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Salivary biomarkers in early detection of oral cancer: A clinical research

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Abstract:

Early, non-invasive detection of oral squamous cell carcinoma remains a major clinical challenge. Therefore, it is of interest to evaluate the seven salivary biomarkers in 100 participants across oral cancer, OPMD and healthy groups. All biomarkers increased significantly from healthy controls to OPMD and oral cancer ($p < 0.001$). IL-6, CD44, defensin-1 and miR-31 showed excellent diagnostic accuracy, with miR-31 best identifying OPMD. Thus, salivary biomarker panels show strong potential for accurate, non-invasive early detection of oral cancer.

Keywords: Salivary biomarkers, oral squamous cell carcinoma, oral potentially malignant disorders (OPMD), interleukin-6, interleukin-8, CD44, microRNA-31, salivary cortisol, early detection, ROC curve analysis

Background:

Oral squamous cell carcinoma remains one of the most common malignancies worldwide and its prognosis is closely tied to the stage at diagnosis, with advanced-stage disease carrying substantially poorer survival than lesions identified early [1]. Screening currently relies on visual and tactile clinical examination followed by tissue biopsy, an approach that is effective once a lesion is clinically apparent but offers little support for detecting molecular change before overt tissue transformation [2]. Saliva bathes the entire oral mucosal surface and has therefore been proposed as a practical, non-invasive "liquid biopsy" medium for oral cancer detection, with inflammatory cytokines such as interleukin-6 and interleukin-8 among the most extensively studied candidate markers [3]. Beyond classical inflammatory mediators, additional salivary analytes have been linked to distinct facets of oral carcinogenesis. Soluble CD44, a marker associated with cancer stem-cell populations, has shown high specificity for distinguishing head and neck squamous cell carcinoma from benign disease [4]. MicroRNA-31, an oncogenic microRNA upregulated in oral tumor tissue, is detectable at increased levels in saliva even in small, early-stage tumors and some evidence suggests it may also mark oral potentially malignant lesions before frank malignant transformation [5]. Dysregulation of the hypothalamic-pituitary-adrenal axis, reflected in elevated salivary cortisol, has additionally been reported in patients with oral cancer and OPMD, potentially linking tumor-associated physiological stress to disease status [6]. Despite this growing body of single-marker evidence, comparatively few studies have evaluated a broad panel of salivary biomarkers together across the full clinical spectrum of healthy mucosa, OPMD and oral cancer within the same cohort [7]. Therefore, it is of interest to

evaluate the salivary IL-8, IL-6, and CD44, miR-31, cortisol, total protein and defensin-1 concentrations across these three diagnostic groups and evaluated their individual diagnostic performance for early detection.

Materials and Methods:

This cross-sectional, case-control clinical study was conducted at Miri Piri Institute of Medical Sciences and Research Centre after institutional ethics committee approval and written informed consent from all participants. Adult patients presenting for evaluation of an oral mucosal lesion, together with age- and sex-comparable individuals with no oral mucosal abnormality on examination, were consecutively screened. Participants were assigned to one of three diagnostic groups on the basis of clinical examination and confirmatory biopsy: oral cancer (Histopathological confirmed squamous cell carcinoma), OPMD/leukoplakia (Histopathological confirmed dysplasia or non-dysplastic leukoplakia), or healthy control (no clinically detectable oral mucosal lesion). Patients with prior head and neck radiotherapy or chemotherapy, active oral infection unrelated to the study lesion, or systemic conditions known to independently alter salivary composition (*e.g.*, uncontrolled diabetes, Sjögren syndrome) were excluded. One hundred eligible participants were enrolled (oral cancer, $n = 34$; OPMD/leukoplakia, $n = 33$; healthy control, $n = 33$). Baseline demographic and behavioral variables were recorded for all participants, including age, sex, smoking status, smokeless tobacco/areca (betel) nut use and regular alcohol use. For the oral cancer and OPMD groups, lesion site, clinical TNM stage (oral cancer only) and histopathological grade or dysplasia grade were additionally recorded. Unstimulated whole saliva (2-3 mL) was collected by the passive drool method between 8:00 and

10:00 a.m., at least one hour after eating, drinking, or oral hygiene procedures and prior to any cancer-directed treatment in the case groups. Samples were centrifuged and the supernatant stored at -80°C until batch analysis. Salivary IL-8, IL-6, soluble CD44, defensin-1 and total protein were quantified by enzyme-linked immunosorbent assay (ELISA); salivary cortisol was quantified by electrochemiluminescence/ELISA; and salivary microRNA-31 expression was quantified by quantitative reverse-transcription PCR and expressed as fold change relative to a healthy - control reference using the comparative Ct method. All assays were performed in a blinded fashion with respect to diagnostic group. Continuous data are expressed as mean $\pm$ standard deviation. Normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Between-group comparisons of continuous variables across the three diagnostic groups were performed using one-way ANOVA, with pairwise post-hoc comparisons by Welch's t-test and Bonferroni correction for multiple comparisons. Categorical variables were compared using the chi-square test. Diagnostic performance of each biomarker was assessed by receiver operating characteristic (ROC) curve analysis, with the area under the curve (AUC) and Youden-index-optimal sensitivity and specificity reported for the oral cancer-vs-healthy-control, OPMD-vs-healthy-control and oral cancer-vs-OPMD comparisons. The significance level was set at $p \leq 0.05$. All analyses were performed with IBM® SPSS® Statistics and Python (SciPy/scikit-learn).

Results:

Baseline demographic and behavioral characteristics of the 100 participants (34 oral cancers, 33 OPMD/leukoplakia, 33 healthy controls) are shown in **Table 1**. Age increased progressively across groups (healthy control 41.79 ± 8.55 years; OPMD 50.91 ± 8.22 years; oral cancer 58.24 ± 8.41 years; ANOVA $p < 0.001$), consistent with the age-related risk profile of oral cancer. Male sex, current smoking, smokeless tobacco/areca nut use and regular alcohol use were each significantly more prevalent in the oral cancer group than in the OPMD and healthy control groups (all $p \leq 0.03$), mirroring the established behavioral risk-factor gradient for oral malignancy. Among the 34 oral cancer patients, the buccal mucosa was the most common lesion site (16 patients,

47.1%), followed by the lateral tongue and gingivobuccal sulcus (6 patients each, 17.6%). Clinical staging showed a predominance of locoregionally advanced disease (stage II, 13 patients; stage III, 8; stage IV, 8) with only 5 patients (14.7%) presenting at stage I and histopathological grading showed moderately differentiated tumors in 16 patients (47.1%), well differentiated in 10 (29.4%) and poorly differentiated in 8 (23.5%). Among the 33 OPMD/leukoplakia patients, the buccal mucosa was again the most frequent site (20 patients, 60.6%); dysplasia grading showed moderate dysplasia in 13 patients (39.4%), mild dysplasia in 10 (30.3%), no dysplasia in 7 (21.2%) and Severe dysplasia in 3 (9.1%). Comparison of salivary biomarker concentrations across the three diagnostic groups is presented in **Table 2**. All seven biomarkers increased in a stepwise gradient from healthy control to OPMD to oral cancer (overall ANOVA $p < 0.001$ for each analyte). Pairwise post-hoc comparisons (Bonferroni-corrected) were significant for every biomarker across all three group pairs (oral cancer vs OPMD, oral cancer vs healthy control and OPMD vs healthy control; all $p < 0.02$), with the gradient most pronounced for IL-8, IL-6, CD44 and salivary miR-31 and comparatively more modest - though still statistically significant - between the oral cancer and OPMD groups for salivary cortisol ($p = 0.013$) and total protein ($p = 0.012$). Diagnostic performance of each biomarker, assessed by ROC curve analysis, is summarized in **Table 3**. For distinguishing oral cancer from healthy controls, IL-6 achieved the highest accuracy (AUC = 1.000), followed closely by CD44 (AUC = 0.996), defensin-1 (AUC = 0.992) and IL-8 (AUC = 0.983), with sensitivity and specificity each exceeding 0.94 at the optimal cutoff. For the clinically important comparison of OPMD/leukoplakia against healthy controls, salivary miR-31 performed best (AUC = 0.980, sensitivity 0.94, specificity 1.00), outperforming the inflammatory cytokines in this earlier, pre-malignant comparison. For distinguishing oral cancer from OPMD, IL-6 (AUC = 0.952), IL-8 (AUC = 0.923), CD44 (AUC = 0.922) and defensin-1 (AUC = 0.911) again showed the strongest discrimination, whereas salivary cortisol and total protein were comparatively weaker discriminators at this transition (AUC = 0.672 and 0.705, respectively).

Table 1: Baseline demographic and behavioral characteristics by diagnostic group (n = 100)

Characteristic	Oral Cancer (n = 34)	OPMD/Leukoplakia (n = 33)	Healthy Control (n = 33)	P value
Age (years), mean $\pm$ SD	58.24 $\pm$ 8.41	50.91 $\pm$ 8.22	41.79 $\pm$ 8.55	<0.001
Female sex, n (%)	6 (17.6)	12 (36.4)	16 (48.5)	0.027
Current smoker, n (%)	21 (61.8)	17 (51.5)	10 (30.3)	0.032
Tobacco chewing/areca nut use, n (%)	27 (79.4)	16 (48.5)	4 (12.1)	<0.001
Regular alcohol use, n (%)	15 (44.1)	11 (33.3)	3 (9.1)	0.005

Table 2: Salivary biomarker concentrations (mean $\pm$ SD) by diagnostic group IL, interleukin; CD44, cluster of differentiation 44

Biomarker	Oral Cancer (n = 34)	OPMD/Leukoplakia (n = 33)	Healthy Control (n = 33)	P value
IL-8 (pg/mL)	405.84 $\pm$ 142.38	181.84 $\pm$ 63.13	84.95 $\pm$ 36.25	<0.001
IL-6 (pg/mL)	65.46 $\pm$ 19.60	27.72 $\pm$ 9.34	10.80 $\pm$ 5.35	<0.001
CD44 (ng/mL)	15.15 $\pm$ 5.22	6.90 $\pm$ 2.71	3.89 $\pm$ 1.16	<0.001
Salivary miR-31 (fold change)	6.30 $\pm$ 2.58	3.43 $\pm$ 1.02	1.02 $\pm$ 0.30	<0.001
Salivary cortisol (ng/mL)	5.54 $\pm$ 1.89	4.37 $\pm$ 1.32	2.44 $\pm$ 1.03	<0.001
Total protein (mg/dL)	395.80 $\pm$ 91.79	335.69 $\pm$ 72.05	249.15 $\pm$ 60.97	<0.001

Salivary defensin-1 (ng/mL)	50.29 ± 14.20	24.80 ± 12.88	14.41 ± 6.28	<0.001
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Table 3: Diagnostic performance (ROC/AUC, Youden-index-optimal cutoff, sensitivity, specificity) of the leading salivary biomarkers for each pairwise diagnostic comparison. AUC, area under the curve; OPMD, oral potentially malignant disorder

Biomarker	Comparison	AUC	Optimal cutoff	Sensitivity	Specificity
IL-8	Oral cancer vs Healthy control	0.983	195.0 pg/mL	0.97	1.00
IL-6	Oral cancer vs Healthy control	1.000	22.3 pg/mL	1.00	1.00
CD44	Oral cancer vs Healthy control	0.996	5.73 ng/mL	0.97	0.97
Defensin-1	Oral cancer vs Healthy control	0.992	34.6 ng/mL	0.94	1.00
miR-31	OPMD vs Healthy control	0.980	1.49-fold	0.94	1.00
IL-6	OPMD vs Healthy control	0.940	21.6 pg/mL	0.76	1.00
IL-8	OPMD vs Healthy control	0.916	153.5 pg/mL	0.73	1.00
IL-6	Oral cancer vs OPMD	0.952	50.6 pg/mL	0.79	1.00
IL-8	Oral cancer vs OPMD	0.923	302.7 pg/mL	0.79	1.00
Defensin-1	Oral cancer vs OPMD	0.911	38.4 ng/mL	0.79	0.91

Discussion:

In this study, all seven salivary biomarkers examined rose in a consistent stepwise gradient from healthy mucosa through OPMD to invasive oral cancer, a pattern compatible with a field of progressive molecular change preceding and accompanying malignant transformation. Salivary IL-6 and IL-8 showed the strongest discrimination of oral cancer from healthy controls, consistent with prior reports that these cytokines are reproducibly elevated in the saliva of OSCC patients and contribute mechanistically to tumor angiogenesis and progression [8]. The near-perfect AUC observed for IL-6 in the present cohort is in line with the high diagnostic accuracy previously reported for salivary IL-6 mRNA and protein in oral cancer patients versus controls [9]. Soluble CD44 and salivary defensin-1 likewise showed excellent discriminatory performance, supporting earlier work identifying salivary solCD44 as a marker capable of detecting head and neck squamous cell carcinoma with high specificity across disease stages, reflecting the marker's association with cancer stem-cell populations [10]. Notably, salivary miR-31 was the strongest single discriminator of OPMD from healthy controls in this cohort, which is consistent with reports that salivary miR-31 is elevated even in very small, early oral tumors and may also mark premalignant change, positioning it as a particularly promising candidate for pre-clinical or screening-level detection rather than confirmation of already-established malignancy [11]. Salivary cortisol was also significantly elevated across the diagnostic spectrum, in keeping with reports of hypothalamic-pituitary-adrenal axis dysregulation and heightened psychological stress in patients with oral cancer and potentially malignant disorders [12]. However, cortisol and total protein showed comparatively weaker discrimination between oral cancer and OPMD than the inflammatory and stem-cell markers, suggesting these two analytes may better reflect a general disease or stress-associated state than the specific transition from premalignant to malignant disease. Taken together, these findings support the concept - echoed in recent systematic reviews - that no single salivary analyte is likely to serve as a standalone screening test, but that a panel spanning inflammatory, stem-cell, epigenetic and stress-related pathways may offer complementary and cumulative diagnostic value across the full spectrum from health to malignancy [13]. The

behavioral risk-factor gradient observed in this cohort - with smoking, smokeless tobacco/areca nut use and alcohol use all significantly more prevalent in the oral cancer group - mirrors the well-established epidemiology of oral cancer risk and is a useful internal validity check on the cohort, but it also means that some portion of the observed biomarker gradient could reflect exposure-related inflammatory or mucosal change rather than malignancy-specific biology per se; this is addressed further below [14-16].

Conclusion:

Salivary IL-8, IL-6, CD44, miR-31, cortisol, total protein and defensin-1 increased from healthy mucosa to OPMD and oral cancer. IL-6, CD44 and defensin-1 showed good accuracy for oral cancer, while miR-31 showed good accuracy for OPMD. These salivary biomarkers may aid non-invasive detection of OPMD and oral cancer.

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