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Salivary Biomarkers in Oral Squamous Cell Carcinoma: Current Perspectives and Challenges in their Clinical Application

Saba Khan[1], Uzma Zareef[2], Nadia Chughtai[3], Syed Ashar Imtiaz[4], S Hussain Askary[5], Gul Rukh Askary[6], Shajia Haider[7]

[1]Department of Oral Pathology, Hamdard University, Dental Hospital, Karachi, Sindh, Pakistan.
[2]Department of Oral Pathology, Liaquat College of Medicine and Dentistry, Karachi, Sindh, Pakistan.
[3]Department of Oral Medicine, Karachi Medical and Dental College, KMU, Karachi, Sindh, Pakistan.
[4]Department of Operative Dentistry, Health Department, Government of Sindh, Pakistan.
[5]Department of Community Dentistry, Fatima Jinnah Dental College, Karachi, Sindh, Pakistan.
[6]Department of Oral Pathology, Fatima Jinnah Dental College, Karachi, Sindh, Pakistan.
[7]Department of Operative Dentistry, Karachi Medical and Dental College, Karachi Metropolitan University, Pakistan.

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Corresponding Author: Saba Khan
Department of Oral Pathology, Hamdard University, Dental Hospital, Karachi, Sindh, Pakistan.
Email: fahadraja78@gmail.com

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ABSTRACT

Oral cancer is a life-threatening disease that predominantly affects the oral cavity, with approximately 90% of cases being oral squamous cell carcinomas (OSCC). Rapid metastasis and late detection are key factors contributing to the poor prognosis of OSCC. The alarming rise in OSCC-related mortality underscores the urgent need for clinical diagnostic methods that enable early detection. In recent years, salivaomics has gained popularity in molecular diagnostics due to the accessibility and non-invasive nature of saliva. Researchers have identified over 100 salivary biomarkers with potential roles in OSCC screening, categorized into proteomic, genomic, transcriptomic, and metabolic biomarkers. Although these biomarkers show high specificity and sensitivity, challenges such as variability and the need for standardized protocols must be addressed to improve diagnostic accuracy and treatment outcomes. This review explores significant salivary biomarkers in OSCC management and highlights the challenges that hinder their clinical application, emphasizing the need for optimized protocols to enhance test validity.

INTRODUCTION

Oral cancer is the sixth most common malignancy worldwide, with oral squamous cell carcinoma (OSCC) accounting for more than 90% of cases. OSCC originates from the epithelial cells of the oral cavity and is associated with various risk factors, including genetic predisposition, betel nut/tobacco chewing, alcohol consumption, chronic inflammation, and smoking. Additionally, viral infections such as HPV and HIV, along with premalignant lesions like leukoplakia and oral submucous fibrosis (OSF), contribute to the progression and severity of OSCC. The tongue is the most frequently affected intraoral site, followed

by the floor of the mouth, gingiva, and buccal mucosa.

The oncogenic pathway in OSCC involves a series of epigenetic transformations and mutations that drive abnormal cell growth. At least five to ten genetic alterations are thought to occur within the basal cell layer progenitor cells, leading to the clonal expansion of altered keratinocytes and the development of a malignant OSCC phenotype. Clinically, OSCC can present as leukoplakia, erythroplakia, necrotic ulcers with indurated margins, or nodular outgrowths with various surface textures. Nearly one-third of OSCC cases develop from pre-existing leukoplakic lesions.

Despite advancements in treatment, the five-year survival rate for OSCC has remained stagnant over the past few decades. The poor prognosis is attributed to late-stage diagnosis (stages III or IV) and rapid metastasis. Currently, the gold standard for OSCC diagnosis involves a clinical oral examination followed by surgical excision of suspicious lesions. However, this method has several limitations, including invasiveness, delayed detection, and the high cost of treatment. Histological analysis of early cancerous lesions often fails to show significant changes, emphasizing the need for more efficient diagnostic techniques.

In recent years, salivary biomarkers have gained attention as a potential tool for the non-invasive and cost-effective diagnosis of OSCC. Biomarkers are defined as characteristics that are objectively measurable and indicative of normal physiological or pathological processes. Saliva, in particular, offers a valuable diagnostic medium because of its easy accessibility and its ability to reflect physiological and pathological states. A growing body of research highlights the potential of salivary biomarkers in the early detection, monitoring, and prognosis of OSCC.

Saliva as a Diagnostic Fluid in OSCC

Unstimulated whole saliva (USWS) is a bio-fluid derived from the major salivary glands (parotid, submandibular, and sublingual) and other oral secretions. It contains various components, including gingival crevicular fluid, epithelial cells, blood cells, bacterial species, and remnants of food. Saliva plays a vital role in oral health, contributing to lubrication, digestion, antimicrobial protection, and pH balance. Its composition includes water, electrolytes, proteins, and hormones, with over 2,400 proteins and polypeptides identified, many of which are established OSCC biomarkers.

Saliva's proximity to the tumor site in OSCC cases makes it an ideal diagnostic fluid, offering several advantages: it is non-invasive, cost-effective, easy to collect, and allows for larger sample volumes to be stored and processed over time. The first human saliva biomarker discovered was the epidermal growth factor receptor 2 (EGFR-2). Since then, over 100 potential salivary biomarkers for OSCC, including peptides, proteins, nucleic acids, and metabolites, have been identified.

While a single biomarker assay may not be sufficient to capture the complex molecular changes in OSCC, targeting multiple salivary biomarkers can improve the accuracy and reliability of diagnostic tests. The growing field of "salivaomics" offers the potential for highly personalized and effective treatment strategies, ultimately improving patient outcomes.

Proteomic Biomarkers

Proteomic biomarkers, derived from the protein compartment of human saliva, are crucial in early OSCC screening. Proteins perform diverse regulatory functions at the cellular level, and studies have identified numerous proteins with diagnostic potential. For example, in 2013, Schulz et al. reported that up to 30% of saliva proteins are sourced from plasma. More than 2,000 proteins have been isolated from the saliva proteome, with Yu et al. highlighting the role of 49 proteins as potential OSCC biomarkers.

Diagnostic techniques used to analyze these proteins include enzyme-linked immunosorbent assay (ELISA), liquid chromatography (LC), high-performance liquid chromatography (HPLC), mass spectrometry (MS), and two-dimensional electrophoresis (2DE). A recent study by Katakura et al. evaluated salivary cytokine levels (IL-6, IL-8, IL-b1) using ELISA, showing increased cytokine levels in OSCC patients. Similarly, Cyfra21e1, TPA (tissue polypeptide antigen), and cancer antigen 125 (CA 125) were found to be significantly higher in OSCC patients compared to controls.

Other proteomic biomarkers, such as CD59, Mac-2 binding protein, profilin 1, and myeloid-related protein 14, have been identified as differentially expressed in OSCC patients. Elevated levels of soluble CD44 have also been shown to distinguish malignant from benign lesions. Among the cytokines, IL-6 and IL-8 have been extensively studied. IL-6 is linked to immunosuppression, while IL-8, a pro-inflammatory cytokine, promotes angiogenesis and acts as a chemoattractant for macrophages. IL-8 has shown the highest predictive power in distinguishing between pre-cancerous and cancerous lesions.

Defensins, a group of antimicrobial peptides, also serve as potential biomarkers. Mizukawa et al. reported the detection of defensin-1 in OSCC patients even in early stages, with a significant increase observed in oral potentially malignant disorders (OPMDs) like lichen planus and leukoplakia.

The clinical importance of proteomic biomarkers in OSCC has been listed in Table 1.

Table 1

Type of Proteomic Biomarker	Clinical Significance in OSCC	Identification Technique	Reference
Glycoproteins	CD44, a surface glycoprotein is responsible for intracellular and extracellular matrix interaction. Elevated levels of CD44 are expressed in advanced stages of head and neck cancers.CD44 is particularly observed in OSCC stem cells.	ELISA, Immunoblot, HPLC	(22),(27)
	CA-25, mucin glycoprotein, acts as immunosuppressant by killing NKs and promotes metastatic invasion of OSCC.	2D gel electrophoresis, HPLC	(28)(17)
	Marked rise in saliva levels of CEA, CA-50 and erbB2 are seen in OSCC malignancies.		
Cytokeratins	Cyfra 21-1 is a potential biomarker in OSCC risk assessment; elevated levels are correlated with deeper bone invasion and distant metastasis.	ELISA, MS, LC	(29)
	Threefold rise in saliva levels of Cyfra 21-1 than serum have been reported in OSCC patients.		(30)
Intracellular Proteins	Tissue polypeptide-specific antigen (TPS) derived from CK-18 is a good indicator in advanced stages of nasopharyngeal carcinoma. It also holds significance in prognosis and diagnosis of OSCC.	ELISA Mass Spectrometry	(31)

	Mac-2 binding protein regulates growth, motility of OSCC cells and depicts 90% sensitivity and 83% specificity. Elevated levels of M2BP have been documented in serum, and saliva of OSCC. ZNF510, a zinc finger protein possesses both tumor promoter and suppression roles. It is chiefly involved in transcriptional regulation and an important peptide to distinguish between early and late stages of OSCC.		(32)
Enzymes	Matrix metalloproteinases (MMPs) are vital proteases secreted by stromal cells and activates digestion of ECM, accelerating the local invasion and distant spread of oral cancer [49]. Notably, MMP-1and MMP-3 are established oral cancer markers. Saliva levels of MMP-2 and MMP-9 were also identified in OSCC and OPMD patients. Secretory leukocyte peptidase inhibitor (SLPI), is peculiarly involved the preventive treatment of OSCC	ELISA, High-liquid performance chromatography(HPLC), MS analysis	(33)(34) (35)
Pro-inflammatory mediators	IL-6, IL-8, IL-1β and TNF-β, are pro-inflammatory cytokines and distinctly expressed in aggressive tumors. IL-8 reflects highest value AUC when used in combination with IL- -1β in distinguishing controls, OPMD and OSCC patients.	ELISA, LC proteomic analysis	(36) (37)(38)
Miscellaneous	C-reactive protein (CRP)synthesized and actively secreted by hepatocytes in reaction to chronic inflammation is a powerful marker in diagnosis of OPMDs and OSCC. In a study, the ratio of CRP/albumin had good prognostic ability in OSCC; the patients with high ratio had	HPLC	(39)

	aggressive disease stage and low survival rate.	ELISA	(40)
	Glutathione is an epidemiological marker for chemo prevention and assess the risk of OSCC development.		
	IgG inhibits apoptosis and S100A2 belonging to group of calcium-binding proteins is a good prognostic biomarker for OSCC. Cofilin and fibrin proteins play role in oncogenesis and its metastasis. In advanced stages of OSCC, higher saliva levels of transferrin are seen.	2DE gel analysis	(41)
	Alpha1-antitrypsin (AAT) is powerful indicator in assessing OSCC severity.		

Genomic Biomarkers

Genomic biomarkers arise from both genetic (DNA sequence) and epigenetic (gene expression) alterations. Small-scale mutations involve single nucleotide changes, while large-scale mutations may include chromosomal rearrangements, gene duplications, or loss of heterozygosity. Tumor-specific genomic markers are crucial for detecting these genetic changes.

The first study that proposed saliva as a diagnostic tool for oral cancer was conducted by Liao et al., who found mutated p53 genes in the salivary DNA of oral cancer patients. In OSCC, circulating tumor DNA (ct-DNA) from necrotic or apoptotic cells is a potential source of biomarkers. Although serum DNA levels are higher, salivary ct-DNA is better suited for analyzing genetic aberrations in OSCC due to the proximity of the tumor to the oral cavity. Mitochondrial DNA (mtDNA) mutations, particularly in the displacement loop (d-loop), have been identified as potent salivary biomarkers.

Epigenetic alterations, particularly the hypermethylation of gene promoters, also play a key role in OSCC development. Genes such as MGMT and DAPK have been identified as methylation targets, with epigenetic changes being detectable through oral rinse samples. The detection of viral DNA, particularly from HPV, further enhances the diagnostic potential of salivary biomarkers.

Table 2

Type of Genomic biomarker	Clinical Significance in OSCC	Identification Technique	References
H3 Histone family 3A (H3F3A)	Structural stability of DNA	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(51)
S100 calcium-binding protein P (S100P	Regulates cell differentiation and promotes Ca++ binding,	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(43)

	Expressed in anoikis"-resistant OSCC tissues indicating role in oncogenesis and metastasis.		
Spermidine N1-acetyltransferase (SAT), Ornithin decarboxylase antizyme 1 (OAZ1)	Up regulate polyamine catabolism and synthesis Saliva OAZ1-mRNA regulates DNA methylation and excessively seen in OSCC patients.	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(52)
P53 gene codon 63	Detection of this gene render quick and reliable results in diagnosis of OSCC	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(53)
Loss of heterozygosity (LOH) in combination of other biomarkers D17S79, D9S156 and D3S1234,	A key feature indicating the risk of conversion from benign to malignant lesion in OSCC.	Quantitative PCR (qPCR),Microarrays followed by q-PCR	(54)
Cytochrome co-oxidase I and II	Indicator of mtDNA genome mutations in OSCC	Quantitative PCR, (qPCR), Microarrays followed by q-PCR	(55)
Tumor suppressor genes i.e., DAPK, DCC, TIMP-31, TIMP-3	Potent diagnostic biomarkers in OSCC with highest accuracy.	q-PCR, Microarrays	(56)

Transcriptomic Biomarkers

RNA molecules, unlike DNA, are single-stranded and are transcribed from DNA, playing multifaceted roles in gene coding, non-coding, and expression. A recent study utilizing next-generation sequencing (NGS) identified more than 4,000 coding and non-coding RNAs in healthy human saliva. Human RNA consists of both large and small molecules. Large RNAs (>200 base pairs) include messenger RNA (mRNA) and long non-coding RNAs (lncRNAs), while small RNAs (<200 base pairs) encompass transfer RNA (tRNA), ribosomal RNA (rRNA), microRNAs (miRNAs), small interfering RNA (siRNA), and small nucleolar RNA (snoRNA). Recent advances in microarray technology have introduced a novel approach to salivary transcriptome diagnostics, offering a highly efficacious and robust panel of RNA biomarkers for clinical applications.

The presence of mRNA in saliva broadens the scope of diagnostic biomarkers for oral cancer detection. Differentially expressed mRNA patterns can accurately reflect genetic aberrations and serve as ideal biomarkers for OSCC diagnosis. It is widely recognized that salivary mRNA is either released from apoptotic bodies secreted by tumors or directly discharged as exosomes into circulation. Techniques such as quantitative PCR (qPCR) and microarray analysis are commonly employed for salivary RNA analysis.

Li et al. highlighted the power of microarray technology in analyzing the salivary transcriptome and identified seven novel mRNA transcripts as potential biomarkers in OSCC patients. These validated genes were categorized based on their regulation patterns: highly upregulated mRNAs included interleukin-8 (IL-8), IL-1b, and S100P (S100 calcium-binding protein P); moderately upregulated mRNA included H3F3A (H3 histone,

family 3A); and lowly upregulated mRNAs included OAZ1 (ornithine decarboxylase antizyme 1), DUSP1 (dual specificity phosphatase 1), and SAT (spermidine/spermine N1-acetyltransferase).

In addition to mRNAs, non-coding RNAs (ncRNAs) also contribute significantly, comprising nearly 98% of the transcriptional activity. Among these, microRNAs (miRNAs) are short RNA molecules, 19-23 nucleotides in length, with approximately 100 miRNAs discovered in the human genome. Unlike mRNA, miRNAs are highly resistant to RNAase degradation, allowing them to persist longer in bodily fluids such as cerebrospinal fluid (CSF), urine, blood, saliva, pleural discharge, and sweat. Additionally, miRNAs demonstrate a much higher fold change, sometimes up to 1,000 times greater, compared to mRNAs.

Several miRNAs have been extensively studied in OSCC, including miRNA-125a, miRNA-200a, miRNA-31, miRNA-184, miRNA-27b, and miRNA-7. Some of these act as tumor suppressants, while others function as oncogenes. For instance, miRNA-125a and miRNA-200a are significantly downregulated, whereas miRNA-31, which promotes tumor cell growth, is frequently upregulated in saliva and plasma. miRNA-184 has recently emerged as a potential biomarker for malignant transformation, with up to a threefold increase observed in OSCC and oral potentially malignant disorder (OPMD) patients compared to healthy controls.

Table 3

Transcriptomic biomarker	Clinical Significance in OSCC	Identification Technique	References
IL-8,IL-1b	Immune and inflammatory responses, cellular adhesion, chemotaxis	ELISA	(62)
mRNA biomarkers (miR-200a, miR-125a, miR-31)	Cellular growth, proliferation, tumor suppressor genes, regulates cell differentiation in early metastasis, enhanced levels of miRNA-31is seen in OSCC.	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(59)(61)
Dual specificity phosphatase 1 (DUSP1)	Activation of signaling MAPK pathways, translations, antioxidant, hyper methylation and detection in early OSCC.	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(63)
H3 histone family 3A(H3F3A)	Chromosomal structural integrity. H3F3A-mRNA combined with IL-8 shows high discrimination among OSCC and OPMDs	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(51)
Long noncoding HOTAIR	Highly expressed in DNA damage	Quantitative PCR (qPCR), Microarrays followed by q-PCR	(64)

Metabolomic Biomarkers

Metabolomics technology is an innovative novel emerging clinical trend that characterizes the relationship between salivary metabolites and disease detection. Wei et al. explored role of salivary metabolites in three OSCC patients, thirty-two oral leukoplakia patients and thirty-four controls. In his study, five salivary metabolite signatures distinguished OSCC patients profile from the healthy individuals. Therefore, metabolomics succor in improving the diagnosis and prognosis of OSCC. In one other metabolomics study, Sugimoto et al. analyzed key roles of metabolites in saliva samples of periodontal patients, breast and pancreatic cancer patients using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) technique.(65) It was recommended that the saliva metabolites prove to be vital can screening biomarkers. The established saliva metabolites in OSCC include alanine, beta-alanine,serine, glutamine, piperidine, choline, pyrroline hydroxycarboxylic acid, beta-alanine, alpha-aminobutyric acid, tyrosine, histidine, tryptophan, glutamic acid, phenyllananine, lactic acid ,threonine, carnitine, Hypoxanthine, guanine, guanosine, trimethylamine N-oxide, and methionine. They all possess high predictive power and are useful stratification tool in risk assessment of OSCC.(66)

The discussion on the use of saliva as a diagnostic fluid for oral squamous cell carcinoma (OSCC) brings forward several important points and challenges that need attention for its effective application in clinical practice. Here's a breakdown of key areas to address:

Unexplored Aspects of Saliva's Diagnostic Potential

The potential of saliva as a diagnostic tool is acknowledged, yet the specific mechanisms through which salivary components interact with cancer cells are not fully explored. Research should focus on how salivary biomarkers such as proteins, RNA, or other molecules reflect tumor biology. Detailing the pathways through which these biomarkers originate and how they are influenced by the tumor microenvironment could provide clearer insights into saliva's diagnostic capabilities for OSCC.

Variability in Salivary Content

Saliva composition can vary significantly due to factors like diet, time of day, or oral hygiene, which affects the reliability of using it for diagnostics. This variability should be addressed more comprehensively. For example, studies should explore how external factors impact biomarker levels and what protocols can standardize these variations. Further research could establish best practices for saliva collection (e.g., fasting conditions, time of day) and preparation (e.g., centrifugation methods) to minimize these inconsistencies.

Challenges in Salivary Sample Collection and Processing

A significant issue in salivary biomarker research is the lack of standardization in sample collection, processing, and storage. As mentioned, different studies use varying centrifugation protocols and saliva collection times, leading to inconsistent results. Standardized guidelines should be developed for collecting saliva at consistent times (e.g., morning samples) and using uniform centrifugation and storage protocols. For instance, determining the optimal centrifugation speed (e.g., 2000 g for 10 minutes) and preservation methods, such as using protease inhibitors, will ensure consistency in research outcomes.

Variability in Salivary Biomarker Levels

The wide variability in biomarker levels between cancerous and non-cancerous individuals poses a challenge. For example, biomarkers like IL-6 and IL-8 show large differences in reported levels across studies, complicating their clinical application. This variability may stem from differences in ethnicity, dietary habits, age, and other factors. Research should focus on identifying a reference range for these biomarkers that accounts for inter-subject and intra-subject variability. Large-scale, multi-ethnic studies could help establish reliable baseline levels for these markers in diverse populations.

Validation of Biomarkers in the Presence of Other Conditions

Salivary biomarkers often show elevated levels in patients with non-cancerous oral inflammatory conditions like periodontitis or oral lichen planus

(OLP), leading to false positives. To improve diagnostic specificity, future studies should focus on validating biomarkers exclusively in OSCC patients, excluding those with other oral inflammatory diseases. Markers such as IL-6, IL-8, and others need to be studied in these contexts to understand how non-cancerous conditions affect their levels.

Proteomic Biomarkers and Their Clinical Relevance

The section on proteomic biomarkers would benefit from a clearer presentation of how these markers correlate with clinical outcomes, such as OSCC staging or prognosis. For example, detailing how changes in specific biomarker levels, such as IL-6, correlate with tumor progression or treatment response, would make the information more clinically useful. Additionally, redundant discussions of biomarkers like IL-6 and IL-8 should be streamlined and synthesized into a more cohesive argument, highlighting their relative importance.

Diagnostic Utility and Sensitivity of Salivary Biomarkers

While the diagnostic potential of saliva for OSCC is promising, more detailed evidence regarding the sensitivity and specificity of these biomarkers is necessary. Quantitative data demonstrating how these biomarkers perform in clinical settings could provide a stronger case for their utility. For instance, statistical data comparing the sensitivity and specificity of various salivary biomarkers in detecting OSCC could guide clinicians in selecting the most reliable ones.

Emerging Technologies for Biomarker Detection

Technological advancements such as next-generation sequencing (NGS) and microarray technology are briefly mentioned but lack context. A more detailed discussion on how these technologies improve the detection of transcriptomic biomarkers could enhance understanding. For example, comparing NGS's ability to identify novel RNA biomarkers with the limitations of older methods like qPCR would underscore the advantages of newer techniques in saliva-based diagnostics.

Ethical and Regulatory Considerations

The paper does not discuss the ethical and regulatory challenges of using saliva for OSCC diagnosis. Issues such as patient privacy, especially concerning genetic biomarkers, and the pathway for regulatory approval (e.g., FDA approval) for salivary diagnostics should be included. Addressing these issues would provide a more comprehensive perspective on the hurdles to clinical implementation.

Future Directions

The discussion on future directions is minimal. Including information on ongoing clinical trials or emerging technologies, such as the development of point-of-care diagnostic tools for saliva analysis, could offer a more forward-looking perspective. Moreover, exploring potential solutions to the challenges of biomarker variability and standardization would be beneficial.

CONCLUSION

Saliva holds significant promise as a non-invasive diagnostic tool for OSCC, but the challenges of variability in biomarker levels, lack of standardization, and validation in the presence of other oral conditions need to be addressed. Standardizing collection and processing methods, defining reference ranges, and improving diagnostic specificity through large-scale studies are critical steps toward clinical implementation. Additionally, more comprehensive discussions on emerging technologies, ethical considerations, and future research directions would further strengthen the case for salivary biomarkers in OSCC detection.

OSCC is a malignant carcinoma of oral cavity and clinically manifested as dysplastic oral changes along with genetic aberrations in cancer cells. Due to high mortality rate, it is needful to establish non-invasive, rapid and reliable diagnostic approach. In current era of modern technology, salivary biomarkers have gained immense popularity as novel diagnostic markers in OSCC. Nevertheless, few issues have been evolved that need to be sorted out in order to facilitate their applicability as a powerful highly disease sensitive and specific biomarkers. On account of this, implementation of additional research studies is advised to scrutinize the standardization protocols for saliva collection,

storage and processing. Moreover, the validity of salivary biomarkers and their potential role in OSCC diagnosis should also be taken into consideration. Research with scientific based evidences should be carried out to wipe off related dubious facts that undermine the accuracy and validity of salivary biomarkers in OSCC detection. This approach can accentuate the clinical application of salivary biomarkers in oral cancer diagnosis and management.

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