

## Autofluorescence spectroscopy of blood plasma in characterization of oral malignancy - a pilot study

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### Abstract

**Introduction:** Oral malignancy has a very high incident rate in India as a result of habitual tobacco chewing and its products. Microscopic examination of the tissue specimen following a biopsy, which is an invasive procedure, forms the gold standard for diagnosing these lesions. Autofluorescence spectroscopy (AF) of blood plasma promises to be a sensitive and non-invasive method for screening and monitoring such cases.

**Aim and Objectives:** To evaluate and compare the spectral emission range of cases from oral squamous cell carcinoma, leukoplakia and control subjects in the visible spectral region.

**Materials and Method:** Blood samples from 5 cases each of oral squamous cell carcinoma, leukoplakia and normal individual were taken and subjected to excitation wavelengths at 405 and 420 nm using autofluorescence spectrometer and emission wavelength were noted.

**Results:** Fluorescent emission spectra of blood plasma of oral malignant lesions exhibited characteristic spectral difference with a shift towards the red spectrum with reference to normal subjects indicating alteration in the internal fluorophore concentration owing to malignant changes.

**Conclusion:** Autofluorescence spectroscopic analysis of blood plasma promises to be a valuable non invasive tool in the diagnosis of oral malignancy from clinically appearing normal tissue.

**Keywords:** Autofluorescence, Oral Cancer, Spectroscopy, Noninvasive.

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### Introduction

Oral carcinogenesis follows a multistep progression model where carcinomas of oral cavity are characterized by high degree of aggression and have a great propensity to metastasize. Most cases of oral cancer are preceded by oral potentially malignant disorders, but they may develop de novo also. Common potentially malignant disorders include oral leukoplakia, oral lichen planus and oral submucous fibrosis. Up to 36% of such lesions may progress to carcinoma making early diagnosis a necessity.<sup>1</sup> Diagnosis of oral malignant lesions involve histological examination of the tissue procured following a biopsy. Biopsy is an invasive procedure, in which, the results of histopathological examination may be subjective, with variation according to the biopsy site and associated inadvertent diagnostic delay. Therefore, there is, a growing need to develop sensitive and less invasive methods for screening of malignant lesions of the oral cavity.<sup>2</sup>

Recently, the use of alternative methods such as fluorescence spectroscopy has been extended to the medical field to characterize various metabolic and pathological changes at cellular and tissue level, as, it is a highly sensitive method for monitoring minor changes in the structure and micro environment of fluorophores. Biomolecules, within the tissue have the ability to give fluorescence emission within the UV-visible region when excited at suitable wavelengths. Cellular and tissue alterations in pathological conditions result in alterations in the fluorescence emission spectrum. Photosensitizing substances such as tryptophan, Nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) preferentially accumulate in premalignant and malignant lesions.<sup>2</sup> The fluorescent emission spectrum is also influenced by the histological organization of the tissue, in particular for multilayered tissue.<sup>3</sup> The technique of autofluorescence has previously shown promising results in other tissues such as breast<sup>4</sup>, cervix<sup>5</sup> and colon<sup>6</sup>. The present pilot study was designed to study and compare fluorescence characteristics in blood plasma of healthy controls, leukoplakia and oral squamous cell carcinoma subjects.

### Materials and Method

5 cases each of clinically healthy controls, leukoplakia and oral squamous cell carcinoma were included in the study. The procedure of autofluorescence spectroscopy was explained to the

individuals and informed written consent was obtained from all the subjects. Blood samples were collected from the controls, leukoplakia and oral squamous cell carcinoma patients. Ethylene diamine tetra acetate (EDTA) coated test tubes were used to store the samples to prevent blood coagulation. The samples were then centrifuged and plasma was removed without disturbing the buffy coat and erythrocyte sediments. The plasma thus separated was stored at -20°C until the assay was performed. For fluorescence measurements, analytical grade acetone was added to plasma. The mixture was centrifuged and the clear supernatant was taken in a quartz cuvette of 1 cm path length for further analysis.

Fluorescence emission spectra of blood plasma-acetone extract were recorded using fluorescence spectrometer. The samples were excited at two different wavelengths, 405 nm and 420 nm and autofluorescence emission spectra were recorded in the region 430-700nm and 450-700nm respectively. The wavelength of 460-500nm attributed to co-enzyme linked nicotinamide adenine dinucleotide phosphate (NADPH) and spectral shoulder at 512-524 nm attributed to FAD.

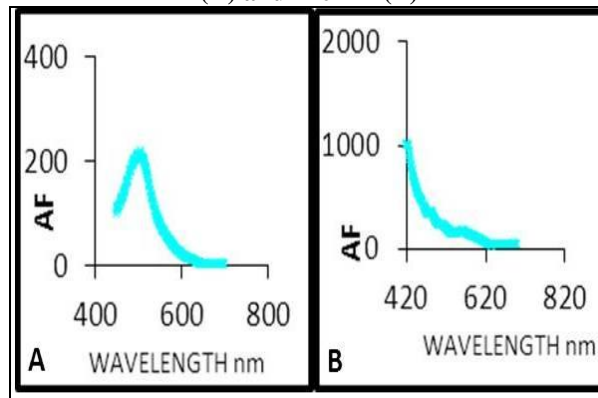
**Results**

The fluorescence emission spectra of blood plasma of control, leukoplakia and oral squamous cell carcinoma is shown in Table 1 at excitation wavelengths of 405 and 420 nm. In our study, oral squamous cell carcinoma showed a characteristic spectral difference when compared to control and leukoplakia cases. Leukoplakia showed spectral emission in the same range as normal cases (Graph. 1 and 2). At 405 nm excitation wavelength, the oral squamous cell carcinoma showed a primary emission peak at 486.5 nm with a shoulder at 495.5-513 nm, whereas, at 420 nm excitation wavelength, it showed a primary spectral emission peak at 484 nm with a shoulder at around 489.5-500 nm and another peak at around 600-620 nm (Graph. 3).

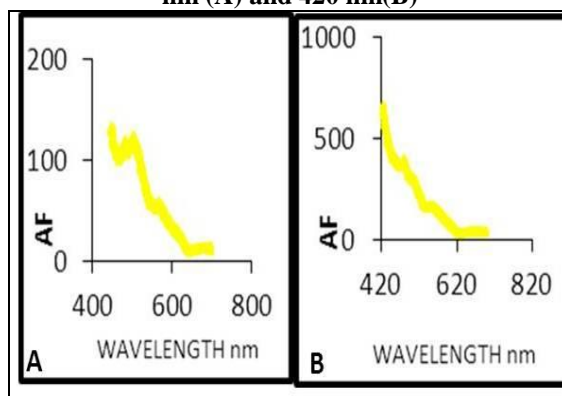
**Table 1: Table showing emission peak and spectral shoulder at 405 nm and 420 nm**

Group	Emission peak	
	405 nm	420 nm
Control	484.5 nm	499.5 nm
Leukoplakia	485 nm	405.5 nm
OSCC	486.5 nm	484 nm
Spectral shoulder	495.5-513	489.5-500nm

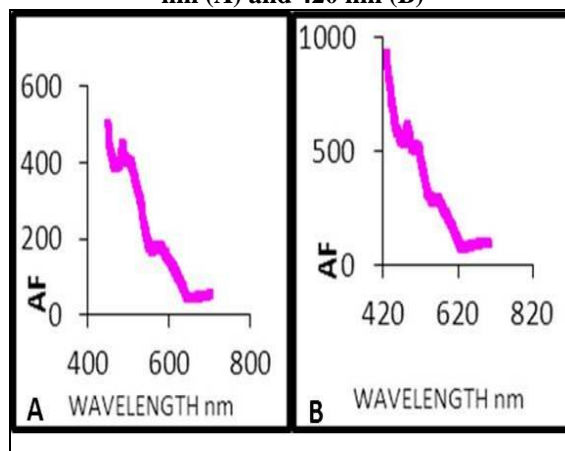
**Graph 1: Spectral Graph showing autofluorescence characteristics of blood plasma in controls at 405nm (A) and 420 nm (B)**



**Graph 2: Spectral Graph showing autofluorescence characteristics of blood plasma in leukoplakia at 405 nm (A) and 420 nm(B)**



**Graph 3: Spectral Graph showing autofluorescence characteristics of blood plasma in oral cancer at 405 nm (A) and 420 nm (B)**



**Discussion**

Most cases of oral cancer are first diagnosed when they become symptomatic. By this stage, approximately two-third of patient would have already developed advanced disease with regional metastasis and have a

consequently diminished prognosis. The subsequent treatment would require surgery and adjunct radiotherapy, with its high rate of morbidity and mortality. If diagnosed and treated at an early stage, both survival rate and quality of life can be improved.<sup>2,7</sup>

The common procedure for detecting malignant oral lesions consists of visual inspection followed by biopsy of any observed suspicious lesion. Recently, there has been increased interest in diagnostics involving optical system using spectroscopy to establish definitive diagnosis. Spectroscopy, was originally the study of interaction between variation and matter as a function of wavelength. Spectrometry is spectroscopic technique used to assess the concentration or amount of a given molecule.<sup>2,3</sup>

Optical spectroscopy explores the optical phenomena resulting from interaction of light with biological tissue. It may be particularly useful for the analysis of differences between normal and cancerous tissue because of major scattering, absorption and fluorescence change which are known to occur during development of cancer. Three optical techniques that are currently utilized in the detection of oral malignancies include autofluorescence, elastic scattering and Raman spectroscopy.<sup>2</sup> Fluorescence spectroscopy is based on autofluorescence or a laser-induced phenomenon and occurs due to the presence of fluorophores like NADPH, collagen, elastin, and cofactors. VELscope is a portable device based on narrow-emission tissue fluorescence which provides light in the range of 400- 460 nm. Under the intense blue light, normal mucosa emits pale green autofluorescence while the suspicious tissue appears dark.<sup>5</sup> Elastic scattering spectroscopy (ESS), generates a wavelength-dependent spectrum that reflects both scattering and absorptive properties of the tissue. ESS, is sensitive for nuclear size, chromatin content, nuclear-cytoplasmic ratio, and cellular crowding, which are all criteria for establishing malignancy. Raman spectroscopy, utilizes a form of elastic scattering which is generated by a shift in the frequency of the incident excitation light. It is most accurate technique but signals are weak. Trimodal spectroscopy, is a combination of all the above-mentioned techniques to increase the accuracy of the technique.<sup>2,8</sup>

The technique used in our study is autofluorescence spectroscopy which uses the principle of fluorescence spectroscopy which was first described by Alfano et al. (1984).<sup>6</sup> It involves using a beam of light, which excites the electrons in molecules of certain compounds and cause them to emit light of lower energy (fluorescence). This can be detected by sensitive spectrometers. All tissues fluoresce due to the presence of internal fluorescence chromophores (fluorophores) within them. Characteristic spectra reflect biochemical changes occurring within the tissue. The resultant spectra not only detect the light that is fluoresced but also are sensitive to the structure that

absorbs light. The commonly detected fluorophores are NADH, collagen, elastin and co factors such as flavins (FAD, FMN).<sup>3,8</sup> The primary emission peak in the range of green fluorescence (480-500 nm) could be attributed to NADPH and those in the higher range towards the red spectrum could be attributed to increased concentration of porphyrins in oral cancer cases.<sup>2</sup>

Blood plasma was used in our study to ascertain the role of internal fluorophores as promising biomarker in oral malignancy. Madhuri S et al. (2003)<sup>3</sup> conducted a study and suggested that the autofluorescence spectroscopy is a useful tool to discriminate oral malignant patients with a sensitivity of greater than 90% from normal healthy subjects. It was observed that the blood plasma of patients with different stages of oral malignancy exhibit significant spectral differences when compared with normal subjects. Gillenwater et al. (1998)<sup>10</sup>, utilized autofluorescence technique in neoplastic and non-neoplastic oral mucosa and observed that fluorescence intensities are less for abnormal than normal sites with a shift towards the red region (635 nm) from blue region of the spectrum (455-490 nm).

In another study, Onizawa et al. (1999)<sup>11</sup> showed that fluorescence photography at 360 nm excitation, with emission above 480 nm, could be used to separate benign and malignant oral tissue with 91.1% sensitivity and 84.3% specificity. Van Staveren HJ et al. (2000)<sup>12</sup> used autofluorescence spectra analysis to distinguish oral leukoplakia from normal mucosa using artificial neural network and revealed a sensitivity and specificity of 86% and 100% respectively. It also helped considerably in classifying homogeneous and non-homogeneous tissue well, with weak or no correlation, between spectral patterns in the grade of dysplasia, hyperplasia and hyperkeratosis.

## Conclusion

The results of the present pilot study have provided a promising platform to carry out a more detailed investigation on a larger population consisting of normal, potentially malignant and malignant disorders for the characterization of various pathological conditions of the body. These new developments in diagnostic technology may greatly improve the ability to screen people for oral malignant lesions. However, intensive research and clinical trial investigations are needed to test the sensitivity, specificity, negative and positive predictive ability of these technologies before they can be widely implemented.

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