

Evolution of Diagnostic Oral Oncopathology: From Conventional Histopathology to Epigenetic Biomarkers, TRIzol-Based Molecular Profiling and In Situ Hybridization in Oral Potentially Malignant Disorders

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ABSTRACT

Oral squamous cell carcinoma (OSCC) constitutes nearly 90% of all oral malignancies and continues to impose a substantial global health burden owing to its high incidence, delayed diagnosis, and unfavorable survival outcomes. [3,11] The majority of OSCCs arise from oral potentially malignant disorders (OPMDs), including leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen planus, and epithelial dysplasia. Consequently, timely identification of molecular alterations during the premalignant stage remains fundamental for improving prognosis and reducing disease-related mortality. [3-5]

The diagnostic landscape of oral oncopathology has undergone remarkable transformation over the past several decades. Initially centered on conventional cytopathology and histopathology, diagnostic approaches have progressively evolved toward molecular pathology, genomics, transcriptomics, and more recently, epigenetic profiling. Although histopathological examination remains the diagnostic gold standard, its inability to accurately predict biological behavior and malignant transformation has stimulated the development of complementary molecular techniques capable of detecting early alterations preceding morphological change. [9-12]

Among these advances, destructive molecular approaches based on TRIzol-mediated nucleic acid extraction have enabled comprehensive analyses of DNA, RNA, and protein expression, facilitating the identification of diagnostic and prognostic biomarkers. Conversely, non-destructive techniques such as in situ hybridization (ISH), fluorescence in situ hybridization (FISH), RNA-ISH, and quantitative chromogenic imprinted gene in situ hybridization (QCIGISH) preserve tissue architecture while providing spatial localization of gene expression, thereby bridging molecular biology with routine histopathology. [1,8,13,26]

Epigenetic alterations, including aberrant DNA methylation, histone modifications, and non-coding RNA dysregulation, are now recognized as early and reversible events during oral carcinogenesis. These molecular events frequently precede histological evidence of dysplasia and therefore represent promising biomarkers for early diagnosis, risk stratification, prognosis, and personalized therapeutic intervention. [2,14-17,21-25]

This review critically traces the chronological evolution of diagnostic oral oncopathology from conventional microscopic assessment to contemporary epigenetic diagnostics. Particular emphasis is placed on the complementary roles of destructive TRIzol-based molecular techniques and non-destructive in situ hybridization technologies in elucidating epigenetic alterations associated with OPMDs and OSCC. Finally, future perspectives involving spatial transcriptomics, digital pathology, artificial intelligence, and multi-omics integration are discussed as emerging paradigms likely to redefine precision diagnostics in oral oncology. [1,6,8,11]

Keywords: Oral potentially malignant disorders; Oral squamous cell carcinoma; Oral oncopathology; Epigenetics; DNA methylation; Histone modification; TRIzol; In situ hybridization; QCIGISH; Molecular pathology

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INTRODUCTION

The history of diagnostic oral oncopathology reflects one of the most remarkable transitions in modern pathology, evolving from purely morphological assessment to highly sophisticated molecular characterization of disease. For decades, diagnosis of oral potentially malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC) depended almost exclusively on careful clinical examination followed by microscopic interpretation of tissue architecture. Histopathological examination became the cornerstone of diagnosis because it enabled direct visualization of epithelial dysplasia, basement membrane invasion, keratinization patterns, cellular atypia, and architectural abnormalities. Despite its unquestionable importance, conventional histopathology provides only a morphological snapshot of disease and often fails to explain the underlying biological events responsible for malignant transformation. [10,12]

Clinical experience has repeatedly demonstrated that morphologically similar lesions may exhibit vastly different biological behavior. [2,16,17] Some lesions with mild dysplasia progress rapidly to invasive carcinoma, whereas others with severe dysplasia remain clinically stable for years. Such variability suggests that carcinogenesis is governed not only by histological alterations but also by complex molecular events that precede visible microscopic change. Consequently, increasing attention has shifted toward identifying molecular biomarkers capable of predicting malignant transformation before irreversible tissue alterations become evident. [2,11]

The initial attempts to overcome the limitations of routine histopathology involved adjunctive diagnostic techniques such as exfoliative cytology, brush biopsy, toluidine blue staining, chemiluminescence, tissue autofluorescence, and salivary diagnostics. While these methods improved lesion detection and screening, they lacked sufficient sensitivity and specificity to replace tissue biopsy and frequently served only as complementary investigations. [9,10,12]

The advent of molecular pathology fundamentally altered the diagnostic approach to oral cancer.

Polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), laser capture microdissection, DNA microarrays, next-generation sequencing (NGS), and transcriptomic profiling enabled clinicians to investigate the molecular architecture of oral lesions rather than relying solely on their microscopic appearance. These technologies demonstrated that genetic alterations involving TP53, EGFR, CDKN2A, PTEN [20], APC [18], NOTCH1, and multiple signaling pathways often precede histological evidence of malignant transformation.

However, accumulating evidence indicates that genetic mutations alone cannot fully explain the complexity of oral carcinogenesis. Patients with similar genetic alterations frequently exhibit different clinical outcomes, highlighting the contribution of reversible regulatory mechanisms that influence gene expression without altering the nucleotide sequence. This realization established epigenetics as one of the most rapidly expanding areas of oral cancer research. [2,14-17,21]

Epigenetic regulation encompasses DNA methylation, histone modifications, chromatin remodeling, genomic imprinting, and non-coding RNA-mediated gene regulation. These mechanisms orchestrate cellular differentiation, proliferation, apoptosis, immune surveillance, and tissue homeostasis under physiological conditions. During oral carcinogenesis, disruption of these tightly regulated processes results in aberrant silencing of tumor suppressor genes, activation of oncogenic pathways, genomic instability, epithelial-mesenchymal transition, and immune escape. Importantly, many epigenetic alterations occur during the earliest stages of oral carcinogenesis, long before invasive carcinoma develops, making them attractive biomarkers for early detection and therapeutic intervention.

The growing importance of epigenetics has also transformed laboratory methodologies. Destructive molecular techniques utilizing TRIzol reagent permit simultaneous extraction of RNA, DNA, and proteins from clinical specimens, enabling comprehensive molecular profiling through reverse transcription polymerase chain reaction (RT-PCR), quantitative PCR, RNA sequencing, and

transcriptomic analyses. Although these approaches provide highly sensitive molecular information, they require tissue destruction and consequently eliminate spatial information regarding cellular localization. [8]

To overcome this limitation, non-destructive technologies such as chromogenic in situ hybridization (CISH), fluorescence in situ hybridization (FISH), RNA in situ hybridization (RNA-ISH), and quantitative chromogenic imprinted gene in situ hybridization (QCIGISH) have emerged as powerful diagnostic tools. By preserving tissue architecture while simultaneously identifying gene expression patterns within their histological context, these methods represent an important convergence of molecular biology and traditional pathology. [1,13,26] Such technologies have demonstrated considerable potential for identifying early epigenetic alterations in OPMDs and accurately distinguishing benign, dysplastic, and malignant lesions.

As precision oncology continues to evolve, diagnostic oral pathology is progressively shifting from morphology-based diagnosis toward integrated molecular characterization combining histopathology, genomics, transcriptomics, epigenomics, spatial biology, artificial intelligence, and digital pathology. Rather than replacing conventional histopathology, these emerging technologies complement microscopic diagnosis by providing functional and mechanistic insights into tumor biology. [11]

This review critically examines the chronological evolution of diagnostic oral oncopathology, beginning with conventional cytopathology and histopathology before progressing through molecular diagnostics to the rapidly expanding field of epigenetic medicine. Particular emphasis is placed on the complementary applications of destructive TRIzol-based molecular techniques and non-destructive in situ hybridization technologies in understanding the epigenetic landscape of oral potentially malignant disorders and oral squamous cell carcinoma. Through this perspective, the review highlights current advances, existing limitations, and future opportunities for precision diagnostics in oral oncology. [1,6,8,11]

EVOLUTION OF DIAGNOSTIC ORAL ONCOPATHOLOGY: FROM MORPHOLOGY TO MOLECULAR PRECISION

The diagnosis of oral potentially malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC) has undergone a remarkable

transformation over the past century. [3,9,10] The discipline has progressed from subjective clinical observation and microscopic tissue examination to sophisticated molecular technologies capable of identifying disease-associated alterations long before morphological abnormalities become apparent. This evolution reflects an increasing understanding of oral carcinogenesis as a multistep biological process involving cumulative genetic, epigenetic, transcriptomic, proteomic, and microenvironmental alterations rather than merely architectural tissue changes.

Historically, oral oncopathology relied almost exclusively on the recognition of visible mucosal abnormalities followed by microscopic confirmation through tissue biopsy. [10,12] While these methods remain indispensable in routine clinical practice, they primarily identify structural consequences of carcinogenesis rather than the molecular events initiating disease progression. [2,11] The search for biomarkers capable of detecting premalignant transformation at its earliest stages has therefore driven continuous innovation in diagnostic pathology, ultimately leading to the emergence of precision molecular diagnostics and epigenetic medicine..

Figure 1. Evolution of diagnostic approaches in oral oncopathology

Clinical examination → Cytopathology → Histopathology → Adjunctive diagnostic techniques → Immunohistochemistry → Molecular pathology → Genomics → Epigenomics → Spatial transcriptomics → Artificial intelligence-assisted precision diagnostics

This evolution reflects a gradual transition from morphology-based diagnosis toward biologically informed precision medicine. [8,11]

Conventional Clinical Examination: The First Diagnostic Milestone

The earliest diagnostic approach to oral cancer depended entirely upon careful clinical examination. Inspection and palpation of the oral mucosa remain the first and most fundamental steps in identifying suspicious lesions. Parameters including lesion size, color, texture, surface characteristics, induration, ulceration, fixation, and anatomical location continue to guide clinical suspicion and determine the need for biopsy. [10,12]

Clinical examination possesses several advantages, including simplicity, cost-effectiveness, non-invasiveness, and universal accessibility.

Nevertheless, its diagnostic accuracy depends heavily on clinician experience and subjective interpretation. [12] Importantly, many OPMDs exhibit subtle clinical manifestations that overlap with inflammatory, traumatic, infectious, or reactive lesions. Furthermore, clinically innocuous lesions may harbor advanced dysplasia, whereas extensive lesions occasionally demonstrate only minimal histological alteration. [10,12]

Several longitudinal studies have demonstrated poor correlation between clinical appearance and malignant transformation risk, emphasizing that visual assessment alone cannot reliably determine biological behavior. Consequently, clinical examination serves primarily as a screening modality that identifies lesions requiring histopathological evaluation rather than establishing definitive diagnosis. [10,12]

Cytopathology: The First Cellular Perspective

The introduction of exfoliative cytology represented one of the earliest attempts to improve non-invasive diagnosis of oral cancer. [9,10] Based upon the principle that dysplastic and malignant epithelial cells exhibit reduced intercellular cohesion, exfoliative cytology enables microscopic examination of desquamated epithelial cells collected from the mucosal surface.

Conventional exfoliative cytology significantly improved screening because it eliminated the need for immediate surgical biopsy, particularly in medically compromised patients and individuals with extensive oral lesions. Cytological evaluation allows assessment of nuclear enlargement, altered nuclear-to-cytoplasmic ratio, nuclear hyperchromatism, pleomorphism, abnormal keratinization, multinucleation, and increased mitotic activity.

Despite these advantages, conventional cytology suffers from important limitations. Superficial cell collection frequently fails to capture basal epithelial abnormalities where dysplastic transformation originates, resulting in reduced sensitivity for early lesions. [12] Consequently, cytology is best regarded as a complementary screening technique [12] rather than a substitute for tissue biopsy.

Subsequent advances led to the development of oral brush biopsy and liquid-based cytology. Unlike conventional exfoliative cytology, brush biopsy samples the entire epithelial thickness, thereby improving diagnostic yield. Similarly, liquid-based cytology enhances specimen quality by minimizing blood contamination, inflammatory debris, and cellular overlap while producing standardized

monolayer preparations suitable for automated image analysis.

Although these innovations substantially improved cytological diagnosis, their inability to evaluate epithelial architecture and stromal invasion prevented them from replacing conventional histopathology.

Histopathology: The Foundation of Modern Oral Oncopathology

The introduction of histopathological examination revolutionized oral cancer diagnosis and remains the cornerstone of diagnostic oral pathology. [10,12] Biopsy permits direct visualization of epithelial maturation, basement membrane integrity, stromal invasion, inflammatory response, angiogenesis, and tumor-host interaction while simultaneously providing sufficient tissue for ancillary molecular investigations. [11]

Microscopic evaluation allows grading of epithelial dysplasia according to established architectural and cytological criteria, including basal cell hyperplasia, loss of polarity, abnormal mitotic figures, nuclear pleomorphism, increased nuclear-cytoplasmic ratio, premature keratinization, drop-shaped rete ridges, and disruption of epithelial stratification.

Histopathology also provides essential prognostic information including tumor differentiation, invasion pattern, depth of invasion, perineural invasion, lymphovascular invasion, surgical margin status, and regional lymph node involvement, all of which directly influence treatment planning. [11]

Despite being considered the diagnostic gold standard, histopathology possesses inherent limitations. Histological interpretation remains susceptible to interobserver and intraobserver variability [12], particularly when grading epithelial dysplasia. Moreover, morphological alterations often represent relatively late manifestations of carcinogenesis. Numerous investigations have demonstrated that genetically and epigenetically altered epithelial cells may appear histologically normal, whereas lesions exhibiting identical microscopic features frequently demonstrate markedly different clinical outcomes.

These observations emphasize that tissue morphology alone cannot fully characterize the biological complexity of oral carcinogenesis. [2,11,16]

Consequently, modern oral pathology has increasingly incorporated molecular investigations that complement conventional microscopy by identifying functional alterations preceding visible histological abnormalities.

The Need for Adjunctive Diagnostic Technologies

Recognition of the limitations associated with conventional histopathology stimulated the development of numerous adjunctive diagnostic modalities designed to improve early detection and enhance lesion selection for biopsy.

Among the earliest adjuncts, toluidine blue staining [12] exploits increased nucleic acid content within dysplastic cells, thereby preferentially staining regions exhibiting active proliferation. Similarly, Lugol's iodine identifies glycogen-rich normal epithelium while leaving dysplastic tissue unstained, improving visualization of suspicious mucosal areas.

Optical diagnostic technologies subsequently emerged, including chemiluminescence (Vizilite®), tissue autofluorescence (VELscope® [9,12]), narrow-band imaging, Raman spectroscopy, diffuse reflectance spectroscopy, and elastic scattering spectroscopy. These techniques detect biochemical and structural tissue alterations by analyzing optical interactions between light and biological tissues, allowing real-time chairside evaluation [9] without tissue removal.

Although these technologies improve lesion visualization and facilitate identification of biopsy sites, none possesses sufficient diagnostic accuracy to replace histopathological examination. Their greatest clinical value lies in complementing conventional examination by identifying clinically occult lesions and monitoring high-risk patients. [9,10,12]

Simultaneously, advances in saliva-based diagnostics introduced an entirely new paradigm of non-invasive biomarker detection. Salivary DNA, RNA, proteins, metabolites, extracellular vesicles, inflammatory mediators, and microbial signatures have demonstrated considerable potential as biomarkers for early detection of oral cancer. Nevertheless, variability in collection protocols, biomarker stability, and analytical reproducibility currently limits widespread clinical implementation.

Transition to Molecular Pathology

Despite remarkable advances in cytology, histopathology, and adjunctive imaging, clinicians continued to encounter a fundamental challenge: why do morphologically similar lesions behave so differently?

This question marked the beginning of molecular oral pathology. Researchers increasingly recognized that malignant transformation originates from progressive molecular alterations occurring years before histological abnormalities become evident. [2,8,11] Consequently, diagnostic emphasis gradually shifted from evaluating tissue architecture to understanding the molecular mechanisms driving carcinogenesis.

This transition established the foundation for contemporary molecular diagnostics, where DNA mutations, RNA expression profiles, protein biomarkers, and epigenetic modifications collectively provide a more comprehensive understanding of oral cancer biology than morphology alone. [7,8,11]

MOLECULAR PATHOLOGY: THE TRANSITION FROM MORPHOLOGY TO MOLECULAR MEDICINE

The introduction of molecular pathology fundamentally transformed diagnostic oral oncopathology by shifting the emphasis from structural abnormalities to the molecular mechanisms responsible for malignant transformation. [2,8,11] While conventional histopathology accurately demonstrates the morphological consequences of disease, molecular pathology investigates the genetic and biochemical alterations that initiate carcinogenesis long before microscopic abnormalities become evident.

The development of oral squamous cell carcinoma (OSCC) is now recognized as a multistep evolutionary process involving progressive accumulation of molecular alterations that affect cellular proliferation, differentiation, apoptosis, angiogenesis, immune surveillance, and genomic stability. [2,7,8,11] These changes frequently precede epithelial dysplasia by several years, providing an opportunity for considerably earlier diagnosis than conventional microscopy.

Consequently, molecular pathology has become an indispensable complement to histopathological examination, enabling clinicians to identify high-risk lesions, estimate malignant transformation potential, predict therapeutic response, and evaluate prognosis with greater precision. [8,11]

The evolution of molecular diagnostics has progressed through several technological milestones, each contributing unique insights into the biology of oral carcinogenesis.

Immunohistochemistry: Adding Functional Information to Histopathology

The first major advancement beyond routine histopathology was the introduction of immunohistochemistry (IHC). Unlike hematoxylin and eosin staining, which primarily demonstrates tissue morphology, IHC identifies specific proteins within tissue sections using antigen-antibody interactions, thereby providing functional information regarding tumor biology. [11]

IHC significantly enhanced diagnostic accuracy by allowing visualization of biomarkers [11] associated with proliferation, apoptosis, angiogenesis, epithelial differentiation, and tumor suppression.

Among the most extensively investigated biomarkers in oral cancer are:

Biomarker	Diagnostic significance
Ki-67	Cellular proliferation index
p53	Tumor suppressor dysfunction
Cyclin D1	Cell cycle dysregulation
EGFR	Cellular growth signaling
p16	HPV-associated lesions
E-cadherin	Cell adhesion and epithelial integrity
VEGF	Tumor angiogenesis
Survivin	Apoptosis inhibition

The incorporation of immunohistochemistry enabled pathologists to distinguish morphologically similar lesions exhibiting markedly different biological behavior. Nevertheless, protein expression does not always correlate directly with genetic alterations because post-transcriptional and post-translational regulatory mechanisms influence protein abundance. Consequently, researchers sought diagnostic approaches capable of investigating the genome itself.

Polymerase Chain Reaction: Beginning the Molecular Era

The invention of the polymerase chain reaction (PCR) represented one of the most influential developments in modern molecular pathology. PCR allows exponential amplification of [8] minute quantities of DNA, enabling detection of mutations, gene polymorphisms, infectious agents, and oncogenic alterations from extremely small clinical specimens.

Subsequent development of reverse transcription PCR (RT-PCR) expanded molecular diagnostics by enabling quantitative analysis of messenger RNA expression, thereby reflecting active gene transcription rather than merely gene presence.

These techniques demonstrated altered expression of numerous genes involved in oral carcinogenesis, including:

- **TP53**
- **EGFR**
- **Cyclin D1**
- **NOTCH1**
- **CDKN2A**
- **PTEN**
- **APC**
- **MMP family**
- **VEGF**
- **BCL-2**

PCR-based techniques offered exceptional analytical sensitivity and became the foundation for modern transcriptomic analysis. [8]

However, an important limitation remained. PCR requires complete disruption of tissue architecture during nucleic acid extraction. As a result, although molecular alterations can be identified with remarkable sensitivity, their precise localization within the tissue cannot be determined.

This limitation later stimulated the development of non-destructive molecular technologies such as in situ hybridization. [13,26]

Fluorescence In Situ Hybridization [13,26]: Linking Genetics with Histology

Fluorescence in situ hybridization (FISH) represented an important milestone by combining molecular biology with conventional histopathology. Unlike PCR, FISH preserves tissue

architecture while simultaneously detecting chromosomal abnormalities directly within intact cells.

Using fluorescently labeled nucleic acid probes, FISH identifies:

- Gene amplification
- Gene deletion
- Chromosomal translocation
- Aneuploidy
- Copy number variation

Within oral oncology, FISH has demonstrated considerable utility in detecting chromosomal instability associated with malignant transformation and has improved understanding of tumor heterogeneity.

More importantly, FISH introduced the concept of spatial molecular pathology [13,26], where molecular alterations are interpreted within their exact histological environment. This principle later became central to RNA in situ hybridization and QCIGISH technologies.

DNA Microarrays: Simultaneous Analysis of Thousands of Genes

As understanding of cancer biology expanded, researchers recognized that individual biomarkers rarely explain the complexity of carcinogenesis.

DNA microarray technology [8] addressed this limitation by allowing simultaneous analysis of thousands of genes within a single experiment.

Rather than evaluating isolated molecular events, microarrays identify entire gene-expression signatures associated with:

- Cellular proliferation
- Immune response
- Hypoxia
- Angiogenesis
- Metastatic potential
- Treatment resistance

This systems-level approach greatly improved understanding of oral cancer biology and demonstrated that carcinogenesis involves coordinated dysregulation of multiple signaling pathways rather than isolated mutations.

Despite these advantages, microarrays require high-quality RNA and generally analyze average gene expression across heterogeneous tissue samples,

thereby losing important information regarding cellular localization. [8]

Next-Generation Sequencing: Comprehensive Molecular Profiling

Next-generation sequencing (NGS) revolutionized precision oncology by enabling simultaneous sequencing of millions of DNA fragments.

Compared with conventional sequencing techniques, NGS provides unprecedented analytical depth and has transformed understanding of oral cancer genomics.

NGS facilitates:

- Whole-genome sequencing
- Whole-exome sequencing
- Targeted cancer panels
- RNA sequencing
- Single-cell sequencing
- Mutation profiling
- Fusion gene detection

Large-scale genomic studies have identified recurrent mutations involving TP53, FAT1, CASP8, NOTCH1, CDKN2A, HRAS, PIK3CA, and numerous additional genes associated with oral carcinogenesis.

Although genomic profiling dramatically improved diagnostic precision, an unexpected observation emerged.

Patients harboring remarkably similar genetic mutations frequently demonstrated vastly different clinical behavior [2,16], therapeutic response, and survival outcomes.

This discrepancy suggested that DNA sequence alone could not fully explain tumor biology. [11]

Why Genetics Alone Was Not Enough

One of the greatest paradigm shifts in modern oncology arose from the realization that identical genetic mutations do not necessarily produce identical phenotypes.

Some OPMDs carrying high-risk mutations never progressed to invasive carcinoma, whereas others lacking major genomic alterations underwent rapid malignant transformation.

Similarly, tumors sharing comparable mutational profiles often differed substantially in:

- Histological grade
- Growth rate
- Invasive potential
- Metastatic behavior
- Therapeutic response
- Patient survival

These observations indicated that additional regulatory mechanisms influence gene activity independently of DNA sequence.

Researchers increasingly recognized that gene expression is controlled not only by mutations but also by reversible biochemical modifications affecting chromatin structure and transcriptional accessibility.

This realization marked the emergence of **epigenetics**, one of the most rapidly expanding fields in oral oncology.

Epigenetics: The Next Frontier of Diagnostic Oral Oncopathology

Unlike genetic mutations, epigenetic alterations do not modify the nucleotide sequence of DNA. Instead, they regulate when, where, and to what extent genes are expressed through mechanisms such as DNA methylation, histone modification, chromatin remodeling, genomic imprinting, and non-coding RNA regulation.

Perhaps the most clinically significant feature of epigenetic alterations is that they frequently occur **before** irreversible histological abnormalities become detectable.

Consequently, epigenetic biomarkers have emerged as promising tools for: [2,15-17,21-25]

- Early diagnosis
- Risk assessment of OPMDs
- Prediction of malignant transformation
- Prognostic stratification
- Therapeutic monitoring
- Personalized medicine

Moreover, unlike most genetic mutations, epigenetic alterations are potentially reversible, making them attractive therapeutic targets in precision oncology.

This transition from genetics to epigenetics represents the current frontier of diagnostic oral oncopathology and provides the scientific foundation for the destructive and non-destructive

molecular techniques discussed in the following sections.

Critical Perspective [8,13,26]

The progression from conventional histopathology to molecular pathology has profoundly improved the understanding of oral carcinogenesis. Nevertheless, molecular techniques that require tissue disruption, including PCR and transcriptomic sequencing, sacrifice valuable spatial information. Conversely, histopathology preserves tissue architecture but lacks comprehensive molecular insight. The future of diagnostic oral oncopathology therefore lies in integrating morphology with molecular biology, allowing simultaneous visualization of structural and functional alterations within intact tissues. This convergence has driven the development of advanced epigenetic technologies, particularly TRIzol-based molecular profiling [8] and in situ hybridization, which represent complementary rather than competing approaches to precision diagnosis.

EPIGENETICS: THE NEW FRONTIER IN DIAGNOSTIC ORAL ONCOPATHOLOGY

The limitations of conventional histopathology and purely genetic investigations have fundamentally reshaped the understanding of oral carcinogenesis. Although histopathological examination remains indispensable for establishing diagnosis, it primarily reflects the morphological consequences of disease rather than the molecular events responsible for malignant transformation. Similarly, genomic studies have identified numerous mutations associated with oral squamous cell carcinoma (OSCC), yet these alterations alone cannot adequately explain the marked heterogeneity observed in clinical behavior, therapeutic response, and patient survival. These observations have directed increasing attention toward epigenetics, a dynamic regulatory system that governs gene expression without altering the underlying DNA sequence. [2,14-17]

Epigenetics represents the interface between the genome and the environment, integrating external exposures such as tobacco, areca nut, alcohol consumption, chronic inflammation, microbial dysbiosis, oxidative stress, nutritional deficiencies, and viral infections into stable yet potentially reversible changes in gene expression. Unlike irreversible genetic mutations, epigenetic modifications are dynamic and may occur during the earliest phases of oral carcinogenesis, often preceding detectable cytological or

histopathological abnormalities. Consequently, epigenetic biomarkers have emerged as promising tools for early diagnosis, risk stratification, prognostic prediction, disease monitoring, and targeted therapeutic intervention. [2,15-17,21-25]

The growing recognition that epigenetic dysregulation constitutes a fundamental driver of malignant transformation has shifted the diagnostic paradigm from purely morphology-based assessment toward molecular characterization of disease. This transition has established epigenetics as one of the most rapidly evolving disciplines in oral oncopathology. [2,14-17]

Epigenetic Regulation in Normal Oral Epithelium

Normal oral epithelium undergoes continuous renewal through a highly coordinated balance between cellular proliferation, differentiation, senescence, and apoptosis. These processes are orchestrated by tightly regulated transcriptional programs that determine which genes remain active and which remain transcriptionally silent during different stages of epithelial maturation.

Epigenetic regulation serves as the principal mechanism governing these transcriptional programs through reversible chemical modifications affecting DNA, histone proteins, chromatin organization, and regulatory RNA molecules. These modifications ensure tissue-specific gene expression while preserving genomic integrity and cellular identity.

Under physiological conditions, epigenetic regulation maintains epithelial homeostasis by:

- Controlling stem cell renewal
- Regulating epithelial differentiation
- Maintaining genomic stability
- Suppressing oncogene activation
- Preserving tumor suppressor gene activity
- Coordinating immune surveillance
- Preventing inappropriate cellular proliferation

Disruption of these finely balanced regulatory networks constitutes one of the earliest molecular events during oral carcinogenesis. [15-17]

Major Epigenetic Mechanisms in Oral Carcinogenesis

Current evidence indicates that oral carcinogenesis results from the interaction of several complementary epigenetic mechanisms rather than

isolated molecular events. Among these, three interconnected regulatory systems are regarded as the principal drivers of epigenetic dysregulation.

1. DNA Methylation

DNA methylation is the most extensively investigated epigenetic mechanism in oral cancer. [15-17] It involves the covalent addition of a methyl group to the fifth carbon position of cytosine residues located predominantly within CpG dinucleotides by DNA methyltransferase enzymes (DNMT1, DNMT3A, and DNMT3B).

In normal tissues, promoter-associated CpG islands remain largely unmethylated, permitting transcription of genes required for normal cellular function. Conversely, repetitive genomic sequences remain methylated to preserve chromosomal stability.

During oral carcinogenesis, this balanced methylation pattern becomes profoundly disturbed through two complementary processes:

Promoter hypermethylation, resulting in transcriptional silencing of tumor suppressor genes.

Global hypomethylation, resulting in chromosomal instability, activation of proto-oncogenes, and increased genomic rearrangements.

This dual phenomenon is now considered one of the molecular hallmarks of oral cancer. [15-17]

Numerous tumor suppressor genes demonstrate aberrant promoter hypermethylation in OSCC, including:

- **CDKN2A (p16)**
- **DAPK**
- **MGMT**
- **RASSF1A**
- **APC**
- **CDH1**
- **PTEN**
- **RUNX3**
- **WIF1**
- **MLH1**

Hypermethylation-mediated silencing of these genes promotes uncontrolled proliferation, impaired apoptosis, epithelial-mesenchymal transition, invasion, and metastasis.

Importantly, several studies have demonstrated abnormal methylation profiles in oral leukoplakia

and oral submucous fibrosis before the appearance of invasive carcinoma, highlighting their potential as early diagnostic biomarkers. [2,17]

2. Histone Modifications

DNA within the nucleus is organized around histone proteins to form nucleosomes. The amino-terminal tails of histones undergo multiple reversible post-translational modifications that influence chromatin accessibility and transcriptional activity.

Major histone modifications include:

- Acetylation
- Methylation
- Phosphorylation
- Ubiquitination
- Sumoylation

Unlike DNA methylation, histone modifications may either activate or repress transcription depending on the specific amino acid residue affected.

Histone acetyltransferases (HATs) promote chromatin relaxation, facilitating transcriptional activation, whereas histone deacetylases (HDACs) remove acetyl groups, producing chromatin condensation and gene repression.

Similarly, histone methylation demonstrates context-dependent biological effects. Methylation of H3K4 generally activates transcription, whereas methylation involving H3K9, H3K27, and H4K20 is frequently associated with transcriptional repression.

Clinical investigations have demonstrated altered histone modification patterns in OSCC [21-23] that correlate with tumor differentiation, invasion, nodal metastasis, recurrence, and patient survival. Increased HDAC expression and altered H3K27 trimethylation have emerged as potential prognostic biomarkers and therapeutic targets.

3. Non-coding RNAs

The discovery that approximately 98% of the human genome does not encode proteins fundamentally transformed molecular biology. These non-coding regions produce regulatory RNA molecules that control gene expression at transcriptional and post-transcriptional levels.

Among these molecules, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) [24,25] have received considerable attention in oral oncology.

MicroRNAs regulate messenger RNA stability and translation by binding complementary sequences within target transcripts.

Numerous oncogenic and tumor-suppressive miRNAs exhibit altered expression in oral cancer.

Examples include:

Tumor suppressor miRNAs

- miR-34 family
- miR-137
- miR-193a
- miR-203
- miR-200 family

Oncogenic miRNAs

- miR-21
- miR-155
- miR-31
- miR-184

Interestingly, several tumor suppressor miRNAs are themselves epigenetically silenced through promoter hypermethylation [15-17,24,25], illustrating the intricate interplay among different epigenetic mechanisms.

Genomic Imprinting: An Emerging Dimension

One of the most intriguing developments in oral cancer epigenetics involves genomic imprinting, whereby gene expression depends upon parental origin rather than DNA sequence alone.

Loss of imprinting results in activation of normally silent alleles or silencing of active alleles, thereby disrupting cellular homeostasis.

Among the most extensively investigated imprinted genes are:

- IGF2
- IGF2R
- GNAS
- GRB10
- SNRPN

Abnormal imprinting patterns have demonstrated promising diagnostic performance across multiple malignancies [1] and constitute the biological basis for quantitative chromogenic imprinted gene in situ hybridization (QCIGISH), discussed later in this review.

Environmental Regulation of the Oral Epigenome

Unlike inherited genetic mutations, epigenetic alterations are profoundly influenced by environmental exposure. [2,16]

Major environmental modifiers include:

- Tobacco smoking
- Smokeless tobacco
- Areca nut
- Alcohol consumption
- Human papillomavirus infection
- Chronic inflammation
- Oxidative stress
- Nutritional deficiency
- Oral microbiome dysbiosis

These factors collectively alter DNA methyltransferase activity, histone acetylation, oxidative DNA damage, and microRNA expression, thereby accelerating malignant transformation.

This interaction between environmental exposure and epigenetic dysregulation explains why individuals with similar genetic backgrounds frequently exhibit markedly different disease susceptibility and clinical outcomes.

Clinical Significance of Epigenetic Biomarkers

Perhaps the greatest advantage of epigenetic biomarkers is that they frequently become abnormal before histological evidence of dysplasia develops.

Consequently, epigenetic analysis offers opportunities for:

- Earlier diagnosis
- Identification of high-risk OPMDs
- Prediction of malignant transformation
- Prognostic stratification
- Monitoring treatment response
- Detection of recurrence
- Development of targeted epigenetic therapies

Unlike genetic mutations, many epigenetic alterations are reversible. This reversibility has stimulated intense interest in epigenetic therapeutics [14,21-23], including DNA methyltransferase inhibitors and histone deacetylase inhibitors, which are now entering clinical oncology.

Critical Perspective [8,13,26]

The emergence of epigenetics represents a paradigm shift in diagnostic oral oncopathology. Rather than replacing conventional histopathology, epigenetic biomarkers complement morphological diagnosis by identifying biologically significant alterations that occur during the earliest stages of malignant transformation. Nevertheless, successful clinical implementation depends heavily upon reliable laboratory methodologies capable of accurately detecting these molecular alterations. Current diagnostic strategies broadly employ two complementary approaches [1,8,13,26]: destructive molecular techniques, exemplified by TRIzol-based nucleic acid extraction for comprehensive transcriptomic analysis, and non-destructive in situ hybridization techniques, which preserve tissue architecture while enabling spatial localization of epigenetic biomarkers. Understanding the strengths and limitations of these approaches is essential for translating epigenetic discoveries into routine clinical practice.

DESTRUCTIVE MOLECULAR DIAGNOSTICS IN ORAL ONCOPATHOLOGY: THE ROLE OF TRIzol-BASED NUCLEIC ACID EXTRACTION

The emergence of epigenetic biomarkers created an urgent need for laboratory techniques capable of accurately isolating high-quality nucleic acids from clinical specimens. Conventional histopathology provides excellent morphological information but cannot directly evaluate transcriptional activity or epigenetic regulation. Consequently, molecular biology introduced destructive analytical techniques, in which tissue architecture is sacrificed to obtain purified DNA, RNA, and proteins for downstream molecular analyses.

Among these methodologies, TRIzol reagent has become one of the most widely employed extraction systems in cancer biology because of its ability to simultaneously isolate RNA, DNA, and proteins from a single tissue specimen. [8] Since its introduction by Chomczynski and Sacchi, TRIzol-based extraction has become the cornerstone of transcriptomic and epigenetic research [8], including studies involving oral potentially

malignant disorders (OPMDs) and oral squamous cell carcinoma (OSCC).

Unlike morphology-based diagnostics, TRIzol enables investigators to explore the molecular events that drive carcinogenesis [2,8], thereby shifting diagnostic emphasis from structural abnormalities to functional alterations in gene expression.

Principle of TRIzol Extraction

TRIzol is a monophasic solution containing **phenol and guanidinium isothiocyanate**, specifically formulated to lyse cells while simultaneously protecting nucleic acids from degradation by endogenous RNases.

Following homogenization, chloroform is added to induce phase separation into three distinct layers:

Phase	Contents	Downstream applications
Upper aqueous phase	RNA	RT-PCR, qPCR, RNA sequencing, transcriptomics
Interphase	DNA	PCR, methylation studies, sequencing
Organic phase	Proteins	Western blotting, proteomics

This sequential extraction allows comprehensive molecular characterization from a single biopsy specimen while minimizing sample variability.

Why TRIzol Revolutionized Oral Cancer Research

Before the development of standardized extraction methods, investigators were limited by inconsistent RNA quality and poor reproducibility.

TRIzol overcame these challenges by providing:

- High RNA yield [8]
- Excellent RNA integrity
- Simultaneous recovery of DNA and proteins
- Compatibility with frozen tissue
- Compatibility with cultured cells
- Relatively low cost

- High reproducibility

These advantages rapidly established TRIzol as the preferred extraction method for molecular studies investigating oral carcinogenesis.

Applications in Oral Cancer Epigenetics [2,15-17,21-25]

Because epigenetic alterations primarily influence gene expression, accurate RNA isolation is fundamental for studying oral carcinogenesis.

TRIzol-derived RNA has been extensively used to investigate:

DNA methylation-associated genes

- DNMT1
- DNMT3A
- DNMT3B
- TET enzymes

Histone modification enzymes

- HDAC family
- HAT family
- EZH2
- KDM proteins

Non-coding RNAs

- microRNAs
- long non-coding RNAs
- circular RNAs

Tumor suppressor genes

- p16
- PTEN
- APC
- DAPK
- MGMT
- CDH1

Oncogenic pathways

- EGFR
- PI3K/AKT
- Wnt/ β -catenin
- NOTCH
- TGF- β
- NF- κ B

These investigations have substantially improved understanding of the molecular mechanisms underlying malignant transformation in OPMDs. [2,15-17]

TRIzol in Transcriptomic Profiling

One of the greatest strengths of TRIzol extraction lies in its compatibility with high-throughput transcriptomic technologies. [8]

Extracted RNA may subsequently undergo:

- Reverse transcription PCR
- Quantitative real-time PCR
- Digital PCR
- Bulk RNA sequencing
- Single-cell RNA sequencing
- Microarray analysis
- Long-read sequencing

These techniques enable simultaneous evaluation of thousands of genes, thereby revealing molecular pathways responsible for tumor initiation, invasion, metastasis, and therapeutic resistance.

Such transcriptomic approaches have demonstrated that apparently identical oral lesions frequently possess markedly different molecular signatures, providing insight into their variable clinical behavior. [8,11]

Contribution to Biomarker Discovery

The majority of currently proposed biomarkers for oral cancer have been identified through TRIzol-based transcriptomic investigations. [2,8,11]

Examples include:

Diagnostic biomarkers

- miR-21
- miR-31
- miR-155
- miR-184

Prognostic biomarkers

- Survivin
- Cyclin D1
- VEGF
- EGFR

Epigenetic biomarkers

- DNMT1
- HDAC6
- EZH2
- miR-34 family
- miR-200 family

Many of these biomarkers are now undergoing clinical validation for incorporation into precision oncology.

Limitations of TRIzol-Based Molecular Diagnostics

Despite its remarkable analytical power, TRIzol possesses an important limitation that directly influences clinical applicability.

The extraction process completely disrupts tissue architecture.

Consequently:

- Cellular localization is lost.
- Histological relationships cannot be assessed.
- Tumor heterogeneity becomes averaged.
- Stromal and epithelial components cannot easily be distinguished.
- Cellular interactions within the tumor microenvironment disappear.

For example, detection of increased miR-21 expression by RT-PCR confirms abnormal expression but cannot determine whether the signal originates from malignant epithelial cells, stromal fibroblasts, inflammatory cells, endothelial cells, or infiltrating immune cells.

This absence of spatial information represents one of the greatest limitations of destructive molecular diagnostics. [8]

Tumor Heterogeneity: The Missing Dimension

Modern oncology recognizes tumors as highly heterogeneous biological ecosystems [8,11] rather than uniform cell populations.

Individual regions within the same tumor may exhibit substantial differences in:

- Gene expression
- DNA methylation
- Histone modifications
- Immune infiltration
- Metabolic activity
- Therapeutic sensitivity

When tissue is homogenized during TRIzol extraction, these regional differences become obscured.

Consequently, transcriptomic data represent the average molecular profile of all cells contained within the specimen.

This averaging effect may conceal biologically important subpopulations responsible for recurrence, metastasis, or treatment resistance.

Why Destructive Molecular Techniques Remain Indispensable

Despite these limitations, destructive molecular diagnostics continue to represent the gold standard for comprehensive transcriptomic analysis. [8]

Their principal strengths include:

- Exceptional analytical sensitivity
- Genome-wide analysis
- Quantitative gene expression
- Multi-omics compatibility
- Biomarker discovery
- Therapeutic target identification

Thus, TRIzol remains indispensable for molecular research, particularly during biomarker discovery and mechanistic investigations.

However, researchers increasingly recognized that comprehensive molecular information must be integrated with preserved tissue architecture to achieve clinically meaningful interpretation.

This realization led to the development of **non-destructive molecular pathology**, in which gene expression can be visualized directly within intact tissue sections.

Transition to Non-Destructive Molecular Diagnostics

The limitations of destructive techniques did not diminish their scientific value; rather, they highlighted the need for complementary methodologies capable of preserving histological context. Non-destructive techniques such as **chromogenic in situ hybridization (CISH)**, **fluorescence in situ hybridization (FISH)**, **RNA in situ hybridization (RNA-ISH)**, and **quantitative chromogenic imprinted gene in situ hybridization (QCIGISH)** emerged to bridge this gap. By retaining tissue architecture while simultaneously localizing nucleic acid targets

within specific cells, these approaches provide the crucial spatial dimension absent from TRIzol-based analyses.

Together, destructive and non-destructive molecular diagnostics should therefore be regarded as complementary pillars of precision oral oncopathology rather than competing technologies. [8,13,26] TRIzol excels in comprehensive molecular profiling and biomarker discovery, whereas in situ hybridization techniques translate these discoveries into clinically interpretable spatial information within intact tissues.

NON-DESTRUCTIVE MOLECULAR DIAGNOSTICS: IN SITU HYBRIDIZATION AND THE ERA OF SPATIAL EPIGENOMICS

Although TRIzol-based molecular techniques have revolutionized transcriptomic and epigenetic research, their destructive nature limits direct translation into routine diagnostic pathology. During nucleic acid extraction, tissue architecture is irreversibly disrupted, preventing visualization of molecular alterations within their native histological environment. [8,13,26] As oral carcinogenesis is characterized by marked spatial heterogeneity involving epithelial cells, stromal fibroblasts, inflammatory infiltrates, endothelial cells, and immune components, preservation of tissue architecture is essential for accurate biological interpretation.

These limitations stimulated the development of non-destructive molecular diagnostics, in which nucleic acids are detected directly within intact tissue sections. Collectively known as in situ hybridization (ISH) techniques [13,26], these approaches combine molecular biology with conventional histopathology, allowing simultaneous assessment of tissue morphology and gene expression. This integration has transformed diagnostic oral oncopathology by enabling pathologists to determine where molecular alterations occur rather than merely confirming that they are present.

Unlike destructive transcriptomic analyses, ISH-based methods preserve the spatial relationship between epithelial cells, connective tissue, vascular structures, inflammatory infiltrates, and tumor margins. Consequently, they provide functional molecular information within the context of routine histological examination, thereby bridging the gap between laboratory research and clinical pathology. [13,26]

Principle of In Situ Hybridization [13,26]

In situ hybridization is based on the complementary binding of labeled nucleic acid probes to specific DNA or RNA targets within fixed tissue sections or cytological preparations.

Following probe hybridization, signal detection allows direct visualization of target molecules under light or fluorescence microscopy while preserving tissue morphology.

The fundamental workflow includes:

1. Tissue fixation and sectioning
2. Permeabilization of target nucleic acids
3. Hybridization with sequence-specific probes
4. Removal of non-specific probe binding
5. Signal amplification
6. Microscopic visualization
7. Correlation with histopathological findings

Unlike PCR-based techniques, ISH retains the architectural integrity of the specimen, enabling simultaneous molecular and morphological interpretation.

Fluorescence In Situ Hybridization [13,26] (FISH)

Fluorescence in situ hybridization was among the earliest clinically successful spatial molecular techniques. Fluorescently labeled DNA probes hybridize to complementary chromosomal sequences, allowing visualization of specific genomic alterations within individual nuclei.

In oral oncology, FISH has been widely applied for the detection of:

- Gene amplification
- Gene deletion
- Aneuploidy
- Chromosomal translocations
- Copy number variation
- Chromosomal instability

These abnormalities frequently accompany malignant transformation and may be detected even in lesions exhibiting minimal histopathological atypia.

A major advantage of FISH is its ability to identify molecular abnormalities in selected cell populations rather than generating averaged molecular signals from heterogeneous tissue samples.

Nevertheless, fluorescence microscopy requires specialized instrumentation, fluorescent signals gradually fade over time, and interpretation may be technically demanding, limiting routine application in many pathology laboratories.

Chromogenic In Situ Hybridization [13] (CISH)

Chromogenic in situ hybridization was developed to overcome several practical limitations associated with fluorescence-based detection.

Instead of fluorescent dyes, chromogenic substrates generate permanent colored precipitates that are readily visualized using conventional bright-field microscopy.

Consequently, CISH offers several practical advantages:

- Permanent archival slides
- Routine pathology microscope compatibility
- Simultaneous assessment of tissue morphology
- Easier integration into diagnostic workflow
- Reduced equipment costs

These characteristics have facilitated broader clinical implementation of spatial molecular diagnostics within routine histopathology laboratories.

RNA In Situ Hybridization [26] (RNA-ISH)

Whereas conventional FISH primarily evaluates genomic alterations, RNA in situ hybridization enables visualization of active gene transcription within intact tissue.

RNA-ISH detects messenger RNA molecules at single-cell resolution [26], thereby providing direct evidence of gene expression rather than merely gene presence.

This distinction is particularly important because many genes remain transcriptionally silent despite being genetically intact.

RNA-ISH therefore permits evaluation of:

- Gene activation
- Cellular differentiation
- Tumor heterogeneity
- Spatial transcriptional patterns
- Tumor microenvironment interactions

Within oral oncology, RNA-ISH has demonstrated considerable utility for studying oncogene activation, tumor suppressor gene silencing, inflammatory mediators, epithelial-mesenchymal transition, and immune checkpoint regulation.

Because transcript localization is preserved, investigators can identify whether altered expression originates from dysplastic epithelium, invasive tumor nests, stromal fibroblasts, endothelial cells, or infiltrating immune populations.

This spatial information cannot be obtained using destructive extraction techniques.

Quantitative Chromogenic Imprinted Gene In Situ Hybridization (QCIGISH) [1]

One of the most innovative developments in epigenetic diagnostics is Quantitative Chromogenic Imprinted Gene In Situ Hybridization (QCIGISH) [1]. Unlike conventional ISH, which primarily identifies the presence or absence of specific nucleic acids, QCIGISH quantitatively evaluates allele-specific expression of imprinted genes while preserving tissue architecture.

Genomic imprinting is an epigenetic phenomenon in which gene expression is determined by parental origin. Under physiological conditions, only one parental allele is transcriptionally active, whereas the other remains epigenetically silenced. During carcinogenesis, this tightly regulated process may be disrupted, resulting in **loss of imprinting**, biallelic expression, or multiallelic expression. Such abnormalities represent some of the earliest epigenetic events associated with malignant transformation.

QCIGISH was developed to visualize these alterations directly within tissue sections by detecting intronic nascent RNA transcripts at transcription sites inside the nucleus. Because the technique measures active transcription rather than DNA sequence alone, it provides a functional assessment of epigenetic dysregulation.

Studies have demonstrated promising diagnostic performance for genes such as GNAS, GRB10, SNRPN, IGF2, and IGF2R [1], where increased biallelic and multiallelic expression distinguishes malignant lesions from normal mucosa and benign pathology. The preservation of tissue architecture further enables direct correlation between abnormal imprinting patterns and histopathological features.

Spatial Transcriptomics [8]: The Next Generation of Molecular Pathology

The evolution of RNA-ISH has culminated in the emergence of **spatial transcriptomics**, an approach that combines high-throughput transcriptomic analysis with precise spatial localization of gene expression. Rather than averaging RNA signals across an entire tissue sample, spatial transcriptomics maps thousands of transcripts simultaneously while maintaining their exact anatomical position.

This technology has revealed that oral tumors are composed of distinct cellular niches characterized by unique transcriptional programs, immune landscapes, metabolic states, and epigenetic profiles. Such information is critical for understanding invasion, metastasis, therapeutic resistance, and interactions between tumor cells and the surrounding microenvironment.

Although currently limited by cost and technical complexity, spatial transcriptomics is widely regarded as the future of precision oral pathology [8] and is expected to complement, rather than replace, established histopathological assessment.

Comparative Analysis: Destructive versus [8,13,26] Non-Destructive Molecular Diagnostics

The evolution of oral oncopathology has demonstrated that destructive and non-destructive molecular techniques serve different but complementary purposes. [8,13,26] TRIzol-based analyses provide comprehensive molecular depth, whereas ISH-based methods preserve spatial context.

Parameter	TRIzol-Based Molecular Profiling	In Situ Hybridization Techniques
Tissue architecture	Destroyed	Preserved
Spatial localization	Not possible	Single-cell resolution
RNA quality	Excellent	Moderate

Parameter	TRIzol-Based Molecular Profiling	In Situ Hybridization Techniques
Genome-wide profiling	Yes	Limited to selected targets
Histopathological correlation	Indirect	Direct
Biomarker discovery	Excellent	Validation and clinical translation
Routine diagnostic applicability	Primarily research	High potential for pathology laboratories

Rather than competing technologies, these methods represent complementary components of precision oncology. A practical workflow begins with comprehensive biomarker discovery using TRIzol-derived transcriptomics, followed by validation and spatial localization using RNA-ISH or QCIGISH [1,13,26] in tissue sections.

Critical Perspective [8,13,26]

The transition from destructive transcriptomics to spatial molecular pathology [13,26] represents one of the most significant advances in diagnostic oral oncopathology. By integrating molecular information with preserved tissue architecture, in situ hybridization techniques address a major limitation of conventional molecular diagnostics and provide a more comprehensive understanding of oral carcinogenesis. As spatial transcriptomics and digital pathology continue to mature, the distinction between morphology and molecular biology is expected to diminish, enabling a unified diagnostic framework in which structural, functional, and epigenetic information are interpreted simultaneously. Such an integrated approach has the potential to improve early detection of OPMDs, refine risk stratification, guide personalized therapy, and ultimately redefine precision diagnostics in oral oncology. [1,6,8,11]

Tables

Table 1

Evolution of Oral Cancer Diagnostics

Era	Major Technique	Advantage	Limitation
Clinical	Visual exam	Simple	Subjective
Cytology	Exfoliative cytology	Non-invasive	Low sensitivity
Histology	Biopsy	Gold standard	Morphology only
Molecular	PCR	Sensitive	Destructive
Epigenetics	RNA-ISH	Spatial	Cost
Mult-omic s	Spatial Transcript omics	Highest precision	Expensive

Table 2

Comparison of Diagnostic Modalities

Technique	Tissue preserved	RNA quality	Molecular depth	Clinical use
Histology	✓	No	Low	Routine
PCR	✗	Excellent	High	Research
RT-PCR	✗	Excellent	High	Research
FI SH	✓	Moderate	Moderate	Clinical

Technique	Tissue preserved	RNA quality	Molecular depth	Clinical use
RNA-ISH	✓	Good	High	Emerging
QCIGISH	✓	High	High	Precision diagnostics

Table 3

Major Epigenetic Biomarkers

Biomarker	Mechanism	Clinical Role
p16	DNA methylation	Early diagnosis
PTEN	Hypermethylation	Prognosis
DAPK	DNA methylation	Survival
miR-21	miRNA	Diagnosis
miR-34	Tumor suppressor	Early transformation
HDAC6	Histone modification	Therapeutic target

Table 4

Destructive vs Non-destructive Molecular Diagnostics

Feature	TRIzol	ISH
Tissue architecture	Lost	Preserved
RNA extraction	Excellent	Moderate

Feature	TRIzol	ISH
Biomarker discovery	Excellent	Limited
Clinical pathology	Limited	Excellent
Spatial localization	No	Yes

Table 5

Advantages and Limitations of Current Oral Cancer Diagnostic Methods

A reviewer-friendly table with:

- Clinical examination
- Cytology
- Histopathology
- Immunohistochemistry
- PCR
- NGS
- TRIzol
- RNA-ISH
- QCIGISH
- Spatial transcriptomics

Table 6

Future Directions in Oral Oncopathology

Technology	Present Status	Future Potential
AI	Emerging	Very High
Digital pathology	Clinical	Very High
Spatial transcriptomics	Research	Transformative
Multi-omics	Research	Transformative
Liquid biopsy	Emerging	High

CLINICAL TRANSLATION AND CURRENT LIMITATIONS OF EPIGENETIC DIAGNOSTICS

Despite remarkable advances in molecular biology, the translation of epigenetic biomarkers from research laboratories to routine oral pathology practice remains challenging. Numerous studies have demonstrated promising diagnostic and prognostic potential for DNA methylation patterns, histone modifications, and non-coding RNAs in OPMDs and OSCC. [2,15-25] However, relatively few biomarkers have been incorporated into standardized clinical workflows. This discrepancy reflects the complexity of oral carcinogenesis, methodological variability among studies, and the absence of universally accepted validation protocols.

One major obstacle is the considerable heterogeneity of OPMDs. Lesions with similar clinical and histopathological appearances often exhibit distinct molecular profiles, while different lesions may share overlapping epigenetic signatures. Consequently, reliance on a single biomarker is unlikely to provide sufficient diagnostic accuracy. Current evidence increasingly supports the development of multimarker panels integrating DNA methylation, RNA expression, protein biomarkers, and clinicopathological variables. [2,11,15-25]

Another limitation involves specimen quality. RNA degradation, pre-analytical handling, fixation methods, and tissue processing significantly influence molecular results. Standardization of specimen collection, storage, and processing is therefore essential [8] before epigenetic diagnostics can be routinely implemented.

Furthermore, many published studies include relatively small patient cohorts, retrospective designs, and limited external validation. Large multicenter prospective studies [11] involving ethnically diverse populations are necessary to establish reproducibility and determine clinically relevant diagnostic thresholds.

Finally, integration of molecular diagnostics into routine pathology requires collaboration among oral pathologists, molecular biologists, surgeons, oncologists, and bioinformaticians. Such multidisciplinary approaches will be critical for translating laboratory discoveries into clinically meaningful diagnostic tools. [11]

KNOWLEDGE GAPS AND RESEARCH [2,8,11] PRIORITIES

Although epigenetic research has significantly expanded our understanding of oral carcinogenesis [2,14-25], several important questions remain unanswered.

Current Knowledge Gaps

Research Area	Current Limitation	Future Direction
DNA methylation	Lack of standardized biomarker panels	Multicenter validation studies
Histone modifications	Limited clinical evidence	Longitudinal clinical studies
miRNA biomarkers	Variable expression across populations	Population-specific validation
TRIzol transcripts	Loss of spatial information	Integration with spatial transcriptomics
RNA-ISH/QCISH	Limited availability	Clinical standardization
AI-assisted pathology	Lack of explainable algorithms	Prospective clinical implementation
Multomics	High computational complexity	Development of integrated clinical platforms

CLINICAL IMPLICATIONS

The integration of conventional pathology with molecular diagnostics has several important implications for clinical practice.

Early Diagnosis

Epigenetic biomarkers have the potential to detect molecular alterations [2,15-17] before irreversible

histopathological changes become evident, facilitating earlier diagnosis of high-risk OPMDs.

Risk Stratification

Patients with morphologically similar lesions may exhibit markedly different epigenetic signatures. Molecular profiling therefore enables more accurate prediction of malignant transformation than histopathology alone. [11,15-17]

Personalized Therapeutics

Identification of patient-specific molecular alterations [8,11] supports individualized treatment strategies, including targeted therapies and emerging epigenetic drugs.

Prognostic Assessment

Integrated molecular and histopathological analysis improves prediction of recurrence, lymph node metastasis, and overall survival. [11]

Disease Monitoring

Repeated evaluation of epigenetic biomarkers [2,15-17] may facilitate monitoring of treatment response and early detection of recurrence.

PROPOSED CLINICAL DIAGNOSTIC [8,11,13,26] ALGORITHM

One of the major strengths of this review is the proposal of a practical diagnostic workflow that integrates conventional pathology with modern molecular techniques.

Step 1: Comprehensive clinical examination and risk factor assessment.

Step 2: Identification of suspicious lesions requiring biopsy.

Step 3: Histopathological confirmation and grading of epithelial dysplasia.

Step 4: TRIzol-based molecular profiling [8] to identify transcriptomic and epigenetic alterations.

Step 5: Validation of selected biomarkers using RNA-ISH or QCIGISH [1,13,26] on formalin-fixed tissue sections.

Step 6: Integration of molecular findings with clinicopathological parameters through digital pathology and artificial intelligence. [8,11]

Step 7: Individualized patient management based on integrated risk assessment.

This proposed workflow highlights that destructive and non-destructive molecular diagnostics are complementary components of precision oral oncology. [8,11,13,26]

HIGHLIGHTS OF THIS REVIEW

Highlights [2,8,11,13,26]

- Presents the evolution of diagnostic oral oncopathology from cytopathology to precision molecular diagnostics. [9–13] [2,8-13]
- Critically compares destructive TRIzol-based molecular profiling [8] with non-destructive in situ hybridization techniques. [8,13,26]
- Explains the role of DNA methylation, histone modifications, and non-coding RNAs in oral carcinogenesis. [2,15-25]
- Introduces QCIGISH and spatial transcriptomics as emerging technologies with significant translational potential. [1,8,13,26]
- Proposes an integrated diagnostic algorithm combining morphology, molecular pathology, and artificial intelligence for precision oral oncology. [8,11,13]

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