ORGINAL RESEARCH ARTICLE

AUTOFLUORESCENCE SPECTROSCOPIC STUDY OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Background: The success of treatment of all forms of carcinoma to a great extend is influenced by the time interval that depends between the genesis of the lesions, its diagnosis and adequate treatment. Since malignancies are often diagnosed at a later stage, the survival rate is markedly reduced in spite of the advanced available treatment. Fortunately early detection of oral malignancies is possible because of easy visualization and accessibility.

As bio-chemical alteration precedes morphological changes, detection of these alterations has potential practical value in early diagnosis of premalignant and malignant lesions with correspondingly increased chance of cure.

Objective: To analyse the differences in the fluorescence spectral characteristics of normal, inflammatory mucosal lesions, benign gingival lesions and oral carcinomas in the visible spectrum of light by making use of the auto fluorescence properties of tissues.

Methodology: The fluorescence spectrum was analysed using a spectrofluorometer, after exciting the samples at 405nm and 420nm separately.

The results were plotted in a graph and a significant difference was found between normal and malignant tissues.

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INTRODUCTION

Cancer is a generic term for a variety of malignant neoplasm's due to unknown and probable multiple causes, arising in all tissues composed of potentially dividing disabling cells, in man and other animals and resulting in adverse effect in the host and other animals resulting in adverse effect in the host through invasive growth and metastasis.[1]

Global statistics show that the oral cavity constitutes approximately 30% of overall malignancy affecting the whole body.[2] Among various types, squamous cell carcinoma represents about 90% of the carcinomas of the oral cavity .[3]

As malignancies are often diagnosed at a later stage, the survival rate is markedly reduced in spite of the available treatment. Fortunately early detection of oral malignancies is possible because of easy visualization and accessibility. Loss of cell differentiation and increased cellular proliferative activity during malignant transformation leads to alteration of the cells biochemical content. As bio-chemical alteration precedes morphological changes, detection of these alterations has potential practical value in early diagnosis of premalignant and malignant lesions with a correspondingly increased chance of cure.

At present many research works are undertaken to localize bio-chemical changes in the cell undergoing malignant transformation. Various markers have been used in studying these biological changes like expression of oncogenes (P53, C-myc)[3], proliferation markers (K-67, AgNoRs, PCNA) [4, 5, 6], intermediate filaments[7], blood associated all surface carbohydrate and growth factors and their receptors[8].

The principal of fluorescent spectroscopy has been extended to medical community both on a fundamental level and tissue diagnostics. Much earlier, fluorescent dyes, were used to probe and obtain information about the environment in biological media. This was extended to detect cancer and cancer therapy using hematoporphyrin derivatives (HPD). It is known since 1940 that certain porphyrins show a preferential retention in malignant tumours and also in embryonic, lymphatic and regenerating tissues.

A major breakthrough occurred when Alfano and Yao used luminescence spectroscopy as diagnostic tool for tooth decay in 1981[9]. They found a difference in the fluorescence spectral profile from carious and healthy normal regions of teeth. The colours emitted from normal and abnormal regions were different. This work initiated from normal and abnormal regions of teeth. This work initiated a surge of activity to use fluorescence spectroscopy as diagnostic tool for various diseases, especially cancer diagnosis.

A great deal of work has been done regarding cancer diagnosis using fluorescence spectroscopy. Majority of the work has been done in animals and humans as an in-vitro study. Only very few workers have done an in vivo study. Dhingra et al have developed a fibro-optic based portable spectro-fluorimeter for in vivo study[10]. He collected fluorescence spectrum from normal and cancerous mucosal lesions. His study demonstrated clear differences in the fluorescence spectra between normal, benign and neoplastic oral mucosa. Though a lot of work has been carried out in cancer diagnosis as an in-vitro and in-vivo study, only a few works has been carried out in relation to oral cancer. Taking these facts into consideration, the present study deals with the in-vitro application of fluorescence technique for its evaluation in the diagnosis of oral cancer.

OBJECTIVES

1. Histological evaluation of normal, clinically diagnosed inflammatory and benign gingival oral mucosal lesions and oral squamous cell carcinoma.
2. Spectroscopic analysis of tissues taken from the above lesions.
3. Comparison of spectroscopic ratio values with tissue diagnosis.
4. Evaluation of spectroscopic study in the diagnosis of oral cancer.

MATERIALS AND METHODS

The subjects for this study were selected at random, from among the patients who had reported at the outpatient department of Tamilnadu Government dental college and hospital, Chennai.

Twenty patients comprising Of 12 males and 8 females were in the age group of 40 to 75 years having Oral cancer. Five patients comprising of 3 males and 2 females in the age group of 20 to 35 years having inflammatory and benign gingival lesions formed the study group. Five apparently normal persons formed the control groups of which 4 were males and 1 female all in the age group of 15 to 30 years. The control group was selected from patients who came for other dental problems.

All the patients selected for this study were subjected to a comprehensive medical examination to rule out the possibility of any associated systemic diseases.

A standard biopsy procedure was followed and the biopsy sample was thoroughly washed in normal saline to remove blood clots. The sample was divided into two parts; one part was then transferred to a labelled bottle containing 10% buffered formalin solution and subjected to histopathological study. The second part of the biopsy tissue was put in a labelled, clean and dry empty bottle and transported in an ice pack to the medical physics department Anna University, Chennai, for further processing and fluorescence spectroscopic analysis.

Tissue Extraction

Tissue extraction of the specimen was done within four hours of taking the biopsy. A suitable quantity of the fresh tissue was thoroughly washed with physiological saline of 0.9% Nacl solution. The washed tissue was minced with mosquito scissors and made into a paste with saline using a mortar and pestle. This paste was diluted to 4ml by using analytical grade acetone of purity 99.9%. The diluted tissue solution was poured into a clean and dry test tube and mixed well using a cyclo mixer and the solution was then centrifuged at 300 rpm for 10 minutes. After centrifugation, the clear supernatant solution was transferred to another clean and dry test tube and used for auto fluorescence spectral analysis.

Recording of Fluorescence Spectra

The tissue extract was taken in a clean and dry, four sided, polished cuvette and kept in position inside the spectrofluorometer. The sample was excited at different wavelengths because of the heterogeneous nature of the tissue. Out of the various wavelengths tried for the excitation of the sample, the excitation wavelengths of 405nm and 420nm showed very good contrast between the peaks and the result highly was reproducible and consistent.

Hence the sample was excited at two different wavelengths of 405nm and 420nm and the resulting fluorescence spectra were recorded from 450nm to 700nm in the form of a graph in each case. The experiment was done at a scan speed of 100nm/minute.

The fluorescence wavelength and the percentage of fluorescence intensities corresponding to the different peaks in the spectrum were noted for 405nm and 420nm excitation separately for all the samples.

RESULTS

The twenty clinically diagnosed cases of oral squamous cell carcinomas were graded according to the WHO-International Histological Classification of tumours as 6 well differentiated squamous cell carcinomas, 9 moderately differentiated squamous cell carcinomas and 5 poorly differentiated squamous cell carcinomas. The 5 clinically diagnosed cases of inflammatory and benign gingival growths were histopathologically confirmed as 2 Fibrous epulis, 2 Fibromas in each category and a case of Gingival Fibromatosis. The histological sections of five normal tissues from the gingival mucosa were used as controls in this study.

The fluorescence spectra of 5 normal samples, 5 cases of inflammatory and benign gingival growth and twenty cases of oral squamous cell carcinoma were recorded from 450nm to 700nm by exciting the samples at 405nm and 420nm.

The specrum for normal tissue

Fluorescence spectra of normal tissues are taken by exciting the sample at 405nm and 420nm. From Fig.1 it is clearly observed that the spectrum 405nm excitation has a prominent peak at 463nm and also the peak has a shoulder (range of values between two peaks) between 463nm and 535nm and the intensity decreases monotonically towards longer wavelengths.

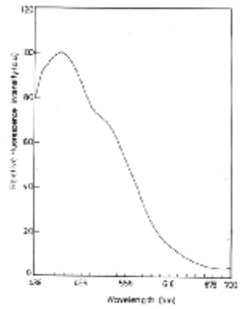

Fig 1.Fluorescence spectrum of averaged Normal oral mucosa at 405 nm excitation

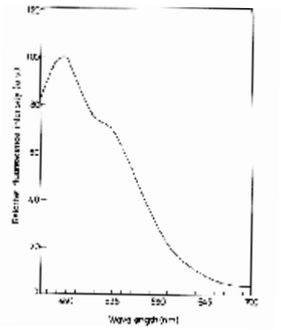

Fig 3. Fluorescence spectrum of fibroma at 405 nm excitation

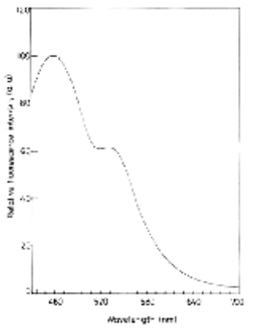

Fig 5.Fluorescence spectrum of fibrous epulis at 405 nm excitation

At 420nm excitation (Fig.2) the prominent peak observed at 483nm and peak has a shoulder between 483nm and 540nm and then the intensity decreases monotonically towards longer wavelength.

The spectrum for inflammatory and benign gingival lesions

The fluorescence spectra of histopathologically diagnosed case of Fibroma.

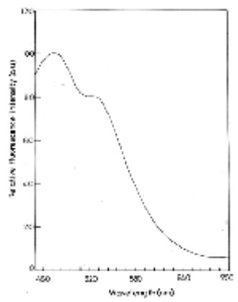

Fig 2.Fluorescence spectrum of averaged Normal oral mucosa at 420 nm excitation

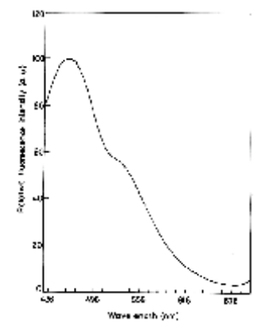

Fig 4.Fluorescence spectrum of fibroma at 420 nm excitation

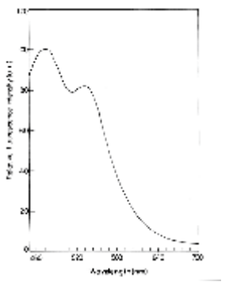

Fig 6.Fluorescence spectrum of fibrous epulis at 420 nm excitation

For 405nm excitation.

(Fig.3) the spectra shows a primary emission peak at 463nm and a additional peak at 535nm and then the intensity decreases towards the longer wavelength.

For 420nm excitation

(Fig.4) the spectra shows a primary emission peak at 463nm and a additional peak at 535nm and then the intensity decreases towards the longer wavelength.

The fluorescence spectra of histopathologically diagnosed case of fibrous epulis

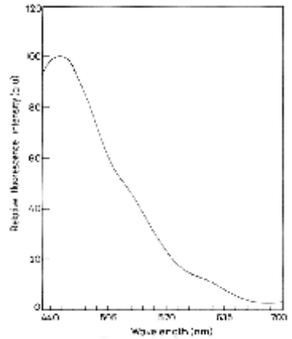

Fig 7.Fluorescence spectrum of gingival fibromatosis at 405 nm excitation

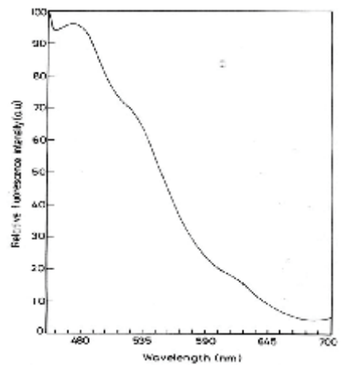

Fig 8.Fluorescence spectrum of gingival fibromatosis at 420 nm excitation

For 405nm excitation

(Fig.5) the spectra shows a primary emission peak at 463nm and a additional peak at 535nm and then the intensity decreases towards the longer wavelength.

For 420nm excitation

(Fig.6) the spectra shows a primary emission peak at 463nm and a additional peak at 535nm and then the intensity decreases towards the longer wavelength

The fluorescence spectra of histopathologically diagnosed case of gingival fibromatoses.

For 405nm excitation

(Fig.7) the spectrum shows a primary emission peak at 463nm and two additional peaks at 530nm and 630nm respectively.

For 420nm excitation

(Fig.8) the spectrum shows a primary emission peak at 463nm and two additional peaks at 530nm and 630nm respectively.

The fluorescence spectrum of malignant tissues

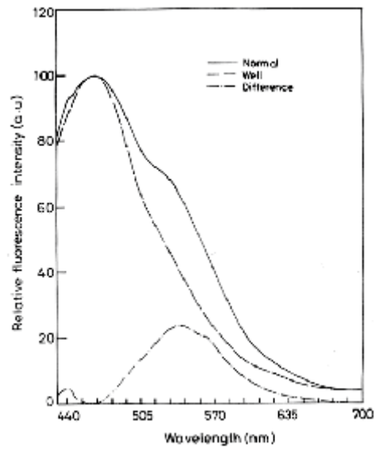

Fig 9.Fluorescence spectrum of averaged normal oral mucosa versus well differentiated squamous cell carcinoma and the difference between them at 405nm excitation

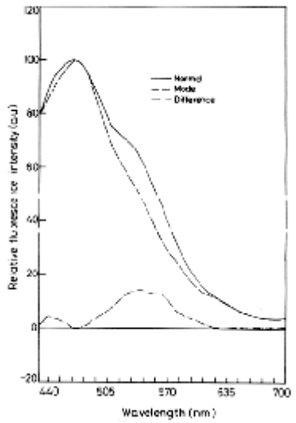

Fig 10.Fluorescence spectrum of averaged normal oral mucosa versus moderately differentiated squamous cell carcinoma and the difference between them at 405nm excitation

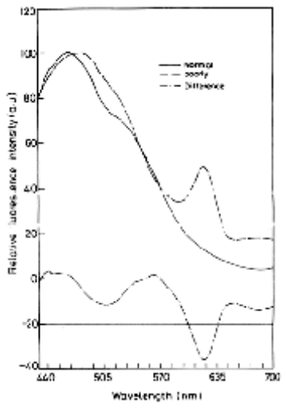

Fig 11.Fluorescence spectrum of averaged normal oral mucosa versus poorly differentiated squamous cell carcinoma and the difference between them at 405nm excitation

For 405nm excitation

The averaged fluorescence spectrum of histo-pathologically proven cases of well differentiated squamous cell carcinomas of oral cavity with respect to normal and their corresponding difference spectrum are shown in Fig.9 for 405nm excitation. It is observed that both normal and well differentiated squamous cell carcinoma spectra show a primary emission peak at 463nm. In addition to the primary peak the averaged emission spectrum of the normal subjects show a shoulder around 535nm which is absent in well differentiated squamous cell carcinoma. The difference fluorescence spectrum of well differentiated squamous cell carcinoma with respect to normal shows two peaks at 441nm and 540nm.

Fig.10 shows the averaged fluorescence spectrum of moderately differentiated squamous cell carcinoma with respect to normal and their corresponding difference spectrum. The primary emission peak of moderately differentiated squamous cell carcinoma is observed at 467nm. The difference spectrum of moderately differentiated squamous cell carcinoma with respect to normal shows three positive peaks at 441nm, 539nm and a minimum (negative peak) around 470nm.

Similarly the average fluorescence spectrum of poorly differentiated squamous cell carcinoma along with that of normal and their corresponding difference spectrum are shown in Fig.11. The spectrum of poorly differentiated squamous cell carcinoma shows a primary emission peaks at 478nm with a distinct secondary peak at 622nm. The difference spectrum shows two positive peaks at 441nm and 563nm and two negative peaks at 506nm and 622nm.

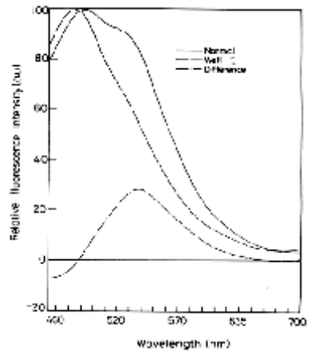

Fig 12.Fluorescence spectrum of averaged normal oral mucosa versus well differentiated squamous cell carcinoma and the difference between them at 420nm excitation

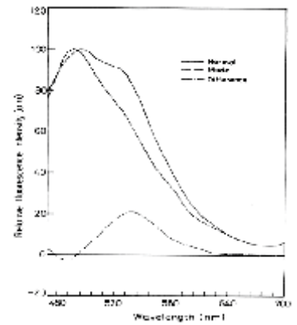

Fig 13.Fluorescence spectrum of averaged normal oral mucosa versus moderately differentiated squamous cell carcinoma and the difference between them at 420nm excitation

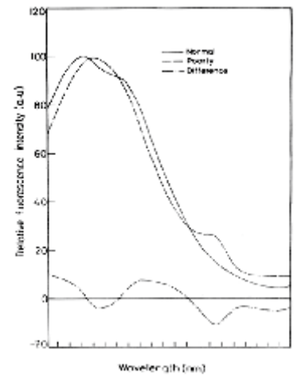

Fig 14. Fluorescence spectrum of averaged normal oral mucosa versus poorly differentiated squamous cell carcinoma and the difference between them at 420nm excitation

For 420nm excitation

The averaged fluorescence spectrum of histo-pathologically proven cases of well differentiated squamous cell carcinoma of oral cavity with respect to normal and their corresponding difference spectrum are shown in Fig.12 for 420nm excitation. It is observed that both normal and well differentiated squamous cell carcinoma spectra show a primary emission peak at 483nm. In addition to the primary peak, the averaged emission spectrum of the normal subjects shows a shoulder around 540nm which is absent in well differentiated squamous cell carcinoma with respect to normal shows one positive peak of 540nm and minimum around 454nm.

Fig.13 shows the averaged fluorescence spectrum of moderately differentiated squamous cell carcinoma with respect to normal and their corresponding difference spectrum. The primary emission peak of moderately differentiated squamous cell carcinoma is observed at 479nm. The difference spectrum of moderately differentiated squamous cell carcinoma with respect to normal shows a positive peak at 538nm and a negative peak around 470nm.

Similarly the averaged fluorescence spectrum of poorly differentiated squamous cell crcinoma along with that of normal and their corresponding difference spectrum are shown in Fig.14. The spectrum of poorly differentiated squamous cell carcinoma shows a primary emission peak at 495nm with a distinct secondary peak around 620nm. The difference spectrum shows a positive peak at 546nm and two negative peaks at 501nm and 620nm.

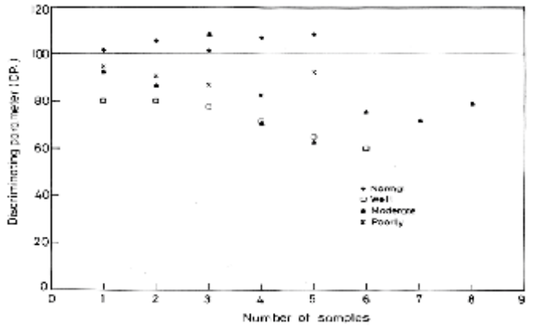

Fig 15. Averaged normal oral mucosa verses different grades of oral squamous cell carcinoma at 405nm excitation

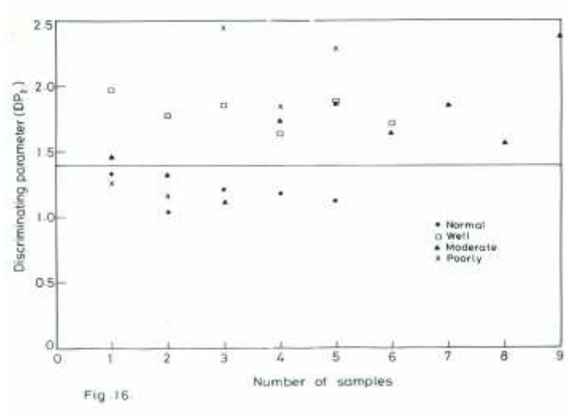

Fig 16.

Fig 16. Averaged normal oral mucosa verses different grades of oral squamous cell carcinoma at 420nm excitation

In order to quantify the results and to check whether there exists any significant difference between normal and malignant subjects, many workers have introduced several discriminating parameters from the measured fluorescence spectra, which could classify normal from abnormal subjects. Based on our results we have also generated different discriminating parameter, by using the fluorescence intensity at different emission wavelength for both 405nm and 420nm excitation. Among them the following two parameters were found to discriminate normal from the malignant subject significantly.

$DP_1 = (I_{441+} I_{539+} I_{559}) - I_{471}$ from fluorescence spectra at 405nm excitation

And

$DP_2 = I_{470}/I_{540}$ from fluorescence spectra at 420nm excitation.

DP_1 has been calculated by adding the emission intensities of the native fluorescence spectra of individual subject at all the positive peak emission wavelength and subtracting those at the negative peak emission wavelengths of the difference fluorescence spectrum. DP_2 is the ratio between the fluorescence intensities at 470nm and 540nm. The distribution of the values of these two discriminating parameters for normal and malignant subjects is shown in Fig.15 & 16 respectively.

DISCUSSION

The fluorescence spectral profiles of fibrous epulis, fibromas and gingival fibromatoses showed a striking similarity with that of normal tissues. At 405nm and 420nm excitation the peak observed at 463nm and 535nm were similar to normal tissues. This is attributed to the presence of the fluorophores NADH and FAD. However the reason for the porphyrin peak at 620nm in a case of gingival fibromatosis is not known.

At 405nm excitation, the primary emission peak of the averaged fluorescence spectra of moderately differentiated squamous cell carcinoma and poorly differentiated squamous cell carcinoma subjects is red shifted (shift towards longer wavelength) with respect to normal. This is not observed in the case of well differentiated squamous cell carcinoma.

At 420nm excitation, the primary emission peak of the averaged fluorescence spectra of well differentiated squamous cell carcinoma and moderately differentiated cell carcinoma is blue shifted (shift towards shorter wavelength) with respect to normal. In the poorly differentiated squamous cell carcinoma it is red shifted with respect to normal. The secondary maximum around 620nm for poorly differentiated squamous cell carcinoma may be attributed to endogenous porphyrins[11].

In our study the altered spectral signature of the different cancerous conditions of the oral tissues with respect to normal and the absence of the shoulder around 535nm in the case of the malignant subjects may be due to some micro-environmental changes of the native fluorophores such as NADH, FAD and endogenous porphyrins present in the

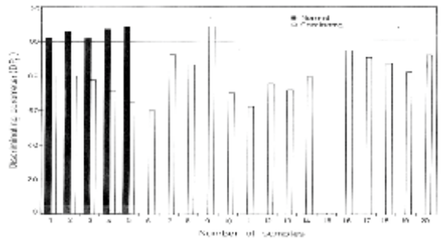

Fig 17. Normal oral mucosa verses oral squamous cell carcinoma at 405nm excitation

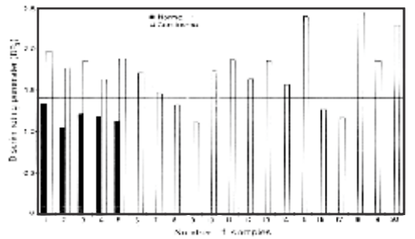

Fig 18. Normal oral mucosa verses oral squamous cell carcinoma at 420nm excitation

tissues which occurs during the transformation of the normal cells into malignant one. Although fluorescence emission around 620nm due to porphyrins is found to be more in the case of poorly differentiated squamous cell carcinoma it is not observed in the case of other grades of malignancy such as well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma.

Based on Fig.17 a critical value of 100 is fixed to discriminate the normal from cancer at 405nm excitation it is found that all the normal samples are classified as normal indicating 100% specificity and out of 20 cancerous tissues only one case is misclassified, indicating a sensitivity of 95%. Similarly for 420nm excitation Fig.18 a critical value of 1.4 is fixed to discriminate the normal from cancer, with the normal subjects, yielding a specificity of 100% and four cases of oral malignant subjects are misclassified resulting in a sensitivity of 80%. Among these two discriminating parameter the values of DP_1 at 405nm excitation is found to be more sensitive in discriminating oral malignancy from normal when compared to DP_2 at 420nm excitation.

In the present study the absence of porphyrin peak in the case of well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma can be attributed to some bio-chemical alteration probably due to keratin formation in these tumours as compared to poorly differentiated squamous cell carcinoma.

Alfano et al in their study of laser induced fluorescence spectroscopy from native cancerous and normal tissues of Rat Prostate, Rat Kidney and Mouse bladder found prominent peak at 521nm region[11]. They attributed this peak to Flavins, which are known to fluorescence between 520nm -535nm. Recently, keratin was found in thymoma tissues. They found that the fluorescence spectra from keratin to be broad with maxima located at 525nm.Hence they attributed the peak at 521nm may also be due to keratin.

In the present study we have observed an overall decrease in the fluorescence intensity of malignant tissues with respect to normal except in the case of poorly differentiated squamous cell carcinoma. P.K.Gupta observed the same features in his study of oral cancers and found this result is in contrast to breast malignancy, breast cancers showing higher fluorescence intensity than normal breast tissues[12]. As keratin formation is unique to oral tumours an extensive study of keratinizing tumours has to be carried out, to make firm conclusions about the role of keratin in bio-chemical alterations, during malignant transformation.

From this study, it is worth to mention that the discriminating parameter DP_1 Fig.17 (100 indicates normal tissues, 100 indicates cancerous tissue) discriminates all grades of malignancy with respect to each other. Hence it is essential to carry out more detailed investigation with more samples especially of dysplastic lesions and detailed statistical analysis has to be made in order to improve this existing technique before utilizing the same in clinical diagnosis.

CONCLUSION

By comparing the visible auto-fluorescence spectra of native cancerous and normal tissues, it has been

found that the auto-fluorescence spectral characteristics of the cancerous tissues are distinct and different from those of normal lesions.

1. Auto-fluorescent spectra of normal tissue and the inflammatory and benign lesions are similar both at 405nm and 420nm excitation.
2. The auto-fluorescence spectral characteristics of the cancer tissues are distinct and different from those of normal lesions.
3. The spectral profiles of Keratinizing tumours such as well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma is different from poorly differentiated squamous cell carcinoma at both 405nm and 420nm excitation.
4. Discriminating parameter (DP_1) clearly distinguish the cancer from normal with a sensitivity of 95%. However, this parameter is unable to differentiate the different grades of squamous cell carcinomas, as the sample size is small.
5. A study of large number of samples of oral squamous cell Carcinoma and dysplastic lesions is suggested and a detailed statistical analysis has to be made in order to improve this existing technique before utilizing the same in clinical diagnosis.
6. If incorporated in a fibro-optic system, this could be used as an in-vivo, non-invasive, simple technique for wide area surveillance of mucosa in individuals at risk of development of oral cancers, in mass screening setup.

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