Application of salivary noncoding microRNAs for the diagnosis of oral cancers

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1 INTRODUCTION

Oral cancer is a late presenting disease linked to a high mortality rate over 5 years. GLOBOCAN estimates that lip, oral cavity, and pharyngeal cancers can be attributed to 5,295,000 new cases and 292,300 deaths worldwide. This accounts for 3.8% of all cancer cases and 3.6% of cancer deaths.1 It is well established that the treatment of early-stage oral cancers achieves higher survival rates with less morbidity. The 5-year survival rate for patients with oral cancer whose disease is detected early is 70%. This is compared to 37% 5-year survival rates in cases of late
diagnosis. These statistics are unfavorable but also define the solution: finding an effective clinical biomarker assay for the early detection of oral cancer will save lives.

The treatment of advanced oral cancers is often associated with high morbidity as it affects organs critical in basic functions such as speech and swallowing. It is also a commonplace that patients requiring surgery undergo major facial reconstruction, which greatly impacts appearance and quality of life. The standard diagnosis is often made by a tissue biopsy, which can cost in the range of USD $1000-$3000. Individuals from lower socioeconomic backgrounds and countries with inaccessible health care systems may not have this choice. Furthermore, it is estimated that patients with advanced oral cancer incur three times more in treatment and care costs, than patients with early-stage cancer.

Saliva is a water-based bodily fluid containing trace amounts of inorganic elements and heterogenous populations of biological particles. Saliva is primarily secreted by three major salivary glands (parotid, submandibular, and sublingual), with an extensive blood-filtering process occurring within highly specialized glandular cells. Post-filtering, an exchange occurs between the circulatory system and saliva, which explains how molecules that are present in plasma are also present in saliva. It is for this reason saliva has been termed the “mirror to the body,” as it reflects local and systemic conditions.

Saliva is needed to maintain oral health, homeostasis, beginning the process of digestion, and provide lubrication and protection for the upper gastrointestinal tract from abrasion and wear. This medium contains important enzymes and has a pH level protecting the oral cavity from disease by inhibiting the growth of bacteria and viruses. Over the last decade, advancement in transcriptomic techniques, quantitative PCR, array analysis, and genomic sequencing has led to an interest in utilizing transcriptomic techniques, quantitative PCR, array analysis, and genomic sequencing has led to an interest in utilizing saliva as a diagnostic tool.

This oral fluid has an abundance of biomarker materials including hormones, interleukins, proteins, and ribonucleic acids (RNA) subclasses. Globally, the common saliva-based biomarkers are hormones which are used for cortisol monitoring. Saliva can also be used for infectious disease testing and substance abuse detection.

Though saliva has been widely suggested as a tool for screening, as it would be easily collected from a broader population, there are no commercially used or reliable U. S. Food and Drug Administration-approved saliva-based diagnostic methods. This review focuses on the research to-date for noncoding RNA (ncRNA) as saliva-based biomarkers in oral cancer. We review the current published approaches in saliva-based RNA methodology and put forward a set of suggested guidelines to standardize methods in collecting and analyzing salivary biomarkers.

2 | THE NONCODING RNA FAMILY

Of the RNA family, ncRNAs are the most abundant type of RNA, equating to around 98% of transcriptional outputs in mammalian cells. These ncRNAs have been widely described as being stable in body fluids, ostensibly protected from RNA degradation, and are classified on the basis of transcript size; small ncRNAs are less than 200 nucleotides and long ncRNAs are more than 200 nucleotides in length. Among all ncRNAs, small ncRNAs are the most investigated and widely described ncRNAs in saliva. Particularly, microRNAs (miRNAs) are an RNA subclass of short, noncoding sequences (19-23-nucleotide long, single-stranded RNA molecule). These miRNAs act as regulators for a diverse range of physiological functions, significantly playing a role in oncogenesis and tumor progression (Figure 1). Their expression in circulation may be indicative of a cancer phenotype and are touted as potential pathological tools for low-invasive cancer staging and prognosis. MicroRNAs have been shown to be stable in blood circulation, but are also resistant to various environmental conditions. Considering that oral cancers occur in the oral cavity, there is a likelihood that potential disease markers may drain into this fluid. To this end, recent studies have suggested the application of salivary miRNAs as biomarkers for cancer diagnosis.

A major advantage of using saliva is the ease of collection, as it is noninvasive and requires no extra costs for trained personnel or complex procedures. There are, however, some caveats; saliva has low RNA abundance, and a small sample collection is common to patients with oral cancer. Of note, saliva contains highly fragmented mRNA and an abundance of bacterial content. Despite these challenges, several groups have investigated the miRNA population within saliva.

3 | CHARACTERIZATION OF SALIVARY miRNAs

Weber and colleagues examined the presence and distribution of miRNAs in 12 human body fluids, including saliva. Notably, saliva, breast milk, and seminal fluid had the highest number of miRNA species. Interestingly, hsa-miR-509-5p, hsa-miR-515-3p, and hsa-miR-335 were among the most abundant miRNAs found and shared among the different fluids, which suggests a common origin or function. In other studies, hsa-miR-223, hsa-miR-191, hsa-miR-16, hsa-miR-203, and hsa-miR-24 were found to be the five most abundantly expressed miRNAs in saliva. Of note, miRNAs in these saliva samples were
found to be stable for 48 hours at room temperature despite the enzymatic nature of saliva.\textsuperscript{17-20} The salivary RNA transcriptome has also been measured in both whole saliva and cell-free supernatant (CFS) using RNA-Seq.\textsuperscript{16} Whole saliva, the unaltered fluid obtained immediately after expectorating, contained more microbial sequences due to the higher bacterial content. In contrast, salivary supernatant obtained through centrifugation had more human transcripts. Although there is a preference to use CFS to reduce microbial RNA, a higher sequencing depth combined with an efficient PCR should also reduce the impact of these microbial RNAs. Other ncRNA families such as piwi-interacting RNAs, circular RNAs, and the transfer RNA-derived RNA fragments were shown to be present in the CFS of saliva.\textsuperscript{21,22}

\textbf{FIGURE 1} The micro-RNA (miRNA) biogenesis pathway. In brief, miRNAs are transcribed by RNA polymerase II to produce the primary miRNA strand. These structures typically contain a hairpin which is then cleaved by Drosha in the nucleus to produce a precursor miRNA. This precursor is shuttled to the cytoplasm whereby it undergoes further processing. The enzyme Dicer cleaves the precursor miRNAs to produce the double-stranded RNA duplex (miRNAs). This miRNA is then loaded onto Ago2 which is part of the RISC unit. This complex separates the two strands of the miRNAs leaving the sense or “guide” strand bound to Ago2. Using the guide strand, complementary binding occurs at the 3’UTR, which results in gene regulation via translation inhibition [Color figure can be viewed at wileyonlinelibrary.com]

\textbf{4 | CURRENT STATUS: SALIVARY miRNAs IN ORAL CANCER}

The accumulated evidence clearly supports the use of miRNAs for diagnosis, but to date, this has not been a clinical reality. For oral cancer, tissue-based profiling has been heavily invested in by both researchers and companies (as reviewed by Troiano et al\textsuperscript{23}). Implementing an miRNA tool into clinical practice has not been effectively disseminated, although many expression signatures have been identified. A direct example is the numerous public domain studies reporting associations between miRNA biomarkers and oral cancer.\textsuperscript{24-39} These studies derived from profiling solid tumors indicate miRNAs as highly stable in tissue. The discovery of blood-based or circulating miRNAs has expanded the search for other diagnostic mediums utilizing miRNAs. Thus, the vital question is can we harness the presence of miRNAs in saliva as a biomarker assay for the diagnosis and prognosis of patients seen with oral cancer?

Saliva-based miRNAs are ideal biomarkers as they can be readily collected without the need of specialized medical equipment.\textsuperscript{40} Due to the direct contact between saliva and the oral cancer lesion, it is possible that disease-related concentration changes of saliva components may provide better clues than systemic blood samples.\textsuperscript{41} Saliva constituents, including proteins \textsuperscript{41-45} and RNAs,\textsuperscript{46} have been profiled in multistage cohorts and several miRNAs such as hsa-miR-187, hsa-miR483-5p, and hsa-miR-9 are
<table>
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<td>Unstimulated whole saliva — Volume not stated</td>
<td>mirVana miRNA isolation kit (Ambion Inc)</td>
<td>NA</td>
<td>qRT-preampqRTPCR (TaqMan)</td>
<td>No</td>
<td>Pre-Amp qRT-PCR (TaqMan)</td>
<td>U6 snRNA</td>
<td>hsa-miR-200a, hsa-miR-125a</td>
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<td>Whole saliva — Collected from floor of mouth after simple mouth rinse with water (5 mL)</td>
<td>mirVana PARIS miRNA isolation kit (Ambion Inc)</td>
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<td>qRT-PCR (TaqMan)</td>
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<td>Not stated</td>
<td>miR-16, hsa-miR-31</td>
<td>Mann-Whitney, Wilcoxon, linear regression, ROC analysis</td>
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<td>Yang et al</td>
<td>miRNA expression in precancerous oral lesions</td>
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<td>n = 22; 8 progressing LGD leukoplasias; 7 nonprogressing LGD leukoplasias; 7 healthy volunteers</td>
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<td>RNeasy Micro Kit (Qiagen)</td>
<td>Nanodrop ND-1000</td>
<td>TaqMan MicroRNA Assay (Applied Biosystems)</td>
<td>No</td>
<td>qRT-PCR (TaqMan)</td>
<td>RNU6</td>
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<td>NucleoSpin miRNA Kit (Qiagen)</td>
<td>NA</td>
<td>miScript miRNA PCR arrays (Qiagen)</td>
<td>No</td>
<td>qRT-PCR (SYBR Green)</td>
<td>SNORD96A</td>
<td>hsa-miR-9, hsa-miR-191, hsa-miR-134</td>
<td>Double delta CT, normalization (using SNORD96A), Mann-Whitney U test, ROC analysis</td>
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<td>Momen-Heravi et al</td>
<td>miR expression in OSCC, OSCC-remission, OLP</td>
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<td>n = 34; 9 OSCC; 8 OSCC-remission; 8 OLP; 9 healthy controls</td>
<td>Whole saliva (unstimulated) — Maximum 8 mL</td>
<td>RNeasy Kit (Qiagen)</td>
<td>Nanodrop ND-2000</td>
<td>NanoString nCounter miRNA Assay (NanoString Technologies)</td>
<td>No</td>
<td>qRT-PCR (TaqMan)</td>
<td>miR-191</td>
<td>hsa-miR-27b</td>
<td>One-way ANOVA, two-tailed Mann-Whitney U test, ROC analysis</td>
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<td>Zahren et al</td>
<td>miRNA expression in OSCC; PMD</td>
<td>Salivary supernatant (Centrifuged at 2500g x 10 min)</td>
<td>n = 100; 20 clinically healthy controls; 40 patients with oral PMDs; 20 with confirmed PMD that had not transformed to OSCC over a least a 3-year period (dysplastic lesions); 20 with PMD with nondysplastic lesions</td>
<td>Whole saliva — Volume not stated</td>
<td>microRNA isolation kit (Qiagen)</td>
<td>Nanodrop ND-1000</td>
<td>qRT-PCR (SYBR Green)</td>
<td>No</td>
<td>Not stated</td>
<td>SNORD68</td>
<td>hsa-miR-21, hsa-miR-34, hsa-miR-145</td>
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</table>
already proposed as diagnostic markers for oral cancer.\textsuperscript{46-48} There is, however, limited studies of oral cancer biomarkers in saliva and their use for patient management.\textsuperscript{49} A summary of the current methodologies and clinical studies is provided in Tables 1 and 2, respectively.

The first investigation into salivary miRNAs found significantly lower levels of hsa-miR-125a and hsa-miR-200a in oral cancer when compared to healthy controls.\textsuperscript{18} This study set the foundation to explore the potential of using salivary miRNAs as diagnostic markers in oral cancers. Lui et al.\textsuperscript{50} reported that hsa-miR-31 was upregulated by 10.2-fold in patients with oral cancer before surgery compared with levels after resection. Interestingly, levels of hsa-miR-31 were more abundant in salivary supernatant than in plasma. Of note was that patients with oral verrucous leukoplakia showed no difference in expression to the control group. In precancerous oral lesions, hsa-miR-10b, hsa-miR-145, hsa-miR-99b, hsa-miR-708, hsa-miR-181c, hsa-miR-30e, hsa-miR-660, and hsa-miR-197 were all upregulated compared to controls.\textsuperscript{51} However, these profiles were measured using stimulated saliva through the use of mouthwash, and it has been suggested that salivary stimulants can promote the release of nonassociated miRNA species from the salivary glands.\textsuperscript{52}

Several studies have investigated whole saliva, identifying hsa-miR-9, hsa-miR-191, and hsa-miR-134.\textsuperscript{54} In contrast, profiles using salivary supernatant from patients with oral cancer identified hsa-miR-27b as having the highest sensitivity and specificity when discriminating patients with oral cancer from the other groups. Notably, the expression of plasma and serum hsa-miR-27b was significantly reduced in patients with oral cancer. This may support the idea that oral cancer cells directly secrete specific miRNAs into the oral cavity. Other studies using salivary supernatant indicated that hsa-miR-21 and hsa-miR-184 are significantly higher in patients with oral cancer and in oral potentially malignant disorders (OPMDs) than in healthy controls. In contrast, hsa-miR-145 was significantly lower in oral squamous cell carcinoma and OPMDs.\textsuperscript{53} Receiver operating characteristic (ROC) analysis demonstrated sensitivity of 65%, 60%, and 80% and specificity of 65%, 70%, and 75%, for these three miRNAs, respectively.\textsuperscript{55} The recent study validated low expression of hsa-miR-139-5p in 50 saliva samples. ROC analysis with this miRNA discriminated between two groups of patients: asymptomatic and pretreatment patients vs postoperative patients, with a value of 0.713.\textsuperscript{56}

From all these studies, there is no general consensus regarding which miRNAs are suitable as biomarkers. Much of this variation is due to the different laboratory practices in collection, processing, and detection of these miRNAs. These are the major obstacles for the application for any salivary-based miRNAs. Standardization coupled with automation will go toward eliminating
<table>
<thead>
<tr>
<th>Authors</th>
<th>Total no. of patients in cohort</th>
<th>No. of patients with HNSCC</th>
<th>No. of control specimens</th>
<th>Other cohorts included and no. of specimens</th>
<th>miRs of interest</th>
<th>Upregulated/Downregulated (compared to healthy controls)</th>
<th>AUC</th>
<th>AUC characteristic scale: Fail-Excellent&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Combined AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<td>Momen-Heravi et al&lt;sup&gt;51&lt;/sup&gt;</td>
<td>34</td>
<td>9</td>
<td>9</td>
<td>8 OSCC remission, 8 OLP</td>
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<td>Zahren et al&lt;sup&gt;52&lt;/sup&gt;</td>
<td>100</td>
<td>20</td>
<td>20</td>
<td>20 PMD with dysplasia, 20 PMD without dysplasia, 20 RAS</td>
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Abbreviations: AUC, area under the curve; HNSCC, head and neck squamous cell carcinoma; LGD, low-grade dysplasia; OLP, oral lichen planus; OSCC, oral squamous cell carcinoma; OVL, oral verrucous leukoplakia; PMD, potentially malignant disorder; TSCC, tongue squamous cell carcinoma.

<sup>a</sup>In accordance with the suggestions from Hanley and McNeil.<sup>54</sup>
variation between laboratories and errors associated with manual handling of the specimen. The field of salivary miRNA biomarker assays should adopt standardized guidelines for specimen collection, processing saliva, and miRNA isolation techniques.

5 | A FRAMEWORK FOR STANDARDIZING miRNAs ISOLATION AND DETECTION FROM SALIVA

There are three forms of collection which are consistently used in the major studies.

a. Typically, unstimulated CFS is the cleanest choice for RNA extraction. Collection is achieved by asking the patient to remain seated with no food or stimulating material, such as mouthwash, for at least 30 minutes prior. The patient is asked to clean their mouth with 3-5 mL of sterile water for 30 seconds, either swallowing it or spitting it out. After pooling saliva in their mouth, the patient or volunteer can then expectorate into a specimen container where upon it is snap frozen. The saliva is centrifuged at 2600g for 15 minutes at 4°C to remove any cells and other debris, and the supernatant is obtained, ready for RNA analysis. Studies which have used this method include Park et al.,18 Liu et al.,50 Momen-Heravi et al.,57 Zahran et al.,53 and Duz et al.56

b. The second collection method uses unstimulated whole saliva, which is not desirable. Similar to unstimulated CFS, saliva is collected without the use of stimulating materials and is pooled in the mouth.

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**FIGURE 2** Procurement of saliva samples:
recommendations for collecting saliva for RNA-based biomarker research

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**Procurement of Saliva Samples**

- No eating, smoking, drinking or mouthwash activity from participant for 1-hour prior.
  
  If collecting from field site, provide questionnaire, particularly to identify whether participant has inflammatory issues or chronic conditions which need to be noted.

- Allow participant to sip 10-15 mLs of distilled water (let participant know they can swallow or spit it out).

- Wait 3-5 minutes, then encourage patients to tilt their heads slightly forward and allow drool to fall into a 50ml sterile tube. Collect 3-5mLs.

- Snap freeze on dry ice. Keep in storage at -20 to -80 degrees Celsius for subsequent processing.

**Notes:**
Reduce specimen stimulation and immediate freezing of saliva.

Collection of the samples is the most vulnerable step in RNA biomarker analysis. If samples are not processed as listed, the change in protocol must be noted and later determined whether it was a confounding factor.
The patient is then asked to expectorate into a specimen container for immediate snap freezing. Whole saliva is processed without centrifugation; however, studies have noted the high presence of microbial RNA within whole saliva that has negative impacts on the sensitivity of human RNA detection.16 Studies which used this method include Park et al.18 and Salazar et al.54

The last approach is to collect stimulated whole saliva. Chemical stimulation of saliva production may contaminate the oral cavity and alter the desired biological population of interest. Only one study51 used this particular method.

Considering all the above, our recommendations for collecting saliva for miRNA biomarker research are summarized in Figure 2.

6 | EXTRACTING RNA FROM SALIVA

There are various approaches to isolating miRNAs ranging from liquid base guanidine isothiocyanate to silica columns.22 All have merits, but a consensus must be reached, ensuring consistency between different cohort studies. Table 1 refers to methodologies used by various research papers. Consistency of RNA extraction is important to eliminate study discrepancy and bias. It has also been shown that silica columns do not efficiently bind RNAs smaller than 200 nucleotides.58

With this in mind, our laboratory has adopted a liquid-based guanidine isothiocyanate approach for the isolation of all RNA species from biofluids. We have optimized this protocol to deliver the highest yield of RNA from bodily fluids.59 Recent studies have shown that RNA profiles from noninvasive fluids such as saliva do in fact exhibit large variations in the RNA content and integrity.60 Whole saliva has elevated but inconsistent levels of RNA content, similar to whole blood. Both mediums, which have high numbers of cells and bacterial content, are at risk of false RNA quantification due to contamination with bacterial cells. Although RNA can be visualized by a distinct peak at 260 nm, due to the low RNA content from saliva, the common profile exhibit is a flat reading over the visible spectrum (Figure S1). The suggested protocol is shown in Figure 3.

7 | LESSONS IN SALIVA ASSAY DEVELOPMENT

Several publications elucidated the role of miRNA transcripts deregulated in the general field of head and neck squamous cell carcinomas.18,34,37,61-63 Following this, numerous articles reported the accuracy levels at which

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**FIGURE 3** Proposed method to standardize salivary RNA extraction for micro-RNA analysis

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<table>
<thead>
<tr>
<th>Salivary miRNA Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) All RNA extraction must be done at 4°C</td>
</tr>
<tr>
<td>2) Thaw whole saliva sample on ice and vortex for 5 seconds</td>
</tr>
<tr>
<td>3) Distribute whole saliva sample (3-5 ml) into multiple 550 μL aliquots</td>
</tr>
<tr>
<td>4) Each 550 μL sample can now be further processed for RNA analysis</td>
</tr>
<tr>
<td>5) Centrifuge the entire sample at 2,600g for 15 minutes at 4°C</td>
</tr>
<tr>
<td>6) Pellet will have formed (Refer to Supplementary Figure 1A)</td>
</tr>
<tr>
<td>7) Without disturbing pellet remove 500μL of supernatant</td>
</tr>
<tr>
<td>8) Add 100μL of Trizol LS (1.5ml) and 100μL BAN to the supernatant. Mix thoroughly</td>
</tr>
<tr>
<td>9) Spin at 12,000g in a pre-spun phase-lock tube at 4°C</td>
</tr>
<tr>
<td>10) Carefully remove supernatant and add to cold 100% isopropanol and 5μg (50mg/mL) of glycogen. Invert the tube and store overnight in -20°C</td>
</tr>
<tr>
<td>11) The next morning, spin at 12,000g for 20 minutes</td>
</tr>
<tr>
<td>12) A lightly coloured pellet will have formed on the lower bottom side of the tube (Refer to Supplementary Figure 1B)</td>
</tr>
<tr>
<td>13) Carefully remove supernatant</td>
</tr>
<tr>
<td>14) Centrifuge at maximum g for 5 minutes at 4°C</td>
</tr>
<tr>
<td>15) Carefully remove supernatant</td>
</tr>
<tr>
<td>16) Wash pellet twice with cold 70% ethanol (Centrifuge for 10 minutes at 10,000g at 4°C between washes)</td>
</tr>
<tr>
<td>17) Suspend in 10μL of RNase free water</td>
</tr>
<tr>
<td>18) Store 9μL of sample in -80°C and utilise 1μL for Nanodrop quantitation (Refer to Supplementary Figure 1C)</td>
</tr>
</tbody>
</table>

**Notes:**
- The 3-5mL of saliva used for processing can be combined in Step (17) after each isolation has been quantified on a Nanodrop. This will increase the concentration for later use.
- Following RNA extraction, once the required samples have been isolated for the microfluidics chip (Bioanalyser) it is important to immediately run the Integrity check prior to the first freezing.
- Specimen thawing must be minimised.

**RNA integrity**
- Always quantitate sample using a Bioanalyser in preparation for downstream miRNA transcriptomic analysis (qPCR or Deep Sequencing).
  a) Use 1-2μL of sample as per Agilent instructions
  b) Analyse the report on the population and size of microRNAs
  c) Reassess for suitability in any downstream application
circulating miRNA biomarkers can precisely characterize cancer states in a diagnostic or prognostic capacity. Rationally, it can be expected that a suite of identified saliva miRNAs is of greater appeal than current techniques, as routine saliva samples are the only requirement for the diagnosis. However, there does not appear to be any consistency with recent studies unable to identify a set of miRNAs for oral cancer diagnosis. This is in part due to the various methods employed at the collection, processing, and detection of these miRNAs.

Adding to these concerns is the difficulty associated with normalizing low miRNA expression in bodily fluids. Although global normalization and reference genes dominate the reporting of qPCR experiments, difficulties are encountered when utilizing samples with low RNA concentrations. Two approaches that are currently reported in the literature include the standardization of qPCR data-handling pipelines and the use of LinRegPCR. Using an iterative algorithm, LinRegPCR determines baseline fluorescence and then creates a window of linearity (W-o-L) for each miRNA target. Following this, it calculates the PCR efficiency per sample. The algorithm also calculates the Cq value and the starting concentration per sample. Utilizing LinRegPCR to measure the constant amplification efficiency in all samples is vital. This allows the user to accurately measure any changes in miRNA expression without normalization of the data to an external housekeeping gene or global normalization. The consolidation of a universal miRNA extraction and detection pipeline will directly impact our ability to translate these miRNAs into the clinic. It is to date the biggest challenge in the field.

### 8 | DISCUSSION AND CONCLUSION

Biomarker assays are important clinical tools for improving the survival rate of patients with head and neck squamous cell carcinoma. Traditionally, tissue, blood, and urine were the preferred mediums for cancer detection. However, the rapid development of genomics and our increased understanding of molecular pathways have broadened the use of biomarkers to ncRNAs. As oral cancers originate in the oral cavity, it may be possible that saliva contains specific biomarkers reflective of this disease. Saliva has been widely suggested as a tool for screening, as it would be easily accessible for a broader population. Given the rising global incidence of oral cancers and the increased use of tobacco and alcohol, there is a requirement to develop better diagnostic tools.

Early detection is paramount for increasing survival, but most patients are seen with advanced TNM stage III or IV tumors. Conventional oral examination (COE) for many decades has been the most widely used and accepted screening method for oral cancers. There are some caveats with COE; the physical examination cannot identify all oral premalignant lesions and cannot predict which lesions will progress to cancer. As the test is conducted by trained individuals, the performance and diagnostic accuracy of COE may be dependent on clinical experience. The discovery and use of salivary miRNAs as a standardized test would add value for the early detection of this cancer. It may be that COE used in combination with a salivary test can increase the early detection rate of these cancers. Beyond the scope of early detection, these miRNAs could also be utilized as markers for disease monitoring, progression, and recurrence.

Disease monitoring may have scope through the use of saliva-based miRNA biomarkers. These miRNAs of interest are evaluated before and after treatment, and during patient follow-up. Salivary hsa-miR-31 was found to be significantly increased in patients with oral cancer at all clinical stages, including very early stages. It was shown to be more abundant in saliva than in plasma, and after tumor surgical removal, its expression was reduced. However, the researchers found no difference between miRNA levels in premalignant lesions and healthy controls, indicating that further studies with larger cohorts are required to elucidate the status of hsa-miR-31 expression in precancerous stages. Despite this, salivary hsa-miR-31 remains a promising marker for early detection and postoperative follow-up and has potential for use in disease monitoring.

The set of salivary miRNAs identified in our review are different between dysplasia and cancer indicating that there are specific miRNA signatures which can denote the progression of the precancerous lesions. Yang et al demonstrated that salivary miRNAs were differentially expressed between low-grade dysplasia (LGD) and high-grade dysplasia (HGD) leukoplakia. This would suggest that specific miRNA patterns can be used to follow disease progression from LGD to HGD which would result in early detection and clinical intervention.

Clinical markers which can predict disease recurrence are needed to effectively treat patients with oral cancer. Approximately 30% to 40% of patients develop recurrent locoregional cancer, whereas between 20% and 30% will develop recurrent metastatic disease. Our review did not identify any salivary miRNAs which have been investigated as markers of recurrence. However, studies using whole blood were able to define cutoff points to determine disease recurrence. In solid tumor studies, hsa-miR-194 and hsa-miR-99 displayed a significant correlation with local recurrence and progression-free survival. As salivary miRNA research is just in its infancy, we believe there is scope to further investigate salivary miRNAs as...
markers of recurrence. This would require longitudinal studies to monitor patients over 3-5 years combined with RNA sequencing to identify these miRNAs.

Our review offers a summary of the current state of salivary miRNAs and its potential applications to oral cancer. There are, however, limited studies in the area of salivary markers. We found that each study adopted a different methodology for processing and measuring salivary miRNAs. This fact alone makes it very difficult to compare studies and determine the accuracy of these reported miRNA biomarkers. This lack of standardization in saliva processing will affect the reproducibility of results. It is clear that standardizing saliva biomarker methodology is paramount for this field of research. Toward this end, we have presented a proposed guide for the collection and processing of miRNAs in saliva. The adaptation of a universal and common methodology would reduce the variations found in all current studies and bring us closer to discovering potential salivary miRNAs for use as clinical tools for oral cancer diagnosis.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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