmation in larger, independent OSCC cohorts before clinical adoption[30].

While DNMT inhibitors are promising, their clinical application is limited by myelosuppression, gastrointestinal discomfort, exhaustion, and risk of infections. These medications are non-specific and may produce worldwide hypomethylation, potentially activating oncogenes or transposable elements. For solid tumors like OSCC, the best dosage regimes are yet unknown, and there are no response-predictive biomarkers.

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Clinical Validation Status of Methylation Biomarkers

The majority of methylation biomarkers in OSCC have been assessed using qMSP, pyrosequencing, or ddPCR in small to intermediate cohorts (usually 40–150 patients). Only a few indicators, such as HOXA9/NID2 in saliva, have been examined in multi-center settings, and even these lack extensive prospective validation trials. Statistical performance indicators such as AUC, sensitivity, and hazard ratios are commonly extrapolated from mixed HNSCC populations. Therefore, the level of evidence remains exploratory rather than practice-changing.

Conclusion and Future Perspective

A crucial step in the development of OSCC is promoter hypermethylation of **TSG,** which has enormous promise as a target for diagnosis and treatment. Although OPSCC and mixed HNSCC cohorts provide the majority of the data, OSCC-specific validation is vital. New salivary testing and epigenetic treatments offer promising prospects, and their combination with cutting-edge technologies like CRISPR-based demethylation and AI-driven methylation profiling could speed up their adoption in clinical settings.

Limitations and Future Challenges

Despite promising outcomes, several barriers hinder the application of methylation markers in therapeutic contexts: Large prospective trials dedicated to OSCC are lacking, and the majority of studies are retrospective and include a variety of HNSCC groups. There is also technical variability and differences in bisulfite conversion efficiency, assay platforms, and cutoff thresholds, which reduce reproducibility. Field cancerization and tumor heterogeneity, like methylation patterns in the oral cavity, differ in time and location. Cost and infrastructure are also important limitations, especially in low-resource settings, where high-sensitivity technologies like ddPCR and NGS are not always accessible.

At this time, there is no approved methylation assay for OSCC as a routine diagnostic test. Standardized procedures, multi-center prospective studies, and integration with AI-driven methylation analysis will be necessary for future progress.

Early Version - Uncorrected

Authors' contributions

Conceptualization, G.S., V.T., Writing - original draft preparation, V.T. and G.S.; writing - review & editing, T.P., K.R., All authors have read and agreed to the published version of the manuscript.

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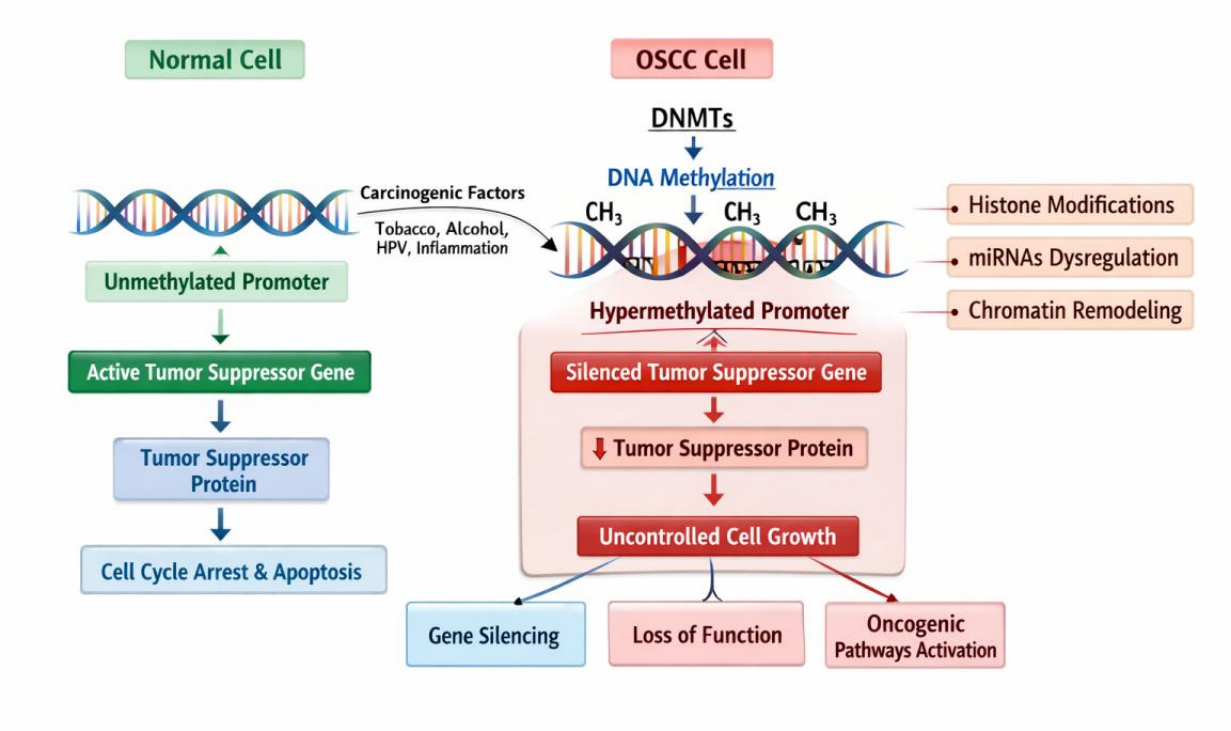


Figure 1 Schematic figure showing the Molecular mechanism of Promoter hypermethylation in OSCC.

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Table 1 Clinically Investigated HPV-Stratified TSG Methylation in OSCC

Gene	Function	HPV Context	Methylation Threshold	Clinical Utility	Performance	References
CDKN2A	Cell cycle inhibitor	HPV-negative	>15%	Poor differentiation, TP53+	AUC 0.88 (OPSCC derived)	[19]
RASSF1A	Ras effector	HPV-positive	>20%	Basaloid subtype, radiosensitivity	AUC 0.91 (OPSCC derived)	[19]
MGMT	DNA repair	Pan-OSCC	>10%	Cisplatin resistance	Sensitivity 78%	[15]
DAPK1	Apoptosis inducer	Pan-OSCC	>12%	Lymph node metastasis	PPV 89% (HNSCC derived)	[4]
HOXA9/NID2	Transcriptional regulation	Pan-OSCC	Panel	Early detection (saliva)	Specificity 95%	[17]
NEIL1/FANCF	DNA repair	Pan-OSCC	Panel	Recurrence prediction	HR 1.8 (OPSCC derived)	[19, 20]

Footnote: Most performance metrics (AUC, HR, PPV) and thresholds are extrapolated from OPSCC/HNSCC cohorts rather than OSCC-specific trials.

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Table 2 Optimized Sample Types, Methylation Detection Methods, and Quality Parameters for Epigenetic Biomarker Analysis in OSCC

Sample	Method	Optimal Markers (most validated)	Key Quality Control	Clinical Utility / Notes	References
FFPE Tissue	Bisulfite Pyrosequencing (qMSP alternative)	CDKN2A, RASSF1A	β-actin internal control; DNA integrity; ≤72 h processing	Gold standard for retrospective studies; useful for validation	[19]
Saliva	Methylation-specific digital droplet PCR (ddPCR)	NID2/HOXA9, MGMT	Centrifugation ≤15 min; cfDNA stability	Non-invasive screening; early detection potential	[19]
Brush Biopsy	Quantitative methylation specific PCR (qMSP)	DAPK1, CDH1	DNA yield >10 ng/μL; Ct ≤35	Minimally invasive; field cancerization assessment	[26]

Footnote: Workflows are derived from study-specific SOPs and exploratory cohorts; not yet universally standardized. Markers listed are those most frequently validated in OSCC or related HNSCC cohorts.

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Table 3 Histologic Criteria for Response to DNMTi Therapy[24]

Feature	Responders	Non-Responders
Keratinization	Paradoxical hyperkeratosis	Reduced keratin
Immune Infiltrate	CD8+/FoxP3+ ratio >2.5	FoxP3+ dominance
Nuclear Morphology	Reduced nuclear:cytoplasmic ratio	Nuclear pleomorphism

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