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Optical Spectroscopy as a Diagnostic Tool in Oropharyngeal Carcinoma & Its Correlation with Clinico-Histological Diagnosis

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Abstarct

Background: There is a rising trend of oropharyngeal cancer, but a easy, safe and non –invasive test to differentiate the benign from malignant lesion is still at an early stage. Bulk fluorescence is an important step in this regards. Auto-fluorescence from biological agents like collagen and NADH (Nicotinamide adenine dinucleotide-reduced form) tissues involves evaluating the biomolecular environment through optical spectroscopy. *Aim of Study:* To study the role of optical spectroscopy in differentiating the benign from the malignant oral lesions by detecting the change in the bulk fluorescence, especially of collagen and NADH. *Method*: A study of 28 patients with oropharyngeal lesion was done. In all 28 freshly excised tissues, bulk fluorescences were measured by optical spectroscopy. Later the same excised tissues were processed for histopathology as gold standard. *Results*: This study showed that bulk fluorescence of collagen is a good method of investigation with 64.29% sensitivity than bulk fluorescence for NADH, which is only 35.70%. *Conclusion*: Optical spectroscopy has a bright future in differentiating the benign and malignant oropharyngeal lesion. Furthermore, it can detect cancer at an early stage.

Keywords: Bulk Fluorescence; Collagen and NADH (Nicotinamide Adenine Dinucleotide-Reduced form); Oropharyngeal Lesions.

Introduction

Development of safe, non –invasive and affordable techniques for early pathological diagnosis of oropharyngeal carcinoma has achieved promising success, though still in its infancy. Being prevalent, but entirely preventable disease and is caused by mostly by tobacco abuse, either with or without alcohol. Because of the crucial role played by the oral cavity structures in articulation and deglutination, treatment of an oral malignancy can have significant impact on patient's quality of life.

The typical demographic profile of oropharyngeal carcinoma is squamous cell carcinoma arising in oropharyngeal region of a man in 5th to 8th decades of the life who is a smoker and a drinker. On the other hand Mendez et al found that patients younger than 40 years of age with early stage disease (stage I or stage II) have a favorable direct survival rate of 76% [13]. So, the early diagnosis of oropharyngeal carcinoma is promptly needed. Apart from clinical acumen with high index of suspicion, non-invasive or minimal invasive tool with high sensitivity is also required. Optical spectroscopy is being used as very promising and sensitive tool for characterization of physical and chemical changes that occur in diseased tissue and for early detection of cancerous changes in body.

The optical spectroscopy for non- invasive probing of human tissue based on the florescence property of certain proteins, amino acids, nucleic acids, coenzymes etc. which changes with the change in architecture of the molecule or changes in its environment. This technique is a new diagnostic modality with the potential to bridge the gap between clinical examination and invasive biopsy. Most cases of the oropharyngeal SCC are associated with life style

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and tobacco being the most common abuse in the form of cigarette or cigar smoking, snuff dipping and tobacco chewing. Other abuses are alcohol and betel chewing with or without slaked lime. The risk of oropharyngeal cancer increases with larger amounts and longer durations of the tobacco. Carcinogens in tobacco are believed to act as initiators, as well as promoters, while alcohol acts as co-carcinogen or promoter. The risk of development of oropharyngeal SCC in the heavy drinker and smoker is 15 times greater than in those who neither smoke nor drink.

The tumours were more likely to have a higher frequency of common genetic changes such as 3p, 4q, 11q13; higher the percentage of chromosomal microsatellite abnormalities and a higher rate of HPV infection. A kindred with a germline mutation of p16 gene, which is normally responsible for a protein inhibitor of cell proliferation, may represent the genetic basis of a familial predisposition to the development of oropharyngeal SCC. *Kamath, Mahato et al* carried out spectral analysis and classification for discrimination of pulsed laser induced autofluorescence spectra [10

Optical Spectroscopy

The initial work was done by *Alfano et al* to establish a difference in the fluorescence spectra of normal and tumor rat tissues, such as kidney, bladder and prostate. Later the study was extended to human tissues and again a difference in fluorophores concentration was observed in healthy normal tissue compared to abnormal one [1,2]

Significant progress has been made in cancer diagnosis using laser induced fluorescence in gastro-intestinal tissues [5]

Neoplastic oral mucosa shows consistent differences in auto florescence spectral intensity when compared with the normal mucosa in the same individual. These early results show that fluorescence spectroscopy may represent a useful technique for non-invasive diagnosis of cancer of the upper digestive tract [8].

It was earlier indicated that fluorescence spectroscopy may be useful in differentiating malignant or premalignant oral tissue from normal oral mucosa [3]. Time-resolved autofluorescence spectroscopy is a also a very sensitive technique for in vivo diagnosis of oral premalignant lesions [4].

Gillenwater et al also found that consistent difference exists between the florescence spectra of abnormal and normal oral mucosa. Therefore florescence spectroscopy has a potential to improve the non-invasive diagnosis of the oral cavity neoplasia. Further studies will better define the role of this technique in the detection of premalignant and early oral cancer lesions [9].

Fluorescence and spectroscopic features may distinguish diseased from healthy tissue and follows an introduction for the clinician to this fascinating and complex field. It is possible to obtain a diagnosis in real time and with the appropriate use of photochemicals and image enhancement. It may be possible to treat and monitor the effects of treatment in real time [14].

Influence of anatomical location was investigated on auto fluorescence of different healthy mucosas and difference was observed in fluorescence intensity between locations. Normalized spectra looked similar for locations, except for the dorsal side of the tongue (DST) and the vermilion border (VB). Porphyrin-like fluorescence was observed frequently especially at DST. No relevant systematic spectral differences have been observed between most locations allowing the use of one large database. For DST and VB separate database is required [6]. Some statistically significant differences between surrounding / contralateral tissues of benign and healthy tissue and of (pre-) malignant lesions are also observed. Healthy mucosa can be separated successfully from cancers. However, auto fluorescence spectroscopy is not able to distinguish from visible (pre-) malignant lesions [7].

Blue excitation light (400 to 460 nm) to excite green-red fluorescence from fluorophores in the oral tissues was also employed. Results from a pilot study of 44 patients were presented. Using histology as the gold standard, the device achieves a sensitivity of 98% and specificity of 100% when discriminating normal mucosa from severe dysplasia / carcinoma in situ (CIS) or invasive carcinoma [11].

Direct multi-class spectroscopic diagnostic algorithm for discrimination of high-grade cancerous tissue sites from low-grade as well as precancerous and normal squamous tissue sites of human oral cavity was also developed. The in vivo auto fluorescence spectral data acquired from patients screened for neoplasm of oral cavity was used to train and validate into algorithm. The diagnostic algorithm based on total principal component regression was found to provide satisfactory performance in classifying the tissue sites in above four different classes [12]

Material & Methods

The patients included in the study presented with

complaint of growth with ulcerative lesion in oral cavity. They were categorized clinically as well as according to FNAC. Total number of cases studied was 28. Morphological and chemical changes that occur when a tissue proliferates in an exaggerated normal or abnormal fashion cause the fluorescence inside the tissue to fluorescence differently as compared to their normal environment. Fluorescence spectroscopy can differentiate the biochemical and morphological changes of normal and diseased tissue. In our study the fluorophores studied were NADH and collagen. When excited at 350 nm they show peak fluorescence at 400 nm and 460 nm respectively. The media itself has scattering and absorption effects, thus distorting the result. Bulk fluorescence is free from the above effects thus permitting better characterization of normal and tumorous tissue.

The Following Protocol was Followed in Each Case:

a. Optical spectroscopic analysis of specimens.
b. Histopathological examination of specimens.

Fluorescence Studies

The pioneer work to recover fluorescence from tissue using NADH as fluorophore was done by *Mayevsky and Chance* [15]. Fresh tissue sample's chunk was obtained immediately after biopsy. Then thoroughly cleaned and washed with normal saline to remove blood and slimy materials. Simultaneously the adjacent normal tissue was also taken for fluorescence analysis. The tissue selected for fluorescence studies was placed in normal saline kept in an ice container and taken immediately for spectroscopic analysis within 4-6 hrs of biopsy procedure. This was done to preserve the normal architecture of cell like a living tissue. During experiments the tissue was kept at room temperature, moist with isotonic saline. The tissue was placed on a quartz plate of size 3cm×1cm×2cm of Fluorolog®-3 spectrfluorometer (Jobin Yvon, USA). The samples were excited with an ozone free xenon lamp of 450 W power, which delivers light from 240 nm to 850 nm for sample excitation, using a emission spectrometer and photo multiplier tube (PMT, Model:R928) and simultaneously recording the elastic scattering spectra. Fluorescence spectra were taken at 290 nm, 350 nm, 436 nm, 470 nm and 488 nm excitation wavelengths. The output of the detector was connected to computer for data acquisition and analysis by Datamx™ software. The 450 W Ozone free Xenon lamp used provides a relatively continuous light output from 240 nm to 850 nm for sample excitation. The Xenon lamp spectrum exhibits a characteristic peak around 467 nm, which can be used to indicate whether the excitation spectrometer is properly calibrated. The lamp has an approximate life of 2000 hours and is Ozone free.

The block diagram of the experimental setup used for the measurement of fluorescence form human tissue is shown in Figures 1. The samples were excited with vertically polarized light. The parallel (V_V) and perpendicular (V_H) components of the fluorescence were collected in the reflection geometry. In the same geometry parallel and perpendicular component of scattered light were also collected.

The fluorescence power per unit area escaping the tissue is related to the distribution of the excitation radiation with the tissue. For a homogenous tissue with uniform structure, the fluorophores are present everywhere inside the tissue. Hence, the path travel by the excitation light (λes) is negligible in comparison to the fluorescence light (λem) and suffers many scattering before coming out of the surface. The excitation polarizer was kept horizontal and emission polarizer was placed at horizontal for co-polarized fluorescence and vertical orientation for measurement of cross polarized fluorescence.

Fluorescence Extraction and Study of Fluorophores in Oral Tissue

The dominant fluorophores found in oral tissue are collagen (peak around 400 nm) and NADH (peak around 460 nm) when excited with a wavelength of 350 nm. Collagen is found in the stromal layer and NADH is found in epithelial layer. Collagen is structural proteins whose cross links break when a healthy tissue develops into a cancerous one. NADH is basically the indicator of the metabolic activities inside a cell. As a healthy tissue develops into abnormal one, cells multiplies, thus increase nuclear – cytoplasmic ratio increases. NADH is found in mitochondria, thus increase in cell density increases NADH in cancer.

Collagen Contribution in Oral Tissue Spectra

Here the absolute contribution of collagen fluorophore in the stroma layer studied for both normal and cancer tissue. As mentioned earlier the collagen peak is around 400 nm. There is generally small shift of this peak in the bulk fluorescence spectra sample wise, hence a band of 20 nm (i.e. 400 ± 10 nm) is chosen to incorporate the collagen peaks and the area under it is calculated. This 20 nm band area is calculated for both normal and cancer from bulk

spectra. Finally the ratio of area under 20 nm collagen band in normal to that in cancer is calculated, taking stroma layer in account since collagen is the dominant fluorophore there.

NADH Contribution in Oral tissue

Taking the area of a 20 nm band around the NADH peak of 440 nm, no significant discrimination is possible between normal and cancerous tissue. However, since an overall decrease in fluorescence intensity is noticed in the cancerous tissue, a normalization of the NADH band with respect to the entire fluorescence may provide information on absolute value of concentration of NADH. It may be noted that this was not required in the case of collagen since the collagen yield is much higher than that of NADH and hence is not affected by the overall fluorescence.

Oral Tissue and Its Fluorescence Spectra

The 350 nm wavelength as excitation wavelength is focused on the epithelial layer of oral tissue kept in the sample compartment of the fluorimeter and the corresponding fluorescence spectra are recorded for both parallel and perpendicular components. Figures (2) show the bulk fluorescence spectra (Co-polarized; V_V) and Cross polarized fluorescence; V_H) from normal oral tissue For histopathological examination corresponding tissue obtained by biopsy were taken, placed in 10% buffered formalin were processed for making paraffin blocks for routine histological diagnosis by H&E method.

Observations

In this study an optical spectroscopy analysis was done in 28 samples to evaluate the changes in fluorescence due to biochemical changes in abnormal tissue. The fluorophores studied were collagen and NADH. After spectroscopic analysis, all 28 samples were sent in 10% formalin for histopathological evaluation, which is the gold standard investigation.

Maximum oral carcinomas (42.86%) were reported in the age group of 51-60 years. Patients in the age group of 21-30 years show a significant number of oral cancer cases (14.29%) that can be explained by increased tobacco use (Table 1). Most of the oral carcinoma affects the lower alveolar ridges (39.29%) while the hard palate, maxillary alveolar ridge and floor of antrum show involvement to a lesser degree (Table 2).

Considering that in emission spectra (bulk fluorescence) collagen peak is around 400 nm and NADH peaks at 460 nm, we have calculated the area under the 20 nm bandwidth at each of those wavelengths i.e. 400 ± 10 nm. This is done for cancerous tissue and its normal counterpart.

Finally, the ratio of area under 20 nm bandwidth in normal to that in cancer is calculated for each fluorophore i.e. collagen and NADH and we get following results. The results of bulk fluorescence were taken into consideration. Where B(X) = Ratio of area under 20 nm bandwidth in normal to that in cancer for collagen and B(Y) = ratio of area under 20 nm bandwidth in normal to that in cancer for NADH. (Table 3)

Here, in case of bulk fluorescence study on collagen, 18 samples show ratio greater than one i.e. detected cancerous while 10 show ratio less than one. Therefore detection rate of cancer by using collagen bulk fluorescence was (=18 /28 x100) 64.29% and in 10 samples, it could not detect the cancer. While study on NADH flurophore only 10 cases show more than one ratio and so detection rate by using NADH bulk fluorescence (=10 /28 x100) 35.71% and it could not detect cancer in 18 samples.

It can be well observed that a disproportionately higher values of bulk fluorescence has been found in two patients i.e. P12 and they are in the age group of 60-70 years. Thus, there might be some age related factors that influences the values of fluorescence in

Table 1: Age wise distribution of oral carcinoma

Age – Group	Number	%age
0-10	-	-
11-20	-	-
21-30	2	14.29
31-40	1	7.14
41-50	4	28.57
51-60	6	42.86
61-70	1	7.14
Total	**14**	**100.00**

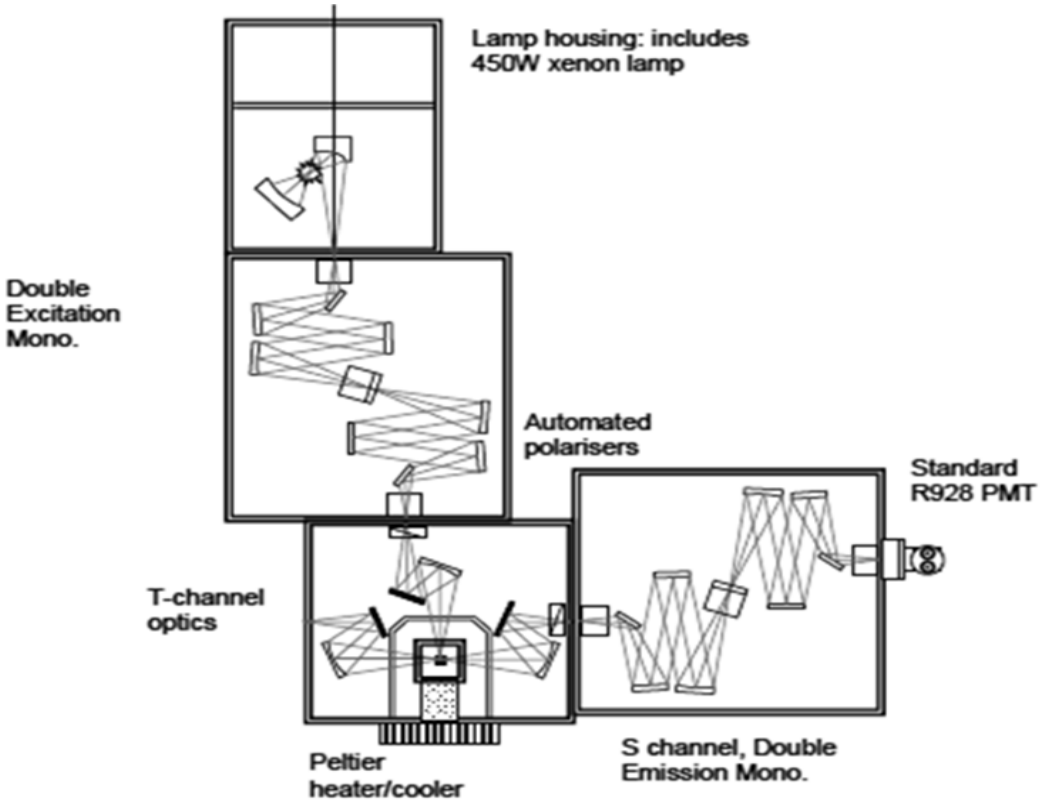

Fig. 1: Experimental Setup Fluorolog®-3 (Model FL3-22)

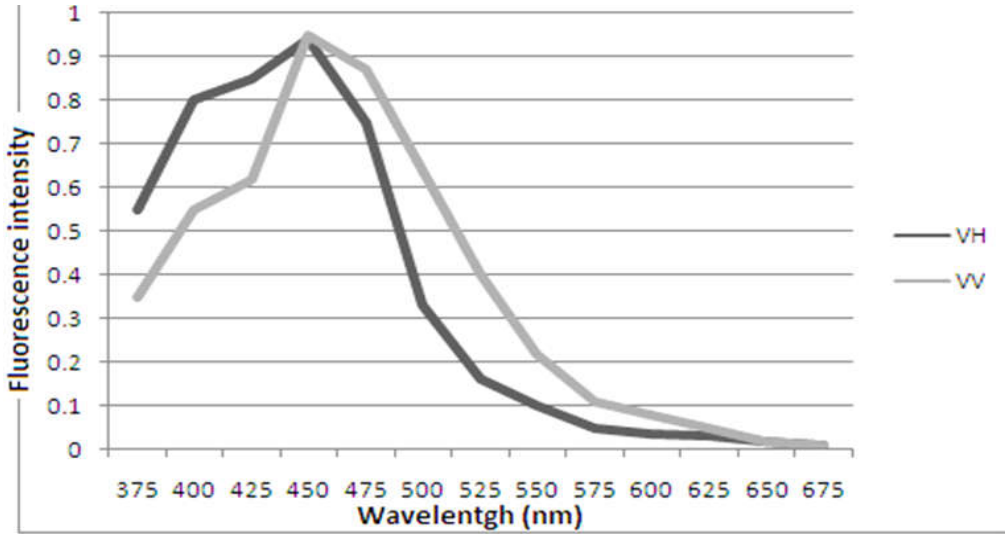

Fig. 2: The bulk fluorescence spectra (Co-polarized; V_V) and Cross polarized fluorescence; V_H) from normal oral tissue

Table 2: Table showing site location of oral carcinoma in oral cavity

Sites	No. of Cases	%age
Tongue	6	21.43
Floor of mouth	4	14.29
Gingival & alveolar ridge (lower alveolar ridge)	11	39.29
The buccal mucosa	6	21.43
The hard palate, maxillary alveolar ridge and floor of antrum	1	3.57
Total	**28**	**100.00**

Table 3: The results of bulk fluorescence

Patients	Bx (Collagen)	Bx (NADH)
P1	1.42	1.28
P2	0.46	0.42
P3	5.41	6.21
P4	1.67	1.10
P5	0.08	0.23
P6	0.54	0.48
P7	3.26	1.97
P8	4.32	6.22
P9	2.17	1.79
P10	0.56	0.49
P11	0.34	0.55
P12	21.27	20.25
P13	3.28	2.29
P14	11.13	11.12
P15	1.33	1.87
P16	0.52	0.59
P17	6.02	6.35
P18	1.57	1.19
P19	0.14	0.80
P20	3.98	4.55
P21	0.68	0.87
P22	6.82	7.84
P23	13.20	12.53
P24	0.61	0.57
P25	0.47	0.74
P26	19.27	20.10
P27	4.08	3.58
P28	2.15	1.88

these patients but this fact can't be strongly proposed as the number of patients here is only two.

Thus, it is obvious that bulk fluorescence of tissues done by using collagen is a more sensitive parameter than NADH for detecting oropharyngeal carcinoma.

On the other hand histopathology revealed all the 28 samples to be malignant, out of which, 18 samples showed well differentiated Squamous cell carcinoma, 4 cases showed moderately well differentiated Squamous cell carcinoma and rest 6 cases were categorized under poorly differentiated squamous cell carcinoma.

As seen above, the overall diagnostic accuracy of optical spectroscopy as compared to histopathology is 64.29%, which could be increased if study was done with more samples.

Discussion

Fluorescence spectroscopy has shown potential as a new diagnostic tool for the detection of oral cancers and precancerous lesions. Auto fluorescence of tissues under excitation with light is produced by several endogenous fluorophores. These include fluorophores from tissue matrix molecules and intracellular molecules like collagen, elastin, keratin and NADH. The presence of disease, changes the concentration of these fluorophores, which makes fluorescence spectroscopy sensitive to tissue alterations. Fluorescence analysis was done on 28 samples. The two fluorophores studied were collagen and NADH. The bulk fluorescence was considered here for these fluorophores.

Optical spectroscopy was done on 28 samples. The results showed 18 samples to be malignant out of 28 based on collagen as fluorophore. After the spectroscopic study, all the specimens were sent in 10% formalin for histopathological examination, which revealed all to be malignant confirming histopathological examination as a gold standard. So malignancy could not be detected in specimens by spectroscopy in 10 cases.

Optical spectroscopy detects these biochemical changes which precede architectural changes. Two unique properties are seen in the molecules-

1. The property to fluorescence that to at a specific wavelength. This wavelength is 400 nm for collagen and 460 nm for NADH, when excited at 350 nm.
2. Fluorescence properties of these molecules changes with change in structure or change in the surrounding environment

The detection of these subtle changes in fluorescence properties of molecules could be considered to be signature of disease causing it.

Conclusion

Following conclusions were made by fluorescence analysis:

1. Bulk fluorescence for collagen is more sensitive i.e. 64.29% than bulk fluorescence for NADH, which is only 35.70%. Thus, collagen is a sensitive fluorophore than NADH for detection of oral cancer.
2. During collection and transportation samples , It may be assumed that NADH might be dissociated into NAD+ and H+, thus leading to decrease in the accuracy of results.
3. There might be some age related factors that can affect the values of bulk fluorescence but this needs further evaluation.
4. Though, the peak incidence of malignancy was in 5th to 6th decade which included 42.86% of all cases. But the incidence is increasing particularly in younger people in 2nd to 3rd decades accounting for 14.29% of all cases (Table 1). The most common site location was found to be lower alveolar ridge, which accounted for 39.29% all cases and hard palate, maxillary alveolar ridge and floor of antrum to be the least common site (Table 2).

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