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Evaluation of salivary circulating tumor DNA for early diagnosis and detection of oral cancer: A case-control study

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Abstract:
Oral squamous cell carcinoma (OSCC) is often diagnosed at an advanced stage, highlighting the need for non-invasive biomarkers for early detection. Therefore, it is of interest to evaluate the salivary circulating tumor DNA (ctDNA), TP53 mutation status and p16 promoter methylation in 100 participants, including 50 OSCC cases and 50 controls. Mean salivary ctDNA was significantly higher in OSCC cases than controls (50.54 ± 16.21 vs 8.99 ± 3.31 ng/µL, $p<0.001$), with an AUC of 0.9996. At a 20 ng/µL cut-off, ctDNA showed 84.0% sensitivity, 96.0% specificity and 90.0% diagnostic accuracy, while TP53 mutations and p16 methylation were significantly more frequent in OSCC cases. Thus, data support salivary ctDNA as a promising minimally invasive biomarker for OSCC detection and warrant validation in larger prospective cohorts, including premalignant lesions.

Keywords: Salivary circulating tumor DNA (ctDNA), oral squamous cell carcinoma (OSCC), liquid biopsy, TP53 mutation, p16 promoter methylation, case-control study

Background:
Oral squamous cell carcinoma (OSCC) accounts for the majority of oral cavity malignancies and remains a major cause of cancer related morbidity and mortality worldwide, with tobacco, alcohol and areca/betel nut use as its principal modifiable risk factors **[1]**. Despite being readily accessible to clinical examination, OSCC is frequently diagnosed at an advanced clinical stage, largely because early lesions are asymptomatic or clinically indistinguishable from benign mucosal change and because definitive diagnosis still depends on incisional tissue biopsy, an invasive procedure that is poorly suited to repeated screening or surveillance **[2]**. Because saliva is in direct and continuous contact with the oral mucosa, it is particularly well suited to capturing tumor-derived material shed from oral cavity lesions **[3]**. Circulating tumor DNA (ctDNA) is the tumor-derived fraction of cell-free DNA released into body fluids through apoptosis, necrosis and active secretion and it carries the mutational and epigenetic signature of the tumor of origin **[4]**. In OSCC, the tumor-suppressor gene TP53 is among the most frequently altered genes and salivary detection of tumor-derived TP53 alterations has repeatedly been shown to distinguish OSCC patients from healthy individuals **[5]**. Promoter hypermethylation of the tumor-suppressor gene p16 (CDKN2A) is a further well-characterized early epigenetic event in oral carcinogenesis and its detection in salivary DNA has been proposed as a complementary biomarker for OSCC **[6]**. A recent panel-based methylation approach has further supported the feasibility of combining tumor-suppressor gene methylation markers for the early detection of oral squamous cell carcinoma **[7]**. While individual salivary genetic and epigenetic markers have shown diagnostic promise, direct quantification of total salivary ctDNA concentration, evaluated alongside TP53 mutation and p16 methylation status within the same case-control cohort, has been comparatively less studied **[8]**. Therefore, it is of interest to evaluate the diagnostic accuracy of salivary ctDNA quantification, using a pre-specified concentration cut-off, together with TP53 mutation status and p16 promoter methylation status, for the early diagnosis of oral cancer in a case-control study design.

Materials and Methods:
Written informed consent was obtained from all participants prior to enrolment. One hundred participants were recruited and allocated to two groups of 50 each: the Case group comprised patients with a histopathologically confirmed diagnosis of oral squamous cell carcinoma and the Control group comprised age- and sex-comparable individuals with a healthy or clinically benign oral mucosa and no history of malignancy. For cases, clinicopathological data were additionally recorded, including anatomical site of the lesion, AJCC/UICC clinical stage and Histopathological grade of differentiation, greatest tumor dimension and regional lymph node status. Unstimulated whole saliva was collected from each participant under standardized conditions prior to any surgical or oncological intervention. Cell-free DNA was extracted from the saliva samples using a standard extraction protocol and the circulating tumor DNA fraction was quantified by fluorometry/qPCR and expressed in nanograms per microliter

(ng/μL). A pre-specified salivary ctDNA cut-off of 20 ng/μL was used to define index test positivity (ctDNA Test Result). TP53 mutation status was assessed by polymerase-chain-reaction-based mutation analysis of the extracted salivary DNA and reported as positive or negative. Promoter methylation status of p16 (CDKN2A) was assessed by methylation-specific PCR and likewise reported as positive or negative. The final histopathological diagnosis (oral squamous cell carcinoma versus normal mucosa/benign) served as the reference standard against which the salivary ctDNA test and molecular markers were evaluated. Data were tabulated in Microsoft Excel and analyzed using standard statistical software. Continuous variables are presented as mean ± standard deviation and were compared between groups using the independent-samples t-test. Categorical variables are presented as frequency and percentage and were compared using the chi-square test. The diagnostic accuracy of the salivary ctDNA test at the 20 ng/μL cut-off was expressed as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall diagnostic accuracy, each with 95% Wilson confidence intervals, along with positive and negative likelihood ratios. A receiver operating characteristic (ROC) curve was constructed for continuous salivary ctDNA concentration to evaluate its overall discriminative ability (area under the curve, AUC) and the Youden index was used to identify the statistically optimal cut-off for comparison with the pre-specified 20 ng/μL threshold. A p-value ≤0.05 was considered statistically significant.

Results:

A total of 100 participants were evaluated, comprising 50 histologically confirmed cases of oral squamous cell carcinoma and 50 controls. Cases were significantly older than controls (57.04 ± 8.38 years vs 50.10 ± 7.71 years, p<0.001). Although a higher proportion of males was observed among cases (37/50) than controls (27/50), this difference did not reach statistical significance (p=0.061). Tobacco use (76.0% vs 46.0%, p=0.004) and areca/betel nut use (70.0% vs 26.0%, p<0.001) were both significantly more prevalent among cases than controls, whereas alcohol use was comparable between groups (50.0% vs 40.0%, p=0.421). **Table 1** summarizes the baseline demographic and risk-factor profile of the two groups. Among the 50 oral squamous cell carcinoma cases, the tongue was the most common site of involvement (32.0%), followed by the gingivobuccal sulcus (20.0%) and buccal mucosa (18.0%). Clinical stage distribution was comparatively even across early and locally advanced disease, with stage II being the most frequent (28.0%). Moderately differentiated tumors predominated (46.0%) and regional lymph node involvement was present in exactly half of the cases (50.0%). Mean tumor size was 2.50 ± 1.08 cm. **Table 2** presents the detailed clinicopathological profile of the case group. Mean salivary ctDNA concentration was markedly and significantly higher in cases than in controls (50.54 ± 16.21 ng/μL vs 8.99 ± 3.31 ng/μL, p<0.001). TP53 mutations were detected in 56.0% of cases compared with only 4.0% of controls (p<0.001) and p16 promoter methylation was detected in 50.0% of cases compared with 4.0% of controls (p<0.001), confirming that both the quantitative ctDNA burden and the molecular alterations it carries were markedly enriched in oral cancer. **Table 3** summarizes these comparisons. Receiver operating characteristic analysis of continuous salivary ctDNA concentration showed near-perfect discrimination between cases and controls, with an area under the curve of 0.9996. The Youden-optimal cut-off (20.82 ng/μL; sensitivity 98.0%, specificity 100%) closely approximated the pre-specified clinical cut-off of 20 ng/μL, supporting the validity of the latter for routine use. At this pre-specified 20 ng/μL cut-off, the salivary ctDNA test correctly identified 42 of 50 cases as positive (true positives) and correctly classified 48 of 50 controls as negative (true negatives), with 8 false negatives and 2 false positives. This corresponded to a sensitivity of 84.0% (95% CI 71.5–91.7), specificity of 96.0% (95% CI 86.5–98.9), positive predictive value of 95.5% (95% CI 84.9–98.7), negative predictive value of 85.7% (95% CI 74.3–92.6) and an overall diagnostic accuracy of 90.0% (95% CI 82.6–94.5); the positive and negative likelihood ratios were 21.0 and 0.17, respectively. **Table 4** details the diagnostic performance characteristics of the salivary ctDNA test. On subgroup analysis within the case group, salivary ctDNA concentration did not differ significantly between early-stage (clinical stage I/II, 49.41 ± 15.62 ng/μL) and locally advanced-stage (clinical stage III/IV, 51.58 ± 16.99 ng/μL) disease (p=0.64), nor between lymph-node-positive (52.74 ± 16.71 ng/μL) and lymph-node-negative cases (48.34 ± 15.73 ng/μL, p=0.34). This indicates that salivary ctDNA elevation was already evident at the earliest clinical stage and was not dependent on nodal spread or advanced tumor stage. When TP53 mutation and p16 methylation positivity were combined into a single composite marker (either marker positive), the combined marker yielded a sensitivity of 78.0% and specificity of 92.0% in distinguishing cases from controls — slightly lower than the sensitivity and specificity achieved by quantitative ctDNA measurement at the 20 ng/μL cut-off.

Table 1: Baseline demographic and risk-factor profile of cases and controls

Variable	Cases (n=50)	Controls (n=50)	p-value
Age, years (mean ± SD)	57.04 ± 8.38	50.10 ± 7.71	<0.001
Sex, Male / Female	37 / 13	27 / 23	0.061
Tobacco use, n (%)	38 (76.0)	23 (46.0)	0.004
Alcohol use, n (%)	25 (50.0)	20 (40.0)	0.421
Areca / betel nut use, n (%)	35 (70.0)	13 (26.0)	<0.001

Table 2: Clinicopathological characteristics of oral squamous cell carcinoma cases (n=50)

Characteristic	n	%
Site of lesion		
Tongue	16	32.0
Gingivobuccal sulcus	10	20.0
Buccal mucosa	9	18.0
Floor of mouth	7	14.0
Palate	5	10.0
Retromolar trigone	3	6.0
Clinical stage		
I	10	20.0
II	14	28.0
III	13	26.0
IV	13	26.0
Histopathological grade		
Well differentiated	17	34.0

Moderately differentiated	23	46.0
Poorly differentiated	10	20.0
Lymph node status		
Positive	25	50.0
Negative	25	50.0
Tumor size, cm (mean ± SD)	2.50 ± 1.08	

Table 3: Salivary ctDNA levels and molecular marker positivity in cases versus controls

Parameter	Cases (n=50)	Controls (n=50)	p-value
Salivary ctDNA, ng/µL (mean ± SD)	50.54 ± 16.21	8.99 ± 3.31	<0.001
TP53 mutation positive, n (%)	28 (56.0)	2 (4.0)	<0.001
P16 promoter methylation positive, n (%)	25 (50.0)	2 (4.0)	<0.001

Table 4: Diagnostic performance of salivary ctDNA (cut-off = 20 ng/µL) for detection of oral squamous cell carcinoma

Diagnostic parameter	Value (%)	95% CI
Sensitivity	84.0	71.5 – 91.7
Specificity	96.0	86.5 – 98.9
Positive predictive value	95.5	84.9 – 98.7
Negative predictive value	85.7	74.3 – 92.6
Diagnostic accuracy	90.0	82.6 – 94.5
Positive likelihood ratio	21.0	—
Negative likelihood ratio	0.17	—
Area under the ROC curve (AUC)	0.9996	—

Discussion:

The present case-control study shows that quantitative salivary ctDNA measurement can discriminate oral squamous cell carcinoma from healthy/benign oral mucosa with a high degree of accuracy, supported by a near-perfect ROC-derived AUC and a Youden-optimal cut-off that closely matched the pre-specified clinical threshold of 20 ng/µL. These findings are consistent with the broader liquid-biopsy literature, in which salivary ctDNA, alongside other salivary biomolecules, has been highlighted as a promising, minimally invasive alternative to tissue biopsy for the detection, characterization and monitoring of oral squamous cell carcinoma **[9]**. TP53 is among the most frequently mutated genes in oral squamous cell carcinoma and its detection in saliva has long been proposed as a molecular marker of tumor presence. D'Cruz *et al.* first showed p53 exon 4 codon 63 mutations in the saliva of oral cancer patients at a substantially higher frequency than in saliva from healthy individuals **[10]** and this finding was later corroborated in an Indian cohort with a high prevalence of betel-quid and tobacco use, where the same salivary p53 alteration was detected in the great majority of oral squamous cell carcinoma cases **[11, 12]**. More recently, gene-panel sequencing of paired tumor and saliva samples confirmed that somatic tumor mutations, most commonly involving TP53, can be recovered from saliva with high sensitivity irrespective of tumor site or stage **[13, 14]**. The TP53 mutation positivity of 56.0% observed among cases in the present study, against a background of only 4.0% positivity in controls, is broadly consistent with this body of evidence, while the exact proportion detected in any single cohort is expected to vary with the specific gene regions interrogated, the mutation-detection assay used and the tobacco/areca-nut exposure profile of the study population. Promoter hypermethylation of p16 (CDKN2A) is a well-established early epigenetic event in oral carcinogenesis. Salivary p16 hypermethylation has previously been shown to be significantly more frequent in oral squamous cell carcinoma than in healthy controls **[15, 16].** A recent methylation-biomarker panel further reinforced the potential of combining tumor-suppressor gene methylation markers for early detection of oral tongue squamous cell carcinoma **[17, 18]**. The 50.0% p16 methylation positivity in cases may reflect the predominance of moderately and poorly differentiated tumors and high tobacco and areca-nut exposure. Quantitative ctDNA at 20 ng/µL outperformed combined TP53/p16 markers for sensitivity (84.0% vs 78.0%) and specificity (96.0% vs 92.0%), suggesting that tumor-derived DNA burden provides additional diagnostic information. Elevated ctDNA in stage I disease and the absence of significant stage-wise differences support its potential for early detection. The case profile was consistent with established tobacco and areca-nut risk factors for OSCC **[19, 20]**. However, the case-control design, modest sample size, single cut-off and absence of premalignant lesions limit generalizability and warrant prospective validation **[21, 22]**.

Conclusion:

Salivary ctDNA quantification at a 20 ng/µL cut-off showed high sensitivity, high specificity and excellent overall discriminative ability (AUC = 0.9996) for distinguishing oral squamous cell carcinoma from healthy/benign controls, with elevated levels evident even in early-stage disease and independent of lymph node status. TP53 mutation and p16 promoter methylation were both significantly more frequent among cases, further supporting the tumor-derived origin of the measured ctDNA. Thus, data support salivary ctDNA as a promising, minimally invasive adjunct for the early diagnosis of oral cancer, warranting validation in larger, prospective studies that include patients with oral potentially malignant disorders.

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