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Autofluorescence Spectroscopy Augmented by Multivariate Analysis as a Potential Noninvasive Tool for Early Diagnosis of Oral Cavity Disorders

C. Venugopal, MDS,[1,*] Shaiju S. Nazeer, MSc, MPhil,[2,*] Anita Balan, MDS,[1] and R.S. Jayasree, PhD[2]

Abstract

Objective: Oral leukoplakia is one of the common potentially malignant lesions encountered worldwide. We report the results of an *in vivo* clinical evaluation of autofluorescence (AF) spectroscopy for differential diagnosis of oral leukoplakia. Multivariate analysis of spectral data has been incorporated to improve the efficacy of the technique. The results of this noninvasive study are expected to provide potential for extending the technique to other disorders. ***Materials and methods:*** A total of 18 patients and 30 normal volunteers participated in this study. AF spectra were acquired from affected sites of patients and from right and left buccal mucosa of normal volunteers. Diagnostic performance was analyzed using spectral intensity ratio (SIR), and principal component analysis followed by linear discriminant analysis (PCA-LDA). ***Results:*** AF spectra of leukoplakic patients showed characteristic emissions from flavin adenine dinucleotide (FAD) and porphyrin at 500 and 630 nm, respectively. But the emission from porphyrin is not very prominent in the case of healthy volunteers. Also, significant decrease in spectral intensity is observed for leukoplakia compared with normal volunteers in the unprocessed spectra. Method of SIR yielded 96% sensitivity and 100% specificity and an overall 100% for PCA-LDA respectively for efficient differentiation of the lesions. ***Conclusions:*** The result of this preliminary study shows that PCA-LDA or SIR applied to AF spectroscopy is a useful tool for the differential diagnosis of oral cavity disorders. This has been demonstrated in leukoplakia in a clinical setting, and it is expected that the technique can be extended to other oral cavity disorders as well.

Introduction

Oral cancer is a serious health concern, as indicated by its high incidence in many parts of the world. The worldwide incidence of oral cancer was estimated to be 275,000 per year and it is the sixth most common cancer in the world.[1,2] Tobacco use in any form, areca nut chewing, and alcohol consumption are major risk factors for oral cancer.[3,4] Most invasive oral cancers are preceded by precancerous lesions that can be identified by visual inspection and various invasive and noninvasive diagnostic procedures inside the oral cavity. Oral cancer is, therefore, potentially amenable to primary and secondary prevention. It has been well recognized since the beginning of this century that oral cancer is one of the most common cancers in India.[5]

In developed countries, premalignant lesions are identified only in a minority of cases, and the majority of oral cancers are considered to be fresh cases. Conversely, in developing countries, the use of tobacco and/or the areca (betel) nut produces potentially malignant disorders (leukoplakia, erythroplakia, and submucous fibrosis) from which majority of oral cancers arise.[6–8]

Oral leukoplakia is one of the most common premalignant or potentially malignant disorders of the oral mucosa. It has a malignant potential of 30%, with a transformation rate ranging from 0.3 to 17.5%.[9–11] It has been recently been redefined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesions, and some oral leukoplakia will transform into cancer. It is one of the common and important potentially malignant

[1]Department of Oral Medicine and Radiology, Dental College, Trivandrum, Kerala, India.
[2]Biophotonics and Imaging Laboratory, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Biomedical Technology Wing, Trivandrum, Kerala, India.
*Contributed equally to this work
Dr. Venugopal's present address is Kerala Health Services, General Hospital, Trivandrum, Kerala, India.
Dr. Balan's present address is Department of Oral Medicine and Radiology, Dental College, Kozhikkode, Kerala, India.

disorders encountered in India.[12,13] The average annual incidence of leukoplakia in Kerala, the southern part of India, is devastating. It has been reported that 17 people per thousand are prone to the occurrence of leukoplakia.[14]

Histopathology followed by excision biopsy is the gold standard for any oral precancer/cancer screening.[15] However, when presented with the need to have an oral or oropharyngeal mucosal biopsy performed, the patient is often reluctant, and at times fearful, of such an invasive procedure. The patient's reluctance may be compounded by the clinician's hesitation to perform a surgical procedure in an unfamiliar location or anatomical site. Moreover, this method is error prone and inordinately delayed because of manual or sampling mistakes, and hence is not adequate to detect biochemical changes in the lesion.[16] Hence, a noninvasive method that can provide biochemical information in real time is essential for effective intervention for the management of oral cancer and the premalignant lesion. Optical spectroscopic techniques such as fluorescence, diffuse reflectance, Raman spectroscopy, and infrared are emerging techniques in *in vivo* diagnostics of oral cancer.[17]

Fluorescence spectroscopy is an upcoming diagnostic modality with the potential to bridge the gap between clinical examination and invasive biopsy. Autofluorescence (AF) of tissues is produced by fluorophores that naturally occur in living cells on excitation with a suitable wavelength. The presence of disease is indicated by the changes in the concentration of the fluorophores as well as the light scattering and absorption properties of the tissues.[18–20]

In this study, the potential of fluorescence spectroscopy to differentiate leukoplakia from normal oral mucosa and to assess the feasibility of this technique as a noninvasive method for early detection of dysplasia has been explored. For exact differentiation, and to collect a database of AF spectra of normal mucosa and leukoplakia, spectral intensity count analysis, spectral intensity ratio (SIR) analysis, and principal component analysis followed by linear discriminant analysis (PCA-LDA) were also performed.

Materials and Methods

Study population

The study was a randomized clinical trial in which suspected leukoplakia patients who visited the outpatient clinic of Oral Medicine and Radiology, Government Dental College, during a period of 2 years were assigned for the spectroscopic study. An informed consent was obtained from each patients and from the volunteers, after explaining the nature of the experiments about to be undertaken. A total of 21 spectra from a group of 18 patients with leukoplakia were considered for this study. A detailed clinical report of the patients involved in this study is given in Table 1. Occupational and lifestyle details of the patients involved in this study are given in Table 2. All patients who engaged in smoking and pan chewing were advised to stop such habits, and all were advised to maintain good oral hygiene. Thirty volunteers with clinically normal oral mucosa were also selected for the study. Age of the volunteers ranged from 50 to 56, the mean age being 52.71 (SD ± 2.05).

Instrumentation

The AF measurements were performed using the Spectrofluorometer (Fluorolog-III; Jobin Yvon Inc., USA), with a fiberoptic accessory for *in vivo* applications (Fig. 1A). The emission spectra from the suspected areas were recorded from the leukoplakia patients irrespective of the sites, and from the right and left buccal mucosa from normal volunteers. The Fluorolog-III is equipped with double grating spectrometers in the excitation and emission positions. Double grating spectrometers offer a significant increase in sensitivity, resolution, and stray light rejection. For remote sensing of fluorescence, the F-3000 fiberoptic mount with fiberoptic bundle of numerical aperture, 0.22 and 1 cm outer diameter, was used (Fig. 1B). The Y–type F-3000 optic probe originating from the spectrometer merges to become a single fiber bundle as it comes in contact with the patient. The desired excitation wavelength was selected, and was transmitted to the site through one arm of the Y type cable, and the received fluorescence signal was directed back to the spectrometer through the other arm. The excitation source was a 450 W Xenon lamp. The excitation wavelength of 410 nm was selected using Datamax™ software (Datamax, Round Rock, TX) and the in-built double-grating monochromator. This excitation wavelength was selected as per the previous studies on oral cavity cancer.[19,21,22] All emission spectra were recorded in the 460–750 nm range in 1 nm increments.

Table 1. Clinical Report of Leukoplakia Patients Involved in this Study

Sl no	*Sex*	*Age*	*Number of sites involved*	*Clinical description*	*Biopsy report*
1	F	48	1	Leukoplakia	Mild dysplasia
2	M	39	1	Leukoplakia	Epithelial hyperplasia
3	M	66	1	Leukoplakia	Epithelial hyperplasia
4	M	42	1	Leukoplakia	Epithelial hyperplasia
5	M	63	2	Leukoplakia	Mild dysplasia
6	M	45	1	Leukoplakia	Epithelial hyperplasia
7	M	53	1	Leukoplakia	Epithelial hyperplasia
8	F	39	2	Leukoplakia	Mild dysplasia
9	M	32	2	Leukoplakia	Mild dysplasia
10	M	44	1	Leukoplakia	Epithelial hyperplasia
11	F	59	1	Leukoplakia	Epithelial hyperplasia
12	M	61	1	Leukoplakia	Mild dysplasia
13	M	62	1	Leukoplakia	Epithelial hyperplasia
14	F	41	1	Leukoplakia	Epithelial hyperplasia
15	M	48	1	Leukoplakia	Epithelial hyperplasia
16	F	45	1	Leukoplakia	Epithelial hyperplasia
17	F	70	1	Leukoplakia	Epithelial hyperplasia
18	M	42	1	Leukoplakia	Mild dysplasia

Data acquisition protocols

Among the 18 patients with leukoplakia, three patients had lesions on the right and left buccal mucosa. Of the remaining 15 patients, 8 had leukoplakia only on the right and 7 had leukoplakia only on the left buccal mucosa. In two patients with bilateral leukoplakia in the buccal mucosa, the tongue was also involved. As the AF from the tongue is reported to be complicated by the presence of emission peaks of bacteria, the data from the leukoplakia lesions on the tongue have been excluded from this study.[19] In all the cases, AF spectra from not fewer than two sites were collected from

Table 2. Lifestyle and Occupational Details of Leukoplakia Patients Involved in this Study

		Occupation			*Lifestyle habits*		
Age group	*Number of patients*	*Manual laborer*	*Agriculture*	*Housewife*	*Chewing*	*Smoking*	*Alcoholism*
30–40	3	0	1	2	3	1	1
40–50	8	4	2	2	6	4	5
50–60	3	3	0	0	3	1	3
>60	4	2	1	1	2	3	3

large leukoplakic lesions, and the average of the intensities was taken for the study (Fig. 1C and D). The suspicious lesions were visually identified before spectral measurements in all cases. In normal volunteers, a minimum of two spectra were acquired from right and left buccal mucosa, and the average was calculated from the average of the intensities. In 10 patients in whom the lesions were either small or involved only on one side, the spectra of normal uninvolved mucosa were also collected and used for comparison with that of the normal volunteers. After spectral acquisition, scalpel biopsy from the suspected sites of leukoplakia patients was performed for histopathological evaluation.

Spectral data analysis

Spectral intensity count. Our initial attempt was to try the differentiation of oral leukoplakia from normal mucosa using the AF spectral data, without further processing. This is in view of the acceptability of the technique among clinicians, because of its ease of use and interpretation of data. It is a fact that clinicians prefer to have the results of the study interpreted as easily and quickly as possible, without the intervention of a spectroscopist. Considering this situation, it is proposed that the initial screening based on spectral intensity and peak shift can be directly interpreted by the clinician at the bedside using a more compact laser-induced fluorescence system (LIFS) supported by the results of the database from previous studies such as the current one. For this, a comparison of the difference in spectral intensity count at 500 and 630 nm has been made between the two cases and evaluated in this study. As an advanced stage of data processing and further to support the results of preliminary study, one way analysis of variance (ANOVA) test was also performed on these intensity count values, using the statistical software package SPSS-17 (SPSS Inc., Chicago, IL).

Processing of spectra. To support the initial findings from the unprocessed spectra, advanced data analysis has been performed. For this, all spectra were baseline corrected using Datamax^{TM} software. Each spectrum were normalized with respect to the maximum intensity wavelength in the 500 ± 10 nm region. From the normalized spectra, intensity corresponding to the 630 nm wavelength was extracted. Using SPSS, ANOVA was performed on the normalized intensity at 630 nm, to find the variation between the groups.

SIR analysis. To obtain the variation from the emissions of different endogenous fluorophores, the mean fluorescence

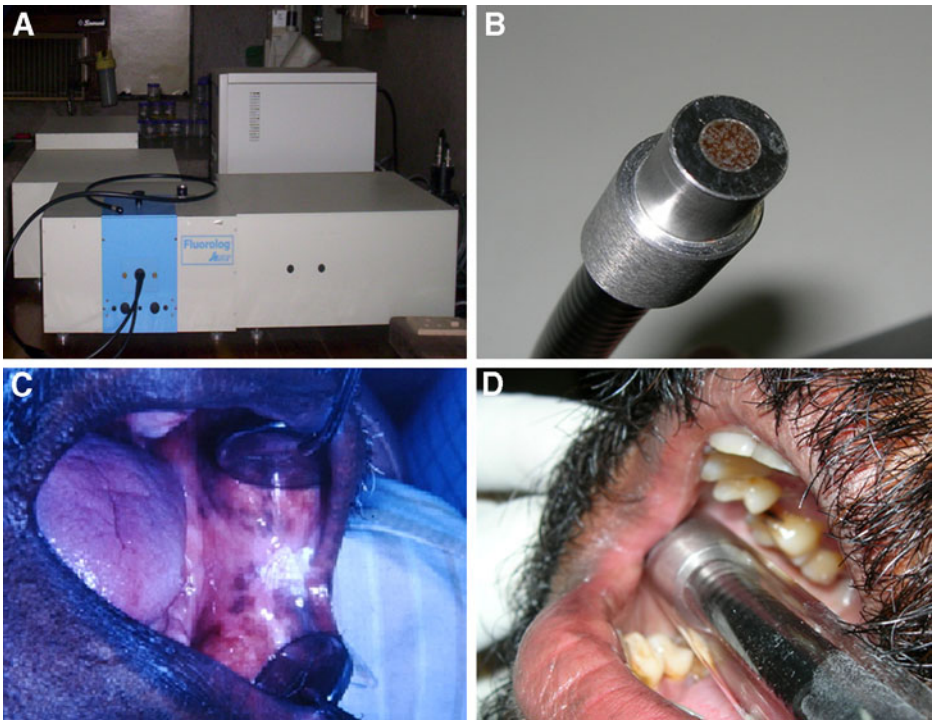

FIG. 1. **(A)** Spectrofluorometer with the fiberoptic probe. **(B)** Fiberoptic probe bundle for *in vivo* analysis. **(C)** Patient with leukoplakia. **(D)** Spectral acquisition from a patient's oral cavity using a fiberoptic probe.

intensity ratio of two fluorophores was calculated. For this, the most prominent emissions observed at ~500 nm and characteristic of the emission from FAD, and the emission from the porphyrin at ~630 nm were selected. Normalized fluorescence intensity (NFI) ratio (NFI 500/NFI 630) values were expressed as scatter plots. Sensitivity, specificity, accuracy, and positive and negative predictive values were calculated from the observed variation in these values.

Multivariate analysis

LDA followed by PCA is a multidimensional statistical tool. It is used to investigate the dependence and connections among variables, helping to reduce and simplify data and to classify objects into groups. In order to avoid the computational complexity in the optimization and implementation of LDA, we have initially performed PCA using SPSS-17, to reduce the dimension of fluorescence spectral data (460–750 nm, 290 intensities). In PCA, dimension reduction is achieved by processing the number of competitiveness indicators into a small number of independent indicators through altering the internal structure of the correlation matrix into a specific number of original indicator variables. These indicator variables were selected as input for the LDA model for tissue classification. LDA was used to determine the discriminant function that maximizes the variances in the data set between groups, while minimizing the variances between members of the same group. In this study, we have performed PCA-LDA on the normalized spectra of each category, using SPSS as per the method described by various groups.[17,19,23] Binary classification results such as sensitivity, specificity, accuracy, and positive and negative predictive values corresponding to PCA-LDA results were also found out.

Receiver operating characteristic (ROC) curves

The ROC curve is a graphic way of relating sensitivity and specificity to different thresholds that separate two populations. ROC curve is obtained by plotting the false positive rate (one specifcity) versus the sensitivity for the different values of thresholds.[24] ROC curve is also helpful in the determination of threshold, if the desired sensitivity and specificity are selected. Here, we have performed ROC curve analysis on the SIR and PCA-LDA scores using SPSS.

Results

Spectral features

The averaged spectra of right and left buccal mucosa of all volunteers, and the suspected sites and uninvolved normal mucosa of leukoplakia patients are shown in Fig. 2. The spectra of normal volunteers and that of the normal mucosa shows strong emission at ~500 nm, with two valleys at ~585 and 630 nm in all cases studied. In the case of leukoplakic lesions, the peak at ~500 nm loses its intensity to about half to that of normal. In comparison with normal, the prominent valley at ~585 nm is missing in leukoplakia samples. In addition, the valley at ~630 nm develops into a prominent peak. These peaks are assigned to the emissions from FAD, phospholipids, and porphyrin.[21,25,26]

Statistical test ANOVA was performed on peak intensities of 500 and 630 nm between normal and leukoplakia of unprocessed spectra. Peak intensity showed a significant difference ($p < 0.05$) between normal and leukoplakia samples for both peaks. Also, in the processed spectra, difference in peak intensity for 630 nm peak was found to be statistically significant ($p < 0.05$).

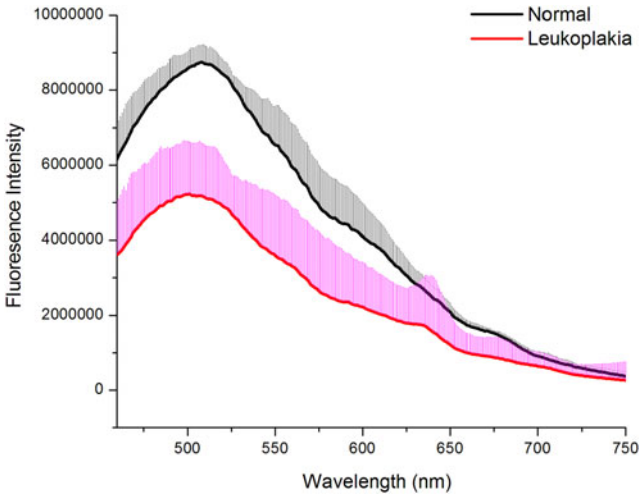

FIG. 2. Average fluorescence emission spectra with standard deviation from oral mucosa of normal volunteers and leukoplakia patients.

Advanced data analysis

For the advanced data analysis, all spectra were baseline corrected and normalized with respect to the peak intensity at 500 nm. Average spectrum based on the processed data is shown in Fig. 3. The appearance of a prominent peak from the emission of porphyrin is also seen at 630 nm in the spectrum from the leukoplakia sites. No other significant change is observed in the emission from other endogenous fluorophores, in the normalized spectra.

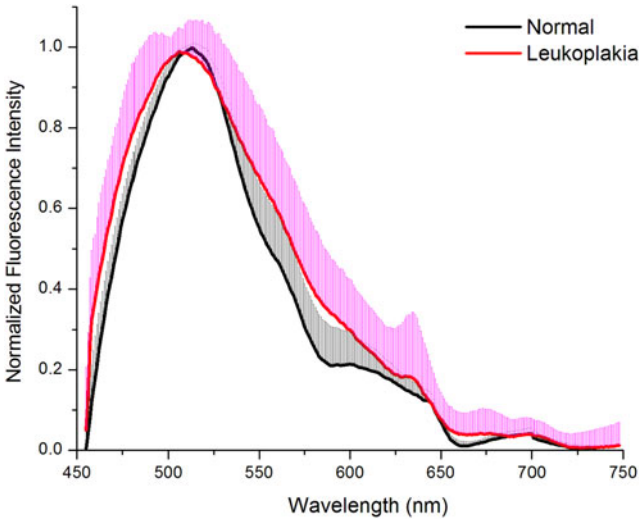

FIG. 3. Baseline-corrected, normalized average spectra with standard deviation from oral mucosa of normal volunteers and leukoplakia patients.

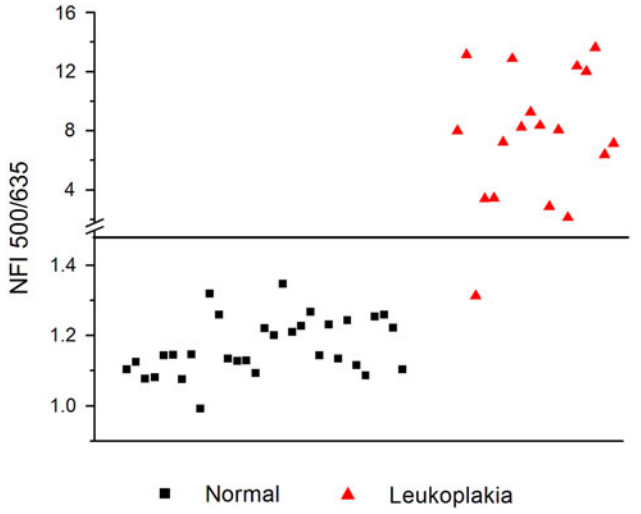

FIG. 4. Spectral intensity ratio plot (normalized fluorescence intensity [NFI] 500/NFI 630) for oral mucosa of normal and leukoplakia patients. The discrimination line at 1.5 gives good discrimination between the groups.

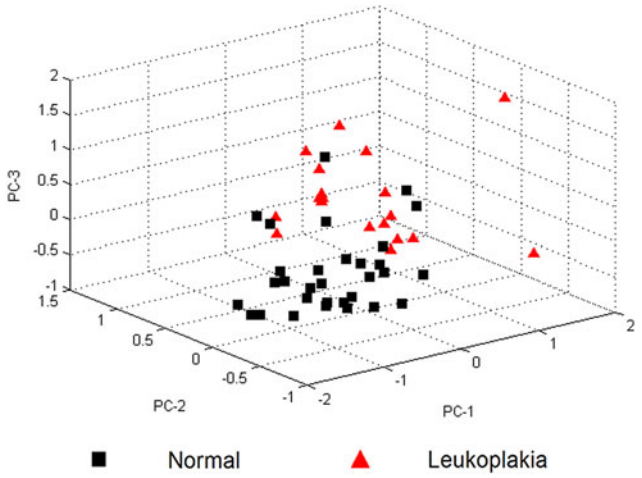

FIG. 5. Principal component score plot of the first three principal components for the spectral data set acquired from normal and leukoplakia patients.

SIR

Scatter plot of normalized fluorescence intensity ratio, NFI 500/NFI 630 from the buccal mucosa of normal volunteers and the leukoplakia lesions is shown in Fig. 4. Discrimination line drawn at 1.5 gives a clear discrimination between normal and leukoplakia samples. Number of positive and negative predictive value obtained from the position for the lesion corresponding to this discrimination line was used to analyze the performance level of this test. All the results of binary classification using SIR are given in Table 3.

Multivariate analysis

PCA of the AF spectral data extracts seven PCs that account for 97.8% of the variance in the entire spectral data (PC1: 48.6%, PC2: 23.5%, PC3: 14.9%, PC4: 6.9%, PC5: 2.1%, PC6: 1.44%, PC7: 0.36%). These PC scores were considered as input variables for LDA. The first three PCs, which constitute 87% of the spectral data, are shown as a three dimensional scatter plot in Fig. 5. The first two discriminant functions obtained from linear discriminant analysis are shown as a scatter plot in Fig. 6. The cutoff value in the scatter plot, which is the weighted mean of the paired values, is used to classify different lesions. These discrimination scores calculated using statistical analysis provide the characteristics of each spectrum. The position of these discriminant value functions was used to obtain positive and negative predictive value, which is used to analyze the performance level of this test. Results of binary classification obtained from PCA-DA are also given in Table 3.

ROC curves analysis

Diagnostic performance of the SIR and PCA-LDA tests was calculated by obtaining the area under the curve (AUC)

Table 3. Overall Diagnostic Accuracies Obtained for Discrimination Between Normal Oral Mucosa and Leukoplakia Using SIR and PCA-LDA

Test conducted	*Sensitivity*	*Specificity*	*Accuracy*	*PPV*	*NPV*
Spectral Intensity ratio	96.77	100	98.03	100	95.24
PCA-LDA	100	100	100	100	100

Sensitivity = True positive/ (true positive + false negative).
Specificity = True negative/ (true negative + false positive).
Accuracy = (true positive + true negative) / (positive + negative).
Positive predictive value (PPV) = True positive/ (true positive + false positive).
Negative predictive value (NPV) = True negative/ (true negative + false negative).
SIR, spectral intensity ratio; PCA-LDA, principal component analysis followed by linear discriminant analysis.

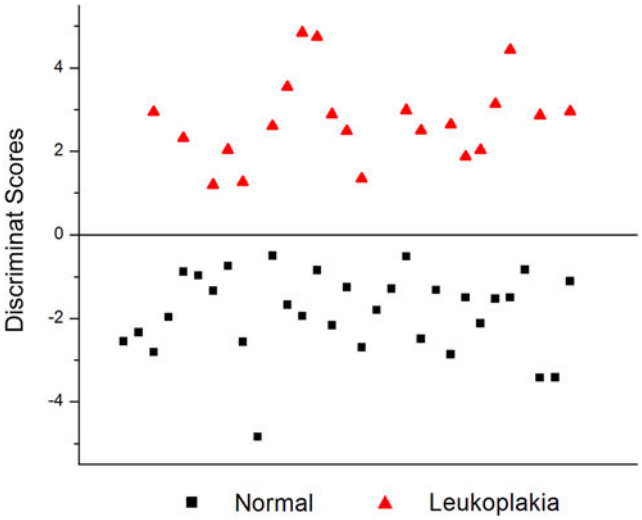

FIG. 6. Scatter plot based on linear discriminant analysis for normal and leukoplakia patients. The discrimination line at 0 gives good discrimination between the groups.

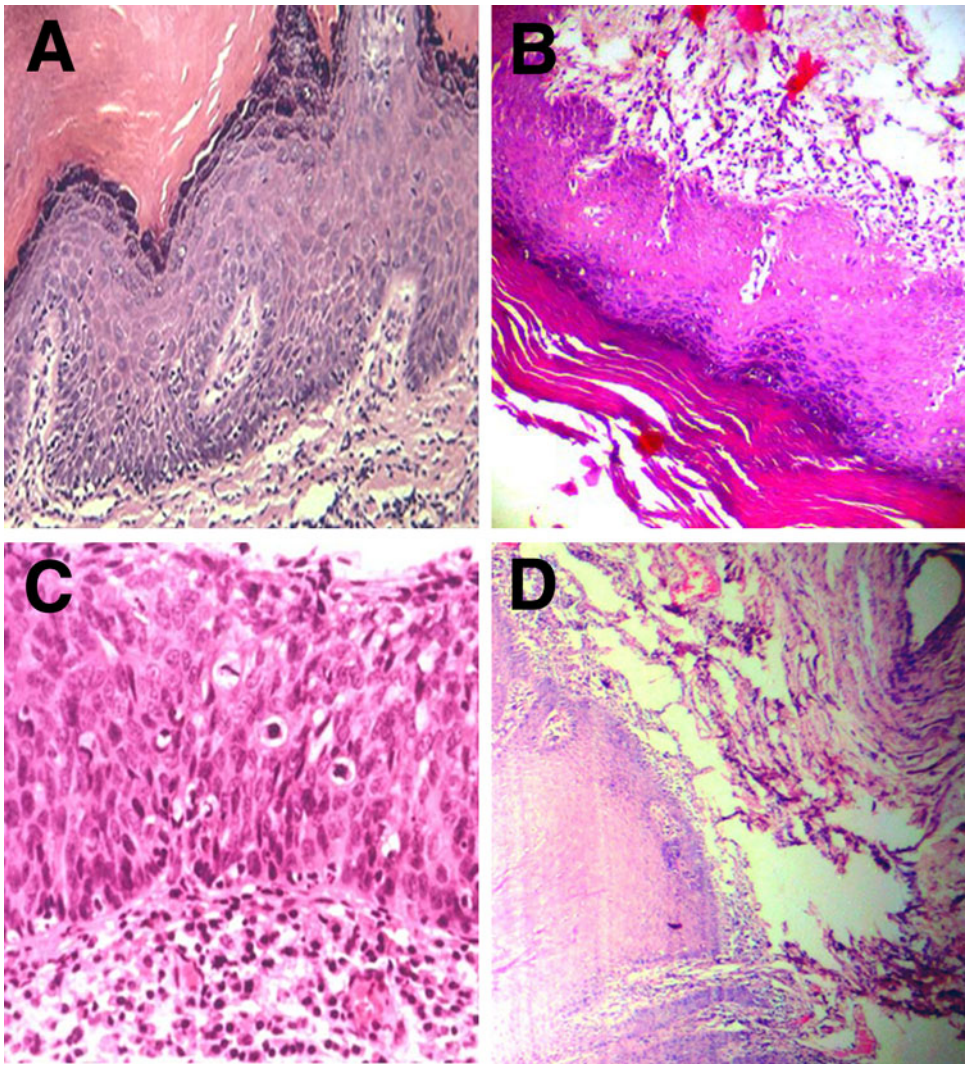

FIG. 7. Hematoxylin and eosin stained histopathological images of suspected sites of leukoplakia patients. **(A, B)** Epithelial hyperplasia. **(C, D)** Mild dysplasia.

using ROC analysis. ROC analysis yielded AUC of 0.997 and 1.000, respectively, for SIR and PCA-LDA analysis.

Discussion

Many groups have reported the use of AF spectroscopy in differentiating different grades of oral cancers and normal mucosa,[17–22] but a systematic approach on the variation observed with respect to different fluorophores has not been addressed seriously so far. In this study, we tried to emphasize the role of AF spectroscopy in discriminating the oral cavity disorders by demonstrating the classification of normal oral mucosa from leukoplakic lesions, clinically. We also utilized diagnostic algorithms such as SIR and PCA-LDA to obtain exact discrimination between the groups.

While considering the raw spectral data, a significant decrease in the total intensity was observed in the case of leukoplakia. When specific fluorophores are considered, significant decrease in the intensity of FAD peak and a considerable increase in the porphyrin peak are observed in the case of leukoplakia. These differences are very clear from the spectra, can be well appreciated and easily interpreted by clinicians, and can be considered as the initial screening for further diagnosis. The difference in these two parameters alone can be considered as an indication of tissue transformation from its normal state. Likewise, an effective preliminary screening is possible using this technique alone. Later, the data may be further processed for advanced analysis such as SIR and PCA-LDA, to reconfirm the results of initial screening.

Overall reduction in fluorescence intensity of leukoplakia compared with normal tissues indicates alterations in both epithelium and stroma. According to de Veld et al., the presence of a disease stage can change the concentration of the fluorophores as well as the light scattering and absorption properties of the tissues, because of changes in blood concentration of fluorophores, nuclear size distribution, collagen content, and epithelial thickness.[27] The contributing factors to the overall reduction in fluorescence intensity in this study are collagen breakdown in stroma and the decrease in the total hemoglobin and metabolic co-enzymes FAD.[21,28] As per the hypothesis of Liu and Vo-Dinh, a red shift in the emission of FAD is expected during tissue transformation associated with the decrease in total hemoglobin concentration,[25] but no such clearly distinguishable shift is observed in the present study. In the case of leukoplakia, decrease in the intensity for phospholipids peak at $\sim$585 nm is observed. This decrease may be the result of

lipid peroxidation in oral cavity tissues caused by the toxic effects of smoking/chewing of tobacco or alcoholism in leukoplakia patients.[17] Enhanced peak observed at ~630 nm in leukoplakia lesions may be ascribed to the accumulation of endogenous fluorophore protoporphyrin IX in the malignant tissues, which is a precursor of heme synthesis.[29,30]

In this clinical trial, we have obtained a sensitivity of 96% and specificity of 100% using SIR, and an overall sensitivity and specificity of 100% using PCA-LDA for the discrimination of leukoplakia and normal oral mucosa. Using artificial neural network analysis, van Staveren et al. have reported an overall sensitivity of 64–100% and a specificity of 82–94%, to differentiate between normal, homogenous, and non-homogenous leukoplakia using AF spectroscopy.[31] Jayanthi et al. reported a sensitivity of 83% and specificity of 100% to differentiate between normal oral mucosa and hyperplasia using LIFS.[19] Later, the same group have reported an improved sensitivity of 93% and a specificity of 100% in differentiating normal and hyperplasic (which includes leukoplakia) oral mucosa using LDA on LIFS data.[21] Using LIFS, Mallia et al. have reported an overall sensitivity and specificity of 89–100% and 74–100%, respectively, in discrimination between normal and hyperplasic oral lesions using SIR analysis.[22] From Table 3, it is clear that sensitivity, specificity, positive and negative predictive value, and accuracy for the differentiation of leukoplakia and normal oral mucosa is much better in this study. Moreover, it is also clear that PCA-LDA provides better discrimination than SIR analysis.

The AUC of ROC gives the measure of accuracy of the diagnostic procedure or analysis. The closer the AUC value to 1.0 or 100%, the better is the accuracy of the procedure. In this study, we have obtained a better AUC value of 1 for PCA-LDA than the 0.997 obtained with SIR analysis. This result is in line with our earlier study on fluorescence spectroscopic variation in oral mucosa of persons with or without lifestyle oral habits.[17] This may be the result of the variation in intensity of the specific AF peak, which may tend to vary from patient to patient; however, in PCA-LDA, the chance of error in discrimination was reduced, because the entire spectral region is considered for analysis.

As with most of the initial study, it is a fact that this study also poses few limitations. Major limitations are the comparatively small patient group and the lack of point to point histopathological correlation from the exact point of spectral acquisition and biopsy. Although we have performed the histopathological analysis from the suspected sites of leukoplakia patients (Fig. 7), a correct correlation with respect to the spectral data is difficult, because of the expected mismatch between the exact point of spectral acquisition and biopsy. These factors definitely would have played a role in the calculation of specificity and sensitivity.

Conclusions

In the present study, fluorophores responsible for the emissions from leukoplakic lesions and the normal oral mucosa have been identified, and the variations in their concentrations have been analyzed. Based on the differences in the emission of various fluorophores, it is shown that an effective classification of leukoplakia from normal oral mucosa is possible using this technique. The major attraction of the study is that the technique of AF spectroscopy can be applied clinically by clinicians, without any further processing, for the initial screening of oral cavity disorders. If the variation in the spectral intensity and peak shift can be considered as changes from normal pathology, the results of this preliminary study can be extended to other oral cavity disorders also. Further, analysis using SIR and PCA-LDA confirms the results of the screening study, with improved sensitivity and specificity compared with previous reports. In summary, AF spectroscopy can be considered as a potential spectroscopic tool for the characterization and classification of oral cavity disorders at an early stage. The comparatively low cost and ease of use of the technique are added advantages to consider it as a screening modality in rural and urban health centers.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
R. S. Jayasree
Biophotonics and Imaging Lab
Biomedical Technology Wing
Sree Chitra Tirunal Institute for Medical Sciences & Technology
Poojappura
Thiruvananthapuram
695 012 Kerala
India

E-mail: jayashreemenon@gmail.com