'SEARCH LESS, VERIFY MORE' – REVIEWING SALIVARY BIOMARKERS IN ORAL CANCER DETECTION

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ABSTRACT

Oral squamous cell carcinoma is one of the commonest head and neck malignancies with approximately 350,000 cases reported annually and a mortality rate of 50% often attributed to late clinical presentation. Due to the close relationship between saliva bio-fluid and tumour lesions, optimizing salivary biomarkers for disease detection and screening provides a major new research

direction in diagnostic oral oncology. As inter- and intra-tumour heterogeneity are common within oral cavity neoplasms, it is unlikely that a single diagnostic or 'risk-stratifying' saliva biomarker will suffice for universal translation to clinical practice. Therefore, this article highlights a number of promising saliva biomarker combinations for oral cavity cancer detection that require further research and validation to determine their true diagnostic potential.

Keywords: oral squamous cell carcinoma; biomarkers; saliva

Introduction

The oral cavity is one of the most accessible regions of the aerodigestive tract, yet early diagnosis of oral squamous cell carcinoma (OSCC) remains a global challenge.[1] The diagnostic process relies on symptomatic patient presentation, frequently associated with an advanced stage of disease progression, leading to increased treatment morbidity and reduced survival rates (<40%).[1,2] Early screening for suspicious oral lesions is an important strategy to improve disease outcome and reduce mortality. Opportunistic screening, which requires simple, cost-effective, valid and reproducible tests with minimal morbidity, remains elusive, however.[3] Histopathologic examination of tissue biopsies, the current diagnostic 'gold standard', is inappropriate as a screening tool due to its invasive nature, cost implication and technique sensitivity. In addition, it requires appropriate recognition of identifiable mucosal precursor lesions, potentially malignant disorders (PMD), to direct biopsy sampling.[4,5] While no consensus regarding screening exists, conventional oral examination remains the most pragmatic method as it is simple and inexpensive, although concerns including subjectivity, validity, and inability to detect occult disease have fuelled the search for diagnostic adjuncts.[6,7]

With the understanding that molecular derangement precedes clinically recognizable lesions, together with advances in molecular biology, research into the identification of OSCC-specific markers in body fluids has intensified. Within the last decade, several candidate biomarkers have been discovered and investigated to determine diagnostic efficacy, with considerable numbers of publications appearing in the literature.[8] It is doubtful, however, that any one biomarker invariably recognises OSCC due to numerous carcinogenic mechanisms, tumour heterogeneity, and substantive risk factor variation. Combinations of biomarkers, therefore, are more likely to deliver improved diagnostic validity. This article reviews existing salivary diagnostic biomarker panels that need further validation and, in addition, highlights single agent disease indicators with potential for future application in multiplex panels.

Saliva as a Premium Biofluid

Saliva, a hypotonic multifunctional biological fluid, is an excellent source for measuring disease-related biomolecules. The non-invasiveness and relative ease of collection, minimal cost, ease of handling and limited expertise required make saliva a preferred screening medium for intraoral and systemic conditions, and prognostic monitoring.[9-11] While only 0.5% of saliva comprises electrolytes, proteins, nucleic acids and epithelial cells in health, molecules exuded from apoptotic

or necrotic tumour cells result in a detectable increase in suspended-molecules; some may be exclusive to malignancy whilst others reference disease-related dysregulation. The application of high-throughput techniques to bio-fluid diagnostics, such as next-generation sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has refined the potential use of whole and gland-specific saliva by identifying several biomarker groups under the term – Salivaomics.[12] Such techniques identify numerous biomarkers, with only those with most potential selected for preliminary validation in clinical diagnostics. Requiring 72 hours laboratory processing, SaliMark[TM] OSCC (PeriRx LLC, Broomall, PA, USA) is one of the few commercially available molecular diagnostic test to predict which oral lesions require scalpel biopsy and histopathologic examinations. With continued developments to optimize the diagnostic potential of saliva, it is hoped that improved efficiency will intensify application to disease stratification, better predict PMD progression and quantify the risk of malignant transformation in high-risk cases.

DNA-based Biomarker Combinations

Gene promoter hypermethylation panels

DNA hypermethylation of cytosine-phosphate-guanine (CpG) within promoter regions of tumour suppressor genes (TSG), proapoptotic genes or DNA repair mechanisms represent early events in OSCC carcinogenesis.[13-15] Following identification of hypermethylated cancer-related genes in saliva samples,[16] subsequent studies have sought to detect other candidate regions with high methylation status; most exhibit low sensitivity (SE) or specificity (SP) however.[17-18] Nagata et al reported upon a quadruple biomarker panel involving promoter hypermethylation of *ECAD, MGMT, RARB and TMEFF2* genes using methylation-specific polymerase chain reaction (MS-PCR) and high-throughput microchip electrophoretic analysis, although the effect of risk factor variability on panel methylation expression remains unclear.[19] More recently, another four-candidate TSG hypermethylation panel of *p16[INK4a], RASSF1A, TIMP3* and *PCQAP/MED15* was shown to yield diagnostic validity parameters above 90%.[20] Though initial trials of similar candidate gene combinations using MS-PCR in head and neck squamous cell carcinoma (HNSCC) yielded lower SE and SP values (71 – 87%), gene hypermethylation panels may be more useful for OSCC discrimination especially in the absence of HPV infection.[21-23] *TIMP3*, the only overlapping candidate gene utilized in studies by Nagata et al[19] and Linayage et al[20], was aberrantly methylated

in 31% and 80% of samples respectively, reflecting ethnic variation in the epigenomic landscape and requiring further validation in different population sub-groups. This may also assist in the verification of tissue-identified methylation markers such as *FLT4, MLH1, DAPK, DCC* and *CCNA1* in saliva.[24-26]

Another promising group of epigenome-based markers in saliva detect unusually methylated genomic loci encoding microRNAs (mgmiRs). Following recognition that miR-137 and miR-193a were downregulated in OSCC cell lines, due to tumour-specific methylation of their genomic loci,[27] similar aberrant promoter hypermethylation of *MIR-375, MIR-200a, MIR-200c-141* and *MIR-137* have been observed in saliva.[28] Cao et al[29] used a seven-member mgMIR panel (mgmiR9-1, mgmiR124-1, mgmiR124-2, mgmiR124-3, mgmiR129-2, mgmiR137, and mgmiR148a) to investigate their use in oral cavity cancer diagnosis (Table 1).

Genome somatic mutation panels

Somatic loss-of-function mutation to TSGs leads to widespread genomic instability underpinning oral carcinogenesis. The ability to identify cell-free tumour DNA within body fluids and their use in delineating tumour heterogeneity has renewed interest in the usefulness of somatic mutation markers.[30] Just as circulating tumour DNA may be used in surveillance and monitoring of treatment efficacy during cancer therapy, Wang et al proposed detecting somatic DNA mutations released as by-products of cell death from the tumour surface within saliva samples.[31] Though specific genomic alterations within individual tumours were matched to corresponding saliva samples, point mutations in one of *TP53, NOTCH1, PIK3CA*, and *CDKN2A* genes as well as translocation mutations in tumour DNA were observed in all OSCC saliva samples (Table 1). This established the hypothesis that OSCC lesions were more likely to shed remnant tumour DNA in saliva than plasma offering promise for early detection of malignant phenotype. While it is recognised that intra- and intertumoral genomic heterogeneity are common in OSCC, and that the mutational landscape will vary with ethnicity and risk factors,[32-33] it is imperative that future studies establish the salient gene alterations for saliva-based application.

mRNA Biomarker Combinations

Saliva coding transcriptomic markers are currently amongst the most frequently researched biomarkers, with the majority deriving from a heptad panel of *IL8, IL1B, H3F3A, DUSP1, OAZ1, S100P,* and *SAT* mRNAs; a number of potential marker algorithms have now been verified, as

summarized in Table 2.[34-37] mRNA markers have been found to be highly efficient and reproducible in OSCC discrimination, irrespective of population differences or assay technique, and may be especially useful when combined with other markers[38-41].

Recent studies have confirmed efficacy in stratifying 'suspect oral lesions' prior to establishing a definitive diagnosis, and demonstrate significant upregulation in OSCC. There is less specificity, however, in identifying epithelial dysplasia in PMD which may limit contemporary application; further investigations are clearly required[42,43].

It is unfortunate that no model is deemed universally appropriate nor are any dysregulation algorithms utilising all 7 mRNAs available, although *OAZ1, DUSP1* and *S100P* may be useful to distinguish lichenoid lesions from OSCC.[44] mRNA upregulation may not be specific to neoplasia, however, because dysregulation profiles have also been observed in chronic periodontitis, with only *S100P* appearing wholly discriminatory for OSCC[45-48].

Tissue and salivary transgelin mRNA levels, which are significantly higher in OSCC patients and demonstrate an inverse relationship with survival, together with upregulation of tissue integrin mRNA expression (*ITGA3, ITGA5* and *ITGB1*) may become relevant tools in the future.[49,50]

MicroRNA Biomarker Combinations

Derangement of noncoding regulatory RNA is seen during initiation, local invasion and subsequent metastasis in several malignancies. Confirmation of miRNA presence and stability in cell-free saliva, as tumour-suppressive (TS) miR-200a and miR-125a, suggests a possible role in OSCC recognition, although further studies are needed to justify clinical application[51-54].

Detectable dysregulation of miR-21, miR-31 and miR-24 oncogenic miRNAs in saliva have helped improve diagnostic panels[55-57]. Prominent amongst these is the exosomal-miRNA signature panel combining miR-21-5p and miR-24a-3p with TS let-7c-5p, miR-99a and miR-100-5p [54]. A binary prediction algorithm comprising individual marker or summative change has been proposed for clinical use, with recent verification confirming diagnostic efficacy with SE and SP between 84 to 89%, as shown in Table 2[58]. These biomarkers differentiated PMD from OSCC, did not vary with risk factor behaviour, and highlighted specific changes during malignant transformation[58].

Gai et al observed miR-302b-3p and miR-517b-3p to be exclusive OSCC biomarkers, whilst miR-412-3p and miR512-3p showed upregulation with significant disease discrimination properties[59]. Other candidate biomarkers with proposed diagnostic validity include oncomiR-27b, TSmiR-136, miR-122-5p, miR-124-3p, miR-146a-5p and miR-92a-3p. [60,61] . A miR-124-3p and miR-146a-5p combination may have ability to differentiate OSCC from other head and neck cancers. Similarly, potential panels such as miR-21-5p+miR-486-3p+miR-139-5p[62], miR-10b-3p+miR-181c+miR-708[63], and miR-129-2-3p+miR-204-5p+miR208b-3p+miR-3065-5p[64] may have value in OSCC diagnosis and PMD malignant transformation prediction.

Proteomic/Peptidomic Biomarker Combinations

Salivary proteases

ECM hydrolases, including A disintegrin and metalloproteinase (ADAM) and matrix metalloproteinases (MMPs), are effectors of malignant cell stromal invasion, tumour metastasis and angiogenesis[65,66]. Quantification using stable isotope standards and capture by anti-peptide antibodies with multiple reaction monitoring mass spectrometry (SISCAPA-MRM-MS) has shown excellent discriminatory power especially for MMP-1, justifying its inclusion in protein biomarker panels[67]. One combination involving MMP-1 as the primary biomarker included KNG-1, ANXA-2 and HSPA-5 as a bi-functional panel to facilitate early OSCC detection and PMD risk assessment; high sensitivity and specificity were recorded in Table 3[68]. In another recent study, MMP-1 was only seen in OSCC patients but when combined with proteins linked to endocrine-related malignancies (KLK-5, CTSV and ADAM-9) produced SE and SP values of 85% and 93% respectively[69].

Gelatinase-B (MMP-9) is another promising salivary protease marker with reported single SE and SP of 100% and 26.7% – 79%, respectively[70,71]. A recent meta-analysis of 9 diagnostic salivary biomarker studies concluded that MMP-9 and chemerin together possessed excellent diagnostic validity and constituted the most sensitive and specific OSCC panel yet[68,72-74].

Salivary antioxidants

Peroxiredoxin (PRDX-2) is an endogenous thiol-dependent antioxidant enzyme involved in the regulation of cell growth, differentiation and apoptosis; salivary PRDX-2 expression is increased in OSCC[75,76]. PRDX-2, ANXA-1 and ZA2G may thus function as putative markers for early

OSCC detection, irrespective of aetiology; Table 3[75-77]. Further verification is required to determine their discriminatory potential, however, especially as salivary PRDX-2 expression may increase in inflammatory oral and systemic disease.

Salivary cytokines and chemokines

There is evidence to suggest that various inflammatory mediator and chemo-attractant levels may be increased in OSCC, due to their protumourigenic effects of proteins via activation of RAS, JAK/STAT, TRADD, Wnt/β-catenin, and NF-κB signalling pathways, together with epigenetic silencing of TSG.[78-80] NF-κB-dependent inflammatory proteins such as IL-1β, IL-6, IL-8 and tumour necrotic factor-alpha (TNF-α) have been most studied, although signalling pathways leading to their activation in OSCC are also shared by inflammatory conditions such as chronic periodontitis and lichen planus raising questions regarding their specificity.[81-83] Nonetheless, recent research investigating the singular diagnostic power of IL-8 and TNF-α against leukoplakia and oral submucous fibrosis have reported AUC values of 0.97 and 0.99 respectively.[84-85] Though unestablished, multiplex cytokine assays in research conducted in disparate populations have suggested IL-6 as a high performer in detecting OSCC.[83,85-87] IL-8 and IL-1β have featured prominently in cross-biomarker panels with transcriptomic biomarkers[38-40], and this may more or less represent the current state of their potential as salivary diagnostic markers.

Single Protein Biomarkers for Future Consideration

Cytokeratin fraction 21-1 (CYFRA 21-1)

CYFRA 21-1 represents the soluble fragment of the acidic intermediate filament cytokeratin -19 (CK-19), released during apoptosis into the immediate milieu from epithelial malignancies. High salivary CYFRA 21-1 expression has been observed in OSCC, and positively correlated with CK19 mRNA expression.[88-89] Using protein immunoassay techniques, SE and SP values obtained for salivary CYFRA 21-1 have ranged from 83.6% – 93.8% and 84.3% – 95%, respectively.[4,89] Furthermore, the marker demonstrated good sensitivity and specificity in discriminating PMD and OSCC, as well as exhibiting significantly lower mean values in non-lesion high-risk individuals.[4,90]

Soluble CD44 (SolCD44)

CD44, a cell adhesion molecule, is a ubiquitous transmembrane glycoprotein normally expressed at basal and para-basal epithelial layers. Abnormal expression is common in dysplastic epithelia, and overexpression of its soluble counterpart released by MMP-aided lysis is observed during tumour progression and metastasis.[91] Promising diagnostic validity and specificity have been reported for OSCC, with SolCD44 applied prospectively to screen high-risk individuals.[92-95] Combinations with CD44 gene hypermethylation, cancer risk factor profiling and total protein levels have been found to enhance marker sensitivity, with possible roles suggested for predicting malignant transformation and identifying occult malignancy.[94,95]

Metabolomic Biomarker Combinations

Altered salivary metabolite levels in OSCC patients reflects preferential utilization of specific biochemical pathways by malignant epithelial cells. Metabolomic approaches to OSCC salivary diagnostics are recent innovations utilising high-throughput mass analyses to identify diagnostic panels including valine + phenylalanine + lactic acid and choline + betaine + pipecolinic acid + L-carnitine, as shown in Table 3.[96,97] In addition, the combination of priopionylcholine, N-acetyl-L-phenylalanine, sphinganine, phytosphingosine and S-carboxymethyl-L-cysteine also offer excellent sensitivity and specificity.[98] Recent investigations have proposed altered levels of s-adenosylmethionine + pipecolinic acid, and the combination of ornithine, o-hydroxybenzoate and ribose-5-phosphate may be early diagnostic markers for OSCC.[99,100] Other candidate markers such as lactate, proline, glycine, citrulline, inositol triphosphate, 2-oxoarginine and glycerate-2-phosphate have all been proposed as putative markers for future validation studies.[101,102]

Conclusions and Future Perspectives

This review has highlighted contemporary OSCC salivary biological marker panels that show promise as diagnostic tools, although further optimization and verification are certainly required. Selective expression and altered biomarker signatures in saliva, believed to be of diagnostic use for OSCC, may not be as heterogeneous as the underlying mechanisms involved in tumour initiation and progression. Whilst the discovery of new diagnostic biomarkers will undoubtedly continue, we propose systematic validation of already-identified panels to ascertain their full

clinical potential. Future work must endeavour to verify panels based on balanced diagnostic validity measures to avoid potential increases in false referral rates, unnecessary biopsies, patient anxiety, post-diagnosis non-compliance and treatment expense.

Conflict of interest: none.
Financial support: none.

Table 1: OSCC DNA-based salivary biomarker panels

Biomarker combinations	Population	Tumour type (by ICD-O classification)	Study design	Saliva sample type	Sample size	Control subject considerations	Molecular assay technique	Multicentre validation (YES/NO)	DAGNOSTIC VALIDITY			Other comments	Reference(s)
									SE[†] (%)	SP[‡] (%)	AUC[§]		
ECAD, TMEFF2, RARβ and MGMT	Japanese	C00 – C03, C05, C06	Case-control	Oral rinse	58	Healthy controls	Methylation specific PCR	NO	100	87.5	NR[¶]		[19]
p16[INK4a], RASSF1A, TIMP3 and PCQAP/MED15	Sri Lankan, Australian, French	C00 – C06	Case-control	Unstimulated saliva	114	Healthy controls	Methylation specific PCR	YES	91.7	92.3	0.92	Panel more effective for HPV-negative tumours	[20-23]
mgmiR9-1, mgmiR124-1, mgmiR124-2, mgmiR124-3, mgmiR129-2, mgmiR137, mgmiR148a	American	C00 – C06, C10, C32	Case-control	Unstimulated saliva	150	Diseased controls[††]	Quantitative methylation specific PCR	NO	NR	NR	NR	AUC value of 0.94 for disparate HNSCCs. Oral carcinoma proportion – 51%	[29]
TP53, PIK3CA, CDKN2A, NOTCH1 somatic mutations	American	C00 – C06	Cross-sectional	Unstimulated saliva and Oral rinse	46	None	Safe-Sequencing system PCR	NO	100	NA[‡‡]	NA	Proof-of-concept study for utilization of cell-free saliva tumour DNA	[31]

[†]SE – Sensitivity; [‡]SP – Specificity; [§]AUC – Area under the receiver operating characteristic curve; [¶]NR – Not reported, [††]Diseased controls include individuals with potentially malignant disorders, oral benign neoplasms, oral inflammatory lesions and conditions; [‡‡]NA – Not applicable, foundational study to confirm cell-free tumour DNAs in body fluids.

Table 2: OSCC RNA-based salivary biomarker panels

Biomarker combinations	Population	Tumour type (by ICD-O classification)	Study design	Saliva sample type	Sample size	Control subject considerations	Molecular assay technique	Multicentre validation (YES/NO)	DAGNOSTIC VALIDITY			Other comments	Reference(s)
									SE† (%)	SP‡ (%)	AUC§		
IL8, IL1B, H3F3A, DUSP1, OAZ1, S100P, and SAT	American, Serbian, Taiwanese, Indian, Greek	Unspecified; generic OSCC term used	Prospective and Retrospective Case-control, Cross-sectional, PRoBE¶ study	Unstimulated saliva	895	Healthy and diseased controls	Reverse transcriptase quantitative PCR	YES	Variable††	Variable	0.74 – 0.93	Markers are cost effective. Occasionally combined with demographic and risk factor history for better accuracy.	[37-44] [48]
miR-21-5p, miR-24a-3p, let-7c-5p and miR-100-5p	Australian	Unspecified; generic OSCC term used	Case-Control	Oral swirls	190	Healthy and diseased controls	Reverse transcriptase quantitative PCR	NO	86.8	81.5	0.87	Time efficient. Processing and analysis in <24 hours	[58]

†SE – Sensitivity; ‡SP – Specificity; §AUC – Area under the receiver operating characteristic curve; ¶PRoBE – Prospective-specimen-collection retrospective-blinded evaluation; ††Variation based on the subset candidate markers specific for population understudied.

Table 3: OSCC protein- and metabolite-based salivary biomarker panels

Biomarker combinations	Population	Tumour type (by ICD-O classification)	Study design	Saliva sample type	Sample size	Control subject considerations	Molecular assay technique	Multicentre validation (YES/NO)	DAGNOSTIC VALIDITY			Comments	Reference(s)
									SE[†] (%)	SP[‡] (%)	AUC[§]		
MMP-1, KNG-1, ANXA-2 and HSPA-5	Taiwanese	Unspecified; generic OSCC term used	Case-control	Unstimulated saliva	460	Healthy and diseased controls	Liquid Chromatography multiple reaction monitoring mass spectrometry	NO	87.5 - 93.4	80.5	0.91 – 0.93	Panel suggested to predict malignancy in PMD with a 77.8% PPV reported	[68]
CTSV, KLK-5, ADAM-9	Chinese	Unspecified; generic OSCC term used	Case-control	Unstimulated saliva	90	Healthy and diseased controls	Protease array and ELISA	NO	90	99.2	0.94		[69]
MMP-9, Chemerin	Egyptian	C01, C02, C04, C06	Case-control	Unstimulated saliva	45	Healthy and diseased controls	Sandwich ELISA	NO	93 - 100	80 - 100	0.88 - 1.00	Serum panel was also found to have high diagnostic discrimination between OSCC and controls	[72]
PRDX-2, ZA2G	Thai	C00 – C06	Case-control	Unspecified	175	Healthy controls	2-dimensional gel electrophoresis, liquid chromatography tandem mass spectrometry and Western blot	NO	100	98.8	0.99	Diagnose OSCC irrespective of viral aetiology. Tumour cells were also confirmed	[77]

												to have high individual protein expression	
Valine, phenylalanine, lactic acid	Chinese	Unspecified; generic OSCC term used	Case-control	Unstimulated saliva	103	Healthy and diseased controls	Ultra-performance liquid chromatography mass spectrometry	NO	86.5 – 94.6	82.4 – 84.4	0.89 – 0.97	Clear link between altered metabolite levels and specific cancer metabolic pathways was shown	[96]
Choline, betaine, pipecolinic acid, L-carnitine	Chinese	Unspecified; generic OSCC term used	Case-control	Unstimulated saliva	60	Healthy controls	Hydrophilic interaction chromatography-ultraperformance liquid chromatography mass spectrometry	NO	100¶	96.7¶	0.99¶	Better efficacy for early than late stage malignancy	[97]
Priopionylcholine, N-acetyl-L-phenylalanine, sphinganine, phytosphingosine and S-carboxymethyl-L-cysteine	Chinese	Unspecified; generic OSCC term used	Case-control	Unstimulated saliva	60††	Healthy controls	Hydrophilic interaction chromatography-ultraperformance liquid chromatography mass spectrometry	NO	100	96.7	0.99		[98]
S-adenosylmethionine, pipecolinic acid	Japanese	Unspecified; generic OSCC term used; two malignant melanoma cases included	Case-control	Unstimulated saliva	68	Healthy controls	Capillary electrophoresis time-of-flight mass spectrometry	NO	NR‡‡	NR	0.83	No difference in early and late stage disease detection.	[99]

| Ornithine, o-hydroxybenzoate, ribose-5-phosphate | American | Unspecified; generic OSCC term used | Prospective specimen collection case-control | Unstimulated saliva | 48 | Diseased controls | Capillary electrophoresis time-of-flight mass spectrometry | NO | NR | NR | 0.87 | Marker proposed to strictly different malignancy and epithelial dysplasia from other benign lesions | [100] |

[†]SE – Sensitivity; [‡]SP – Specificity; [§]AUC – Area under the receiver operating characteristic curve; [¶]Diagnostic validity only reported for discrimination between healthy controls and stages I and II malignancy; [††]Duplicate cohort used previously for marker verification; [‡‡]NR – Not reported

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