Article published online: 2022-08-22

Head and Neck Cancer

Significance of Fluorescent Spectroscopy in Screening Oral Potentially Malignant Disorders and Oral Cancer by Characterization of Salivary DNA Using Ethidium Bromide—A Comparative Study

Sadaksharam Jayachnadran[1,2] Aruna Prakasarao[3] Sangeetha Ramamoorthy[4] Yuvaraj Manoharan[3,5]

[1] Department of Oral Medicine and Radiology, Tamil Nadu Government Dental College and Hospital, Chennai, Tamil Nadu, India
[2] Tamil Nadu Dr. MGR Medical University, Chennai, Tamil Nadu, India
[3] Department of Medical Physics, College of Engineering Anna University, Chennai, Tamil Nadu, India
[4] Department of Dental Surgery, Government Hospital, Valangaiman, Thiruvarur District, Tamil Nadu, India
[5] Department of Biochemistry, SRM Arts and Science College, Kattankulathur, Chengalpattu, Tamil Nadu, India

Address for correspondence Sangeetha Ramamoorthy, MDS, Department of Dental Surgery, Government Hospital, Valangaiman, Thiruvarur District, Tamil Nadu 612804, India (e-mail: sangii03@gmail.com).

South Asian J Cancer 2023;12(2):159–165.

Abstract

Sangeetha Ramamoorthy

Background Optical diagnosis is one of the upcoming methods in screening and diagnosing oral cancer at the earlier stage. Currently, DNA-based diagnosis is used along with light-based imaging methods to diagnose oral cancer rapidly.

Aim The aim of this study was to discriminate oral cancer and oral potentially malignant disorders from normal patient with fluorescence spectroscopic characterization of salivary DNA using ethidium bromide dye.

Materials and Methods A total of 40 patients with average age of 20 to 60 years in both the genders were selected and divided into three groups. Group A included clinically proven cases of oral cancer, group B1 included clinically diagnosed cases of leukoplakia, group B2 included clinically diagnosed cases of oral submucous fibrosis, and group C included controls. Salivary DNA fluorescence spectrum obtained after adding ethidium bromide was analyzed using FluoroLog spectrophotometer at 480 nm wavelength.

Results The discriminant analysis of fluorescent emission of salivary DNA shows predictive accuracy of 90% between group C and group A, 95% between group C and group B1, and 65% observed between group C and group B2.

Conclusion From this study, screening of oral cancer can be done at the earliest with the help of fluorescence spectroscopic characterization of salivary DNA. This method can be done rapidly and noninvasively.

Keywords
- oral cancer
- salivary DNA
- fluorescence spectroscopy
- ethidium bromide

DOI https://doi.org/10.1055/s-0042-1750186 ISSN 2278-330X

How to cite this article: Jayachnadran S, Prakasarao A. Ramamoorthy S. et al. Significance of Fluorescent Spectroscopy in Screening Oral Potentially Malignant Disorders and Oral Cancer by Characterization of Salivary DNA Using Ethidium Bromide—A Comparative Study. South Asian J Cancer 2023;12(2):159–165.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Introduction

Oral cancer is the sixth most common cancer worldwide in which 95% are of squamous cell carcinoma.[1] Incidence of oral cancer varies globally. High incidence is reported in South Asian countries such as India and Sri Lanka. In India, oral cancer remains the major cause of mortality in both the males and females.[2] Mostly, oral squamous cell carcinoma (OSCC) is developed from oral potentially malignant disorder (OPMDs) such as leukoplakia (LPA) oral submucous fibrosis (OSMF) and erythroplakia, palatal lesions in reverse smokers, oral lichen planus, and oral lichenoid reactions.[3] The survival and quality of life of these high-risk people depend upon the detection of OPMD and OSCC at an earlier stage. Early diagnosis of OPMD and OSCC is the corner stone to provide prompt treatment and better prognosis.[4] The standard method to identify oral cancer is conventional oral examination followed by scalpel biopsy to confirm the diagnosis. Histopathological test is the gold standard method to confirm oral cancer that is an invasive procedure and requires adequate training.[5]

Light-based imaging of the tissues and biofluids detects slightest changes such as (i) cell microanatomy, e.g., nuclear/cytoplasmic ratio; (ii) redox status; (iii) expression of specific biomarkers; (iv) tissue architecture and composition; (v) chemical changes; and (vi) vascularity/angiogenesis and perfusion. These characters are appropriate to detect minimal (early) changes, for assessing the margins of lesions and possibly the presence of subclinical changes beyond the clinical margins, for repeated noninvasive monitoring of existing lesions, and for rapidly examining at-risk populations.[6] Among the various optical diagnostic methods, fluorescence is one of the most sensitive tools in exploring the subtle changes in tissue conditions by changes in its energy, wavelength, polarization, and direction.[7]

Many researchers have explored the diagnostic potential of fluorescence spectroscopy and imaging in the characterization of tissue and biofluids.[8–10] The altered metabolic function and pathological conditions induce significant changes in both biofluids and tissue samples of patients that were noticed from some of the intrinsic fluorophores such as the aromatic amino acids—phenylalanine, tryptophan, and tyrosine, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), pyridoxine derivatives, structural proteins like collagen, elastin and their cross-links, and endogenous porphyrins present in biofluids and tissues.[8,11,12]

DNA biomarkers specific to OSCC have been identified in saliva that can be used for diagnosis and prognosis.[13,14] Initially, isolation and detection of DNA present in saliva were done using electrophoretic technique and it was a precise method, but long and cumbersome technique. DNA present in the biofluids cannot be detected with fluorescence spectroscopy because of its low quantum yield but by introducing a fluorescent dye like ethidium bromide, DNA can intercalate with a dye and its fluorescent characteristics can be studied that can be used to discriminate disease. Jiang et al reported increased amount of mitochondrial DNA in head and neck cancer patients than normal patients based on quantitative polymerase chain reaction test.[15] From the literature, there was not much studies done to characterize DNA based on fluorescence spectroscopy to discriminate oral cancer and oral potentially malignant disorders. Yuvaraj et al studied about the fluorescence spectroscopic characterization of salivary DNA in oral cancer and normal patients and observed a significant spectral change between the groups.[16]

So, this current study focused to discriminate OSCC and oral potentially malignant disorders rapidly at the earliest with fluorescence spectroscopic characterization of salivary DNA using a fluorescent dye ethidium bromide.

Materials and Methods

Methodology

The study was approved by the Institutional Review Board (IRB Ref.No:7/IRB/2017). The study was performed under the Helsinki Declaration 2013. This was a collaborative study conducted at Department of Oral Medicine and Radiology, Tamil Nadu Government Dental College and Hospital, Chennai, where the patients were recruited and the Department of Medical Physics, Anna University, Chennai, provided the laboratory support. The total sample ($n = 40$) was divided into three groups in which group A ($n = 10$) included OSCC cases, group B1 ($n = 10$) included oral LPA cases, group B2 ($n = 10$) included oral submucous fibrosis cases, and group C ($n = 10$) included healthy controls. The study participants were clearly informed about the purpose of the study and verbal and written informed consent was obtained from all the participants.

Selection Criteria

Patients clinically diagnosed with OSCC, LPA, and oral submucous fibrosis were included in the study. The parameters for group A were presence of ulcer, ulceroproliferative raised lesion with induration, with or without palpable lymphnodes,[17,18] for group B1 presence of nonscrable, leathery white patch,[19] and for group B2 presence of stiff and blanched oral mucosa with palpable fibrotic bands and reduced mouth opening.[20]. Volunteers without any habits were included in the group C.

The immunocompromised patients and recurrent cases of OSCC and previously treated cases of oral submucous fibrosis and oral LPA were excluded from the study.

Saliva Collection

Whole unstimulated saliva was collected from all the participants during the morning hours and it was done before biopsy. All the participants were asked to refrain from eating, drinking any form of liquids or perform oral hygiene procedures for at least 1 hour prior to saliva collection. Prior to saliva collection, the participants were asked to rinse their mouth with 10 mL distilled water. Unstimulated whole saliva was collected and the participants were asked to spit approximately 5mL into a 40 mL sterile container. The saliva was subjected to fluorescence spectroscopic analysis within 2 to 3 hours and the excited emission spectrum was recorded.

Fluorescence Spectroscopic Characterization

The autofluorescence spectroscopic characterization of saliva was performed in spectrofluorometer model Fluorolog-3 (HORIBA JobinYvon, INC, Edison, New Jersey, United States). The light source is provided by a monochromator with 450W ozone free xenon lamp. The preferred excitation wavelength and the emission spectrum were determined by PC-controlled monochromator. All the functions of Fluorolog-3 are maintained by the Datamax software that communicates between a PC-compatible computer and Fluorolog-3. The whole saliva was taken in a cuvette and mounted in projection unit of the spectrometer. For salivary DNA fluoroscopic characterization, 100 µL of ethidium bromide dye is added to 1 mL of saliva. The steady-state fluorescence emission spectra were measured in the emission range from 500 to 750 nm at 480 nm excitation.

Statistical Analysis

The statistical analysis was done using IBM SPSS (IBM Corp. Released 2011.IBM Statistics for windows, version 20.0., IBM Corp, Armonk, New York, United States). The demographic variables such as age and gender were calculated. Descriptive statistics was performed for all the four groups. One-way analysis of variance (ANOVA) was done to compare the average fluorescence emission intensity of salivary DNA among the OSCC, OSMF, and oral LPA and healthy control groups. Post-hoc test of Bonferroni was done to find which group is significant from the other group for the average fluorescence emission intensity. The *p*-value was set as 0.005. Discriminant analysis was done to assess the predictive accuracy of the control and malignancy.

Results

Among the 40 patients, the mean age for group A, B1, B2, and C were 48, 41, 36, and 32 years, respectively. Group A included 8 males and 2 females, group B1 included 10 males, group B2 included 8 males and 2 females, and group C included 2 males and 8 females.

Excitation Emission Spectrum

The fluorescence of salivary DNA was obtained by adding the ethidium bromide dye that binds with DNA and showed a fluorescence emission. The saliva was excited at 480 nm and the fluorescence emission spectrum was recorded from 500 to 750 nm in 1 mm increments and the shape of the spectrum appeared similar in all groups with peak intensity at around 605 nm and the average fluorescence intensity was measured (►Figs. 1 and 2). The fluorescent intensity of normal group is lesser than the other three groups and the group B1 showed a maximum intensity (►Table 1).

One-way ANOVA test was done to compare the DNA fluorescence spectrum among the groups and showed a significant difference with *p*-value of 0.00 as shown in ►Table 2. Post-hoc Bonferroni multiple comparisons showed that the group A showed a significant difference with group C and group B1 and B2 with *p*-values of 0.00, 0.07, and 0.04 respectively. No statistically significant difference was observed between the group C and group B2 (►Table 3).

►Fig. 3 shows the scatter plot of discriminant score for group C and group A. From this analysis, it showed that the original cases and cross-validated cases were classified correctly with 90% of predictive accuracy. The classification is given in ►Table 4.

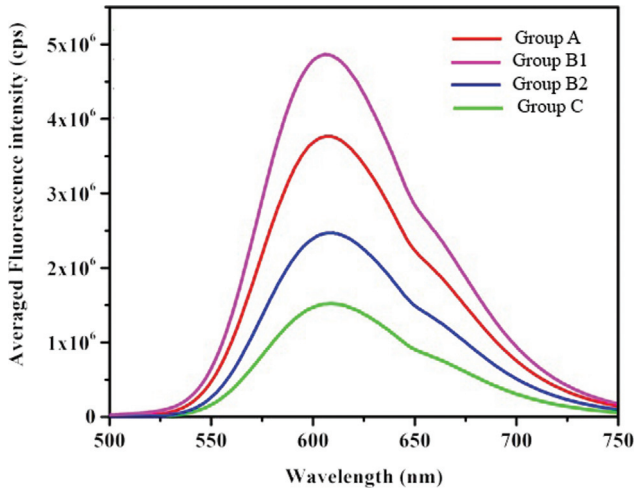

Fig. 1 Average fluorescence emission intensity of salivary DNA at 480 nm excitation. Group B1 and A showed a maximum intensity than group C.

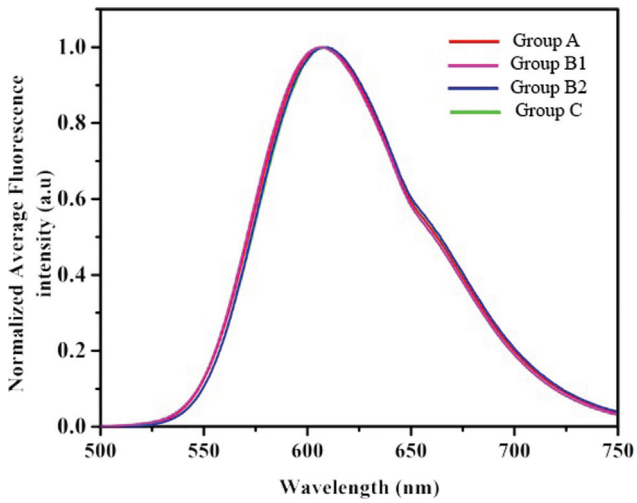

Fig. 2 Normalized fluorescence intensity of salivary DNA.

Table 1 Descriptive statistics (average fluorescence emission intensity in cycles per second of salivary DNA)

Variables	Groups	Mean	Standard deviation
Salivary DNA	Group A	3771135.50	616089.94
	Group B1	5369227.45	1203353.92
	Group B2	2472118.89	1210762.48
	Group C	1524847.56	874305.8

Table 2 One-way ANOVA test to compare the average salivary DNA fluorescence intensity among the groups

Group A Mean + SD	Group B1 Mean + SD	Group B2 Mean + SD	Group C Mean + SD	*p*-Value
3771135.5 ±	5369227.45 ± 1203353.92	2472118.8 ± 1210762.4	1524847.5 ± 874305.8	0.000

Abbreviations: ANOVA, analysis of variance; SD, standard deviation.

Table 3 Post-hoc Bonferroni test for multiple comparisons between the groups for salivary DNA

Dependent variable	Group	Group	Mean difference	*p*-Value
Salivary DNA	Control	Leukoplakia	−3844379.89	0.000
		OSMF	−947271.33	0.255
		OSCC	−2246287.94	0.000
	Leukoplakia	Control	3844379.89	0.000
		OSMF	2897108.55	0.000
		OSCC	1598091.95	0.007
	OSMF	Control	947271.33	0.255
		Leukoplakia	−2897108.55	0.000
		OSCC	−1299016.6	0.040
	OSCC	Control	2246287.94	0.000
		Leukoplakia	−1598091.95	0.007
		OSMF	1299016.6	0.040

Abbreviations: OSCC, oral squamous cell carcinoma; OSMF, oral submucous fibrosis.

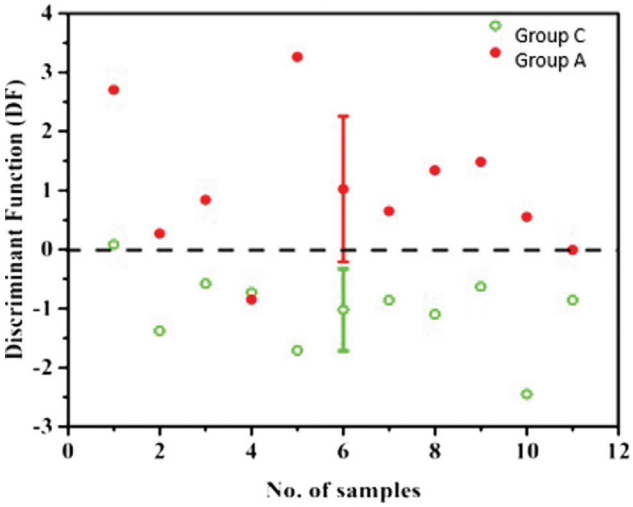

Fig. 3 Scatter plot showing the discriminant function of salivary DNA fluorescence between group C and group A.

The discriminant function of group C and group B1 is shown in ►**Fig. 4** and the classification is given in ►**Table 5**. This analysis showed that 95% of predictive accuracy as the original cases and cross-validated cases were correctly classified.

►**Fig. 5** shows the scatter plot of discriminant score for group C and group B2 and the classification is shown in ►**Table 6**. From this analysis, it is revealed that original and cross-validated cases were correctly classified with predictive accuracy of 65%.

Discussion

In spite of many advancements in the treatment for oral cancer, 5-year survival rate remains the same without any significant variation.[21] Currently, many research projects are mainly done to detect OSCC and OPMD at earlier stage based on optical diagnosis with salivary biomarkers. These salivary biomarkers play a noninvasive role in the diagnosis and inspection of OSCC and OPMDs making it a most sensitive and specific screening method in early detection, staging, and prognosis.[22] Molecular markers for the diagnosis of OSCC include initial changes in the cellular DNA, which result in altered mRNA transcripts, leading to altered protein contents.[23] Literature search reveals many in vitro and in vivo studies done on fluorescence spectroscopy and imaging based on the fluorophores like NADH, FAD, proteins, and protoporphyrin. Udhyakumar et al[11] studied about the normal and malignant tissues using native fluorescence and and time resolved fluorescence spectroscopy. They observed a statistically significant difference in the excitation spectra at 280nm between the normal and malignant tissues because of the fluorophore tryptophan. Vedeswari et al[24] studied autofluorescence intensity of pre- and post-treated patients with OSMF and reported variations in the intensity of fluorescence at 385 and 440 nm intensity region

Table 4 PCA–LDA for salivary DNA fluorescence group C and group A

		VAR00001	Predicted group membership		Total
			1.00	2.00	
Original[a]	Count	Normal	9	1	10
		OSCC	2	8	10
	%	Normal	90.0	10.0	100.0
		OSCC	20.0	80.0	100.0
Cross-validated[b,c]	Count	Normal	9	1	10
		OSCC	2	8	10
	%	Normal	90.0	10.0	100.0
		OSCC	20.0	80.0	100.0

Abbreviations: OSCC, oral squamous cell carcinoma; PCA, principal component analysis; LDA, linear discrimant analysis.
[a]85.0% of original grouped cases correctly classified.
[b]Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.
[c]85.0% of cross-validated grouped cases correctly classified.

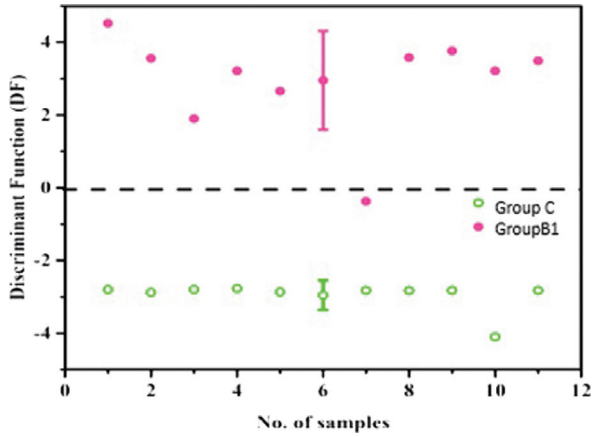

Fig. 4 Scatter plot showing the discriminant function of salivary DNA fluorescence between group C and group B1.

corresponding to NADH. The average fluorescence spectrum of the post-treated OSMF mucosa had a lesser intensity around 385 nm and a higher intensity around 440 nm than that of the pretreated OSMF mucosa, thereby mimicking the normal oral mucosa. Raja Pappu et al[12] studied about the in vivo native fluorescence spectroscopic characterization of oral tissue and saliva in normal patients and oral carcinoma patients and observed that diagnosis of cancer with the help of saliva has better sensitivity than tissue.[12]

DNA-based diagnosis is highly sensitive and accurate but isolation of DNA and analysis are tedious. Certain procedures like DNA microarrays and DNA amplification with polymerase chain reaction are highly laborious and expensive and time consuming. The intrinsic fluorescence of DNA is extremely weak because of its short decay times. Florescent bases can be used as a probe for DNA–protein interaction that can increase the quantum yield which in turn reveals the characteristics of the DNA.[25] Hoechst 33258 fluorochrome, ethidium bromide, and PicoGreen dsDNA quantitation reagent are some of the probes that can be used to study DNA fluorescence. Ethidium bromide dye is widely used as a probe in spectrometric analysis that strongly binds with nucleic acids and enhances its fluorescence which is also

Table 5 PCA–LDA for salivary DNA fluorescence between group C and group B1

		VAR00001	Predicted group membership		Total
			1.00	2.00	
Original[a]	Count	Normal	10	0	10
		LPA	1	9	10
	%	Normal	100.0	0.0	100.0
		LPA	10.0	90.0	100.0
Cross-validated[b,c]	Count	Normal	10	0	10
		LPA	1	9	10
	%	Normal	100.0	0.0	100.0
		LPA	10.0	90.0	100.0

Abbreviations: LPA, leukoplakia; PCA, principal component analysis; LDA, linear discriminant analysis.
[a]95.0% of original grouped cases correctly classified.
[b]Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.
[c]95.0% of cross-validated grouped cases correctly classified.

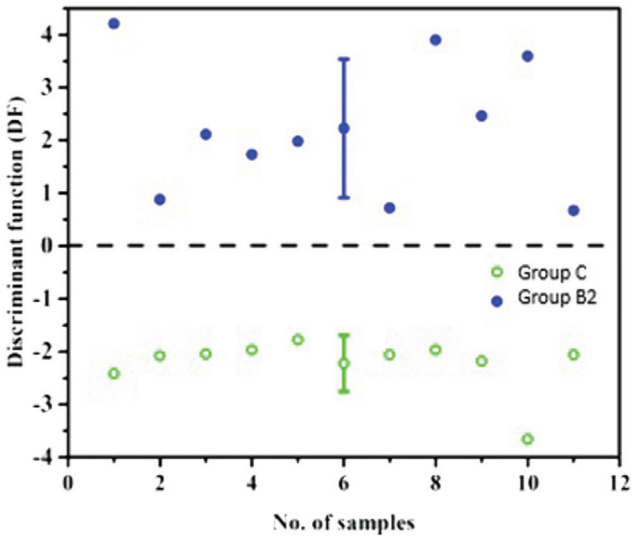

Fig. 5 Scatter plot showing the discriminant function of salivary DNA fluorescence between group C and group B2.

reported in many literatures.[26,27] Yuvaraj et al in his study observed that the fluorescence emission of ethidium bromide and DNA complex is better than the emission of ethidium bromide alone.[16] Based on these concepts, we structured this study to discriminate the OSCC and OPMDs with DNA fluorescence that is rapid, simple, and noninvasive and it can even identify a small fragment of DNA. Gallagher and Desjardins studied the quantification of DNA and RNA and mentioned that minimum DNA concentration of 1000 - ng/mL is required for ethidium bromide assay.[26]

In this study, the salivary samples are excited at 480 nm and emission spectrum is recorded from 500 to 750 nm and the maximum peak obtained in a range from 600 to 610nm. When compared with group C, other groups showed higher intensity in an ascending order of group C (1.6×10^6 cps)> group B2 (2.5×10^6 cps) > group A (3.7×10^6 cps) > group B1 (4.9×10^6 cps) as shown in ►Fig. 1. The maximum intensity is observed in LPA and OSCC because of the continuous cell proliferation that probably results in increased availability of DNA. The fluorescence intensity in OSMF is less than the OSCC but more than the normal, probably because of less availability of DNA. Quantification of DNA should have been done to validate these results. The results of our study are similar to the study done by Yuvaraj et al where they observed the excitation emission spectra of OSCC were higher than the normal.[13] Here in this study, we also included the oral potentially malignant disorders that also showed a higher fluorescence emission than normal.

The test results were analyzed statistically that showed a significant difference between group C and group A ($p = 0.00$) and also between group C and group B1 ($p = 0.00$). The discriminant analysis also showed a predictive accuracy of 95% for oral LPA, 90% for OSCC, and 65% predictive accuracy for oral submucous fibrosis.

Though we achieved a statistically significant results, the sample size was small. Further studies are required with larger sample size. Quantification of the DNA was not done that is necessary to provide accurate amount of DNA required for fluorescence spectroscopy studies. This pilot study may elucidate the potential of screening significance of fluorescence spectroscopy of salivary DNA using ethidium bromide.

Conclusion

In conclusion, the present study revealed that salivary DNA characterization with the help of fluorescent spectroscopy can be used to screen oral cancer and oral potentially malignant disorders at the earliest. It is a rapid, noninvasive method, accurate and used for mass screening. This is the first of its kind to assess the fluorescence spectroscopic characteristics of salivary DNA with ethidium bromide dye in OPMDs and OSCC. Statistically significant difference was

Table 6 PCA–LDA for salivary DNA fluorescence between group C and group B2

		VAR00001	Predicted group membership		Total
			1.00	2.00	
Original[a]	Count	Normal	10	0	10
		OSMF	0	10	10
	%	Normal	100.0	0.0	100.0
		OSMF	0.0	100.0	100.0
Cross-validated[b,c]	Count	Normal	10	0	10
		OSMF	0	10	10
	%	Normal	100.0	0.0	100.0
		OSMF	0.0	100.0	100.0

Abbreviations: OSMF, oral submucous fibrosis; PCA, principal component analysis; LDA, linear discriminant analysis.
[a]100.0% of original grouped cases correctly classified.
[b]Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.
[c]100.0% of cross-validated grouped cases correctly classified.

observed in OSCC and OPMDs from group C. However, further multicentric studies are required with more study samples, various age groups, different genders, different stages of OSMF, LPA, and OSCC, and various sites to validate our research findings.

Conflict of Interest

None declared.

References

1 Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009;45(4-5):309–316
2 IARC. 1 354 051 855. nternational Agency for Research on Cancer. 468, 2018–2019
3 Warnakulasuriya S. Clinical features and presentation of oral potentially malignant disorders. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125(06):582–590
4 Jaychandran S, Meenapriya PK, Ganesan S. Raman spectroscopic analysis of blood, urine, saliva and tissue of oral potentially malignant disorders and malignancy-a diagnostic study. Int J Oral Craniofac Sci 2016;2:11–14
5 Kaur J, Jacobs R, Huang Y, Salvo N, Politis C. Salivary biomarkers for oral cancer and pre-cancer screening: a review. Clin Oral Investig 2018;22(02):633–640
6 Wilder-Smith p, Holtzman J, Epstein J, Le A. Optical diagnostics in the oral cavity: an overview. Oral Dis 2010;16(08):717–28
7 Pradhan A, Pandey PK, Singh P. Overview of Fluorescence Spectroscopy and Imaging for Early Cancer Detection. Neurophotonics and Biomedical Spectroscopy. Elsevier Ltd; 2019. Doi: 10.1016/b978-0-323-48067-3.00012-3
8 Rekha P, Aruna PR, Ganesan S. Study on discrimination of oral cancer from normal using blood plasma based on fluorescence steady and excited state at excitation wavelength 280 nm. InOptical Biopsy XIV: Toward Real-Time Spectroscopic Imaging and Diagnosis 2016 Mar 7 (Vol. 9703, pp. 216–219). SPIE
9 Francisco ALN, Correr WR, Azevedo LH, et al. Fluorescence spectroscopy for the detection of potentially malignant disorders and squamous cell carcinoma of the oral cavity. Photodiagn Photodyn Ther 2014;11(02):82–90
10 Madhuri S, et al. Native Fluorescence Spectroscopy of Blood Plasma in the Characterization of Oral Malignancy 2003;78: 197–204
11 Udayakumar K, Yuvaraj M, Awad F, et al. Native fluorescence and time resolved fluorescence spectroscopic characterization of normal and malignant oral tissues under UV excitation - an in vitro study. J Fluoresc 2014;24(02):613–623
12 Pappu Raja, et al. Correlation of metabolites in saliva and in vivo tissue of oral cancer patients based on fluorescence spectral deconvolution. InOptical Biopsy XVIII: Toward Real-Time Spectroscopic Imaging and Diagnosis 2020 Feb 21 (Vol. 11234, p. 112341D). International Society for Optics and Photonics
13 El-Naggar AK, Mao L, Staerkel G, et al. Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implications in molecular diagnosis and screening. J Mol Diagn 2001;3 (04):164–170
14 Matthews AM, Kaur H, Dodd M, et al. Saliva collection methods for DNA biomarker analysis in oral cancer patients. Br J Oral Maxillofac Surg 2013;51(05):394–398
15 Jiang Wei-Wen. et al. Increased mitochondrial DNA content in saliva associated with head and neck cancer. Clinical Cancer Research 11.7 (2005):2486–2491
16 Yuvaraj M, Aruna P, Koteeswaran D, Tamilkumar P, Ganesan S. Rapid fluorescence spectroscopic characterization of salivary DNA of normal subjects and OSCC patients using ethidium bromide. J Fluoresc 2015;25(01):79–85
17 Burket. ORAL Medicine - Diagnosis and Treatment. 11th edition. BC Decker Inc; Hamilton 2008
18 Amin MB, Greene FL, et al. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more “personalized” approach to cancer staging. CA Cancer J Clin 2017 Mar; 67(2):93–99
19 van der Waal I, Schepman KP, van der Meij EH. A modified classification and staging system for oral leukoplakia. Oral Oncol 2000;36(03):264–266
20 Haider SM, Merchant AT, Fikree FF, Rahbar MH. Clinical and functional staging of oral submucous fibrosis. Br J Oral Maxillofac Surg 2000;38(01):12–15
21 Khan MSR, et al. Diagnosing oral squamous cell carcinoma using salivary biomarkers. Bangabandhu Sheikh Mujib Med. Univ. J. 2018;11:1
22 Shree KH, Ramani P, Sherlin H, et al. S. R. Saliva as a diagnostic tool in oral squamous cell carcinoma – a systematic review with meta-analysis. Pathol Oncol Res 2019;15(25):447–453
23 Markopoulos AK, Michailidou EZ, Tzimagiorgis G. Salivary markers for oral cancer detection. Open Dent J 2010;4:172–178
24 Vedeswari CP, Jayachandran S, Ganesan S. In vivo autofluorescence characteristics of pre- and post-treated oral submucous fibrosis: a pilot study. Indian J Dent Res 2009;20(03):261–267
25 Millar DP. Fluorescence studies of DNA and RNA structure and dynamics. Curr Opin Struct Biol 1996;6(03):322–326
26 Gallagher SR, Desjardins PR. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. Curr Protoc Protein Sci 2008;3(Appendix):4K. Doi: 10.1002/0471140864.psa04ks52
27 Olmsted J III, Kearns DR. Mechanism of ethidium bromide fluorescence enhancement on binding to nucleic acids. Biochemistry 1977;16(16):3647–3654

