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IN-VIVO AUTOFLUORESCENCE SPECTROSCOPY IN ORAL CANCER DIAGNOSIS: A SYSTEMATIC REVIEW

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ABSTRACT

Oral cancer remains a major health problem in South and South East Asian countries. Despite the therapeutic advancements, the 5 year disease free survival rate has been around 50% only. The main problems associated with poor prognosis are late diagnosis of cancer and recurrence of the cancer. This systematic review aims in the assessment of the reliability of autofluorescence spectroscopy in oral cancer diagnosis. Autofluorescence spectroscopy is a non-invasive diagnostic tool. It can assess the molecular level changes which affect the optical properties of tissues. In this review a total of 9 studies have been assessed for their diagnostic accuracy. The review shows that porphyrin as a fluorophore has a good potential in diagnosis of oral cancer.

KEYWORDS: Autofluorescence spectroscopy, fluorophore, native fluorescence spectroscopy, oral cancer, non-invasive diagnosis

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INTRODUCTION

Oral cancer has become a global health burden and accounts for the 6th most common malignancy worldwide. The increased usage of tobacco and related products in India has lead oral and oropharyngeal malignancies to account for more than half of all the malignancies in this part of the world. Estimates have revealed more than 90% of oral cancers are of the squamous cell carcinoma type.[1] The literature search reveals that oral cancers diagnosed in stage I (early stage cancers) have a 5 year disease free survival rate of 80% after treatment, while cancers diagnosed in stage IV have a 5 year survival rate of 20%. Some of the early stage lesions are innocuous and easily deceive a routine clinical examination.[2] Currently the gold standard for cancer diagnosis is through incisional biopsy. But the process of biopsy has a few pitfalls when diagnosing early stage lesions. The site of tissue that is incised in case of early stage lesions is decided on clinical grounds. But in early stage lesions the entire lesion may not show dysplastic features. In these lesions the tissues exhibit precancerous changes and interspersed with dysplastic changes in some sites. Hence if the tissue incised shows only precancerous changes the treatment plan changes which may allow the dysplastic changes to remain recalcitrant to the treatment and cause it to spread further.[3,4] Hence it will be prudent to look for molecular markers or other changes at the cellular level to detect early changes which in turn improves patient prognosis and reduces morbidity. The process of carcinogenesis is multistep and is filled with a lot of structural and biochemical changes in the epithelium and underlying stroma which causes an alteration in the optical properties of the tissues. The biochemical changes precede the structural(morphological) changes. The prominent changes are a rise of reduced Nicotinamide Adenine Dinucleotide (NADH), altered or reduced Collagen and, increased porphyrins which are a constituent of hemoglobin.[5,6] The rise in NADH, a constituent of electron transport chain, is attributed to the increased energy requirements by a dysplastic cell due to the rapidity of the divisions.[5-8] With regards to porphyrin there is a rise in concentration of porphyrin due to increased microbial synthesis: A cancerous tissue with its thickened or disrupted epithelium attracts opportunistic pathogens like candidiasis which leads to an increased production of porphyrins,[9-12] increased vascularity of the cancerous tissue causes an increased concentration of hemoglobin[9,12,14] & accumulation of protoporphyrin IX due to the lack of ferrochelatase in the cancerous cells.[9] These biomolecules which are optically sensitive are referred to as fluorophore. Each fluorophore has a particular excitation wavelength and it undergoes a process of fluorescence and emits a light of a particular wavelength. The fluorophore has a tendency to absorb light at its particular excitation wavelength and reemit the light at its emission wavelength for a specific period of time referred to as the lifetime of fluorescence.[7] Spectroscopy is a method in which an instrument with a fibreoptic probe is connected to two spectrometers viz the excitation and emission spectrometers. Depending on the molecule to be assayed the wavelength of the spectrometers are set and the fluorescence intensity is recorded. Spectrometers can measure the intensity by fixing either the excitation or emission wavelengths. Modern day spectrometers can also assay the life time of fluorescence. Optical Spectroscopy can be used as a diagnostic tool to measure the biochemical changes occurring inside a cell in a non-invasive way. Autofluorescence spectroscopy is a method where the fluorescence properties are studied, without the application of any dye. The excitation and emission wavelengths of the fluorophores which have been studied in oral cancer are listed in table 1.[7,8]

Table 1
Molecules which can be used in the diagnosis of oral cancer with their excitation and emission wavelengths

Endogenous fluorophore	Excitation maxima	Emission maxima
NADH	337	450-455
Porphyrin	404-410	635
Collagen	350-60	380-400

AIM

The aim of this systematic review article is to assess on the reliability of autofluorescence spectroscopy for the diagnosis of oral cancer. A systematic search of the literature for studies based on autofluorescence spectroscopy in the diagnosis of oral cancer was carried out to analyse the studies in terms of diagnostic accuracy based on their sensitivity and specificity for each fluorophore.

SEARCH METHODOLOGY

A systematic search of the published articles was done with the electronic database. The database that were used for the search are PubMed, Sciencedirect and Cochrane database. The search strategy was carried with the keywords of "autofluorescence spectrscopy", "native fluorescence spectroscopy", "oral cancer", "oral malignancy", "squamous cell carcinoma". The primary filters that were employed for the selection of abstracts were, human studies and language of the articles in English. The search yielded an abstract count of 53 in pubmed, 24 from science direct and 0 from Cochrane database. A detailed count of articles is given in table 2. A final count of 59 articles were included for the primary stage of filtration after the elimination of overlapping articles.

Table 2
Search results

Keywords	PubMed	Science Direct	Cochrane
Autofluorescence spectroscopy AND oral cancer	50	22	0
Nativefluorescence spectroscopy AND oral cancer	6	0	0
Autofluorescence spectroscopy AND oral malignancy	44	12	0
Nativefluorescence spectroscopy AND oral malignancy	6	0	0
Autofluorescence spectroscopy AND oral squamous cell carcinoma	23	7	0
Nativefluorescence spectroscopy AND oral squamous cell carcinoma	7	0	0

From the available abstracts the primary selection of clinical trials were done. The criteria employed in case of primary filtration included the usage of biopsy to confirm the diagnosis. Studies which confirmed oral malignancy based on the clinical observation parameters and exfoliative cytology specimens were excluded from the review. The full text of the 24 articles that were selected from the primary stage was obtained. Here based on the aim of this systematic review the index test was the usage of autofluorescence spectroscopy and reference test was biopsy.

The following inclusion criteria were adopted:

1) Clinical trials with informed consent and ethical clearance on humans.
2) Clinical trials where the samples have been subjected to the index test and the results blinded and the samples then subjected to the reference test.
3) Those clinical trials which had reported the results in terms of sensitivity, specificity or the area under the Receiver Operator Characteristics (ROC) curve were included.

The exclusion criteria that were chosen are:

1) Clinical trials which had excluded normal tissue from non-tobacco users as healthy controls.
2) The clinical trials with no detailed explanation of the excitation and emission wavelengths were not included.

RESULTS

The full text of the 24 articles were subjected to the secondary stage of article selection using the aforementioned inclusion and exclusion criteria. This had yielded a total of 11 studies for the data extraction.[5,15-22] Within this group of 11 clinical trials 2 clinical trials had used principal component analysis to develop algorithms for diagnosis. Since they were outside the aims specified for the review they were excluded. Hence 9 clinical trials were included in this systematic review and the results of the 9 clinical trials are listed in table 3.

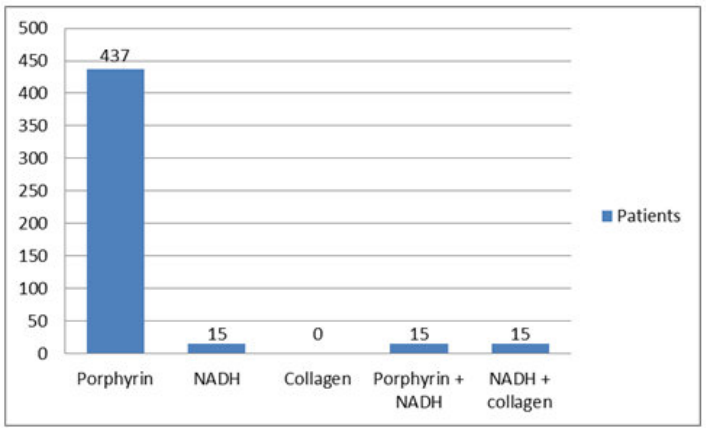
Table 3
Results

Study	Study design Sample size	Spectroscopy parameters	Data analysis	Results
Gillenwater et al (1998)[5]	In vivo 33 sites from15 patients 8 healthy volunteers	Emission spectra after excitation 337 nm (NADH) 410 nm (Porphyrin)	Emission spectra showed a rise in 455 nm(NADH) and a rise in 635 nm (Porphyrin) 635/455	Sensitivity of 82% Specificity of 100%
Inaguma et al (1999)[15]	In vivo 78 patients 30 Normals	Emission spectra after excitation 410 nm (porpyhrin)	Emission peak at 635 nm observed with a higher intensity in cancer patients compared to normals	85% sensitivity 90% specificity
Muller et al(2003) [16]	In vivo 53 sites from 15 patients 38 sites from 8 healthy volunteers	Emission spectra after excitation 337 nm (NADH) 358 nm (Collagen)	Rise in 450 nm (NADH) spectra Fall in 390 nm (Collagen) Spectra	Sensitivity of 96% Specificity of 96%
Tsai et al (2003)[17]	In vivo 15 patients 15 healthy volunteers	Emission spectra after excitation 330 nm (NADH)	Emission peak at 480 nm and raised peaks at 360 nm compared to normals.Spectral intensity ratio of 480/360	sensitivity 81% Specificity 87%
de Veld et al (2004)[18]	in-vivo 155 patients 96 healthy volunteers	Emission spectra after excitation 365 nm, 385 nm, 405 nm, 420 nm, 435 nm, 450 nm	Emission peak at 635 nm only in malignancy cases (corresponds to porphyrin) other emission wavelengths produced no significant effect	ROC - AUC cancer vs healthy 0.9 to 0.97 ROC-AUC pre cancer vs healthy 0.5 to 0.7
Mallia et al (2008)[19]	In vivo 44 patients 35 healthy volunteers	Emission spectra after excitation 404 nm (porphyrin)	Peak at 500 nm in case of malignancy shifted to 520 nm. A prominent peak at 635 nm only in malignancy cases. Spectral intensity 635/500	sensitivity and specificity of 100%
Chaturvedi et al (2010)[20]	In vivo 97 patients with oral cancer 20 patients with oral submucous fibrosis 27 patients with leukoplakia 26 healthy volunteers	Emission spectra after excitation 404 nm (porphyrin)	390 nm(collagen)peak characteristic in Oral submucous fibrosis Fall at 450 nm(NADH) peak in malignancy cases Peak at 635 nm (porphyrin)characteristic of malignancy cases	Spectral ratio for the peak at 635 nm Sensitivity of 98% Specificity of 100%
Meir et al (2010)[21]	In Vivo 53 patients 30 normals	Life time fluorescence 410 nm (porphyrin)	1.7 +/- 0.06 ns for normals 1.3 +/- 0.06 ns for cancer	p<0.0025
Ebenezer et al(2012) et al[22]	In vivo 10 patients 15 healthy volunteers	Excitation spectra after excitation 635 nm (porphyrin)	Emission peak at 410 nm only in malignancy cases (corresponds to porphyrin)	Spectral peak at 635 nm Sensitivity of 100% Specificity of 100%

DISCUSSION

The most common way of detecting a malignant lesion has been a clinical examination which consists of an inspection and a palpation. Then to confirm the diagnosis the gold standard of biopsy was performed. The site of the biopsy has to be best representative of the disease which is mostly decided by the clinical examination.[1] However, in several early stage lesions and in premalignant lesions with early dysplasia, it is difficult even for an experienced clinician to decide on the ideal site of biopsy.[9] In this situations, it would be better if an adjunct is used to arrive at a most probable site for biopsy. In screening camps for oral cancer in addition to the oral examination, if these adjuncts are used, it will be helpful in early diagnosis. Thus an adjunct should have the attributes of being reliable, objective and non-invasive. These adjuncts when used can bridge the gap between under diagnosis and the need for repeated biopsies.[1] The various adjuncts that are used in the diagnosis of oral cancer are velscope, vizilite, toluidine blue, oral brush biopsy, salivary biomarkers. Autofluorescence spectroscopy has been an emerging tool which has been used as an adjunct in oral cancer diagnosis.[5] Autofluorescence was used in two main forms viz autofluorescence imaging and autofluorescence spectroscopy. Autofluorescence imaging was the first used modality among the two.[9] The principle of autofluorescence had its origin from the therapeutic concept of photodynamic therapy.[23,24] In photodynamic therapy a photo sensitizer was applied to the tissue. The dysplastic tissue retained the dye and later upon activation liberated singlet oxygen molecules which caused cell death.[24] This initiated the idea that a photo sensitizer (exogenous fluorophore)which can be applied which is selectively retained by the dysplastic tissue. Later upon excitation with a specific wavelength of light the photo sensitizer emits a light which will help in identifying the tissue. Thus the dysplastic tissue became visible to naked eye. However the application of exogenous dyes was associated with a holding time where the tissues have to take up the dye and it also made the patient temporarily sensitive to light which may affect the normal routine life of the patient.[25] Hence research started focussing on optically sensitive molecules (fluorophores) located within the cell. This method was first employed in 1977 in lung cancer diagnosis through bronchoscopy. The same principle when used in the oral cavity yielded positive results when used in the oral cavity for diagnosis.[9,26,27] However the optical imaging suffered from the selective nature of the observer. When spectroscopy was combined with auto fluorescence it became objective and fluorescence intensity, wavelength of the emitted light could be assessed along with the duration of fluorescence (lifetime).[8] The principle of auto fluorescence was based on the complex morphological and biochemical changes in the cell. The morphological changes results in an increase in the thickness of the epithelium, hyperchromatism, increased nuclear-cytoplasmic ratio and increase in micro vascularity. These morphological changes affect the way the cell responds to light. The above principle is used in autofluorescence imaging. The biochemical changes that occur are an increase in NADH, a decrease in FAD, altered elastin, keratin and collagen composition. Further the increase in vascularity causes changes in porphyrin fluorescence which is a constituent of hemoglobin.[23] In the 9 clinical trials , 7 had used porphyrin as a fluorophore either independently or in combination with NADH.[5,15,18-22] 2 clinical trials had used NADH either alone or in combination.[16,17] Porphyrin is a molecule which is a constituent of hemoglobin. A total of 482 patients in this 9 clinical trials .had been subjected to autofluorescence spectroscopy. Among them 452 patients were assessed for changes in porphyrin fluorescence.[5,15,19-22] Graph 1. 4 clinical trials had taken only porphyrin as a fluorophore.[15,18,19,21,22] 2 clinical trials had compared the fluorescence profiles of NADH and porphyrin.[5,16] Among the 7 clinical trials 5 had assessed the emission characteristics of the molecule[5,15,18-20], 1 clinical trial assessed the excitation characteristic[22] and 1 clinical trial assessed the life time of fluorescence[21] of porphyrin molecule.

Graph 1
No of patients assessed with each fluorophore



Emission spectra refers to a spectra where the sample is excited at a particular wavelength and the light emitted is screened over a wide range of wavelengths upto two times the excitation wavelength.[8] It is the most common autofluorescence spectroscopy test that can be done in-vivo since the time required to perform the test is small.[8] The wavelength used for excitation ranged from 404-410 nm. de Veld et al[18] had used a variety of excitation wavelengths of 365 nm, 385 nm, 405 nm, 420 nm, 435 nm, 450 nm on 155 cancer patients individually. He had observed that emission peak at 635nm was the greatest when the excitation wavelength was 405 nm. Among the 5 studies that assessed the emission characteristics, 5 clinical trials had expressed their results in terms of sensitivity and specificity[5,15,19,20,22] and 1 clinical trial alone had expressed the result as Area Under the Curve(AUC) of the Receiver Operator Characteristics (ROC) curve[18]. The sensitivities ranged from 82% to 100%.[5,15,19,20,22] The specificities ranged from 90-100%.[5,15,19,20,22] The study by de Veld et al[18] had reported the AUC of ROC valued greater than 0.97 to distinguish between cancer and normal patients. Among the 5 clinical trials only 2, by de Veld et al[18] and Chaturvedi et al[20] had used potentially malignant disorders as a secondary group with oral cancer and normal controls. Both the studies had reported that not much significant differences with porphyrin was demonstrable between cancer and potentially malignant disorders. However both had observed that there is a decrease in porphyrin fluorescence intensity when recorded over the fibrous bands. This has been explained as the presence of fibrosis leads to decreased penetration of light to the deeper blood vessels from where the porphyrin fluorescence is recorded. The clinical trials by Tsai et al[17] and chaturvedi et al[20] reported the results in terms of spectral intensity ratios between the emission peaks of porphyrin and NADH. Both the ratios are significantly higher than the normals. This indicates that there is a rise in porphyrin intensity to a greater extent than with NADH. In the clinical trial by Ebenezer et al[22], they have evaluated the excitation characteristics after subjecting the normal and cancer tissue to a variable length excitation light source after fixing the emission spectrometer at the wavelength of 635 nm. In this study they observed that the highest peak at 635 nm was recorded from cancerous tissue when the excitation light source was 410nm. Using spectral intensity ratio analysis they had found a sensitivity and specificity of 100% in distinguishing cancer tissue from normal tissue. This is the only study in this systematic review which had assessed the excitation characteristics of the molecule. The observation of the excitation characteristics lends additional confirmative information to the molecule concerned.[8] The observations of this study was helpful to fix the excitation and emission wavelength of the molecule. The study by Jeremy et al[21] was unique as it is the only study found assessing the life time of fluorescence of the porphyrin molecule. They

fixed the excitation wavelength at 410 nm and emission wavelength at 635 nm and recorded the kinetic fluorescence spectroscopy. Life time of the fluorescence denotes the duration of the fluorescence by the molecule. In life time analysis of the molecule reveals if there is any structural variation in the molecule or changes in the micro environment of the molecule.[8] In this clinical trial they had observed a statistically significant drop in the fluorescence lifetime of porphyrin between normals and cancer patients. The study had concluded by saying that the probable drop of the fluorescence lifetime may be due to the changes in the micro environment of the porphyrin molecule in cancer patients.[21] Porphyrin as a fluorophore has been thoroughly studied and the steady state kinetics, excitation and emission spectra, with the life time of the fluorophore has been thoroughly evaluated. From the above studies the excitation and emission wavelengths of porphyrin molecule has been established and that there is no change in the wavelengths in diseased states was also observed and recorded. A total of 45 patients from 3 studies[5,16,17] were subjected to autofluorescence analysis using NADH as a fluorophore. NADH being an indicator of electron transport chain gives an account of the metabolic activity. The study by Gillenwater et al[5] had assessed 33 sites from 15 patients and have used separate emission spectra for NADH and porphyrin. When reporting the results they have reported in terms of spectral intensity ratios of porphyrin peak at 635 nm and 455 nm for NADH. Hence the diagnostic accuracy separately based on NADH is not available. The study by Muller et al[16] had compared between the fluorophore of NADH and Collagen with separate excitation wavelengths. They have also reported the results in terms of spectral intensity ratios only. The study by Tsai et al[17] had used NADH as an exclusive fluorophore and had carried out the analysis. There is only 1 study by Muller et al[16] done on collagen as a fluorophore. Collagen which is a component of subepithelial connective tissue is not much affected in malignancies of the oral epithelium. In later stages with the increasing thickness of epithelium there is a decreased fluorescence intensity due to the reduced penetration of light. With regards to NADH and collagen as a fluorophore, there are no studies to assess the excitation characteristics and life time of fluorescence. The number of studies are also few compared to collagen. All the studies have been comparing the spectral intensity ratios with another molecule. Independent studies assessing the diagnostic accuracy to the particular molecule of NADH and collagen are lacking Metabolic redox ratios which is an indicator of the metabolic activity can be determined by autofluorescence spectroscopy. They are calculated based on Flavin Adenine Dinucleotide and NADH intensity ratios. A study by Jayanth et al[28] had indicated that there is a rise in the redox ratios in oral submucous fibrosis, however such studies are not available pertaining to oral cancer. A study by Vedeswari et al[29] compared the fluorescence intensity ratios of collagen and NADH in 20 patients with oral submucous fibrosis before and after treatment and had found significant differences with a fall in collagen intensity and a rise in NADH intensity after treatment. Such studies on the prognostic changes in oral cancer are lacking. There is only 1 study which had included potentially malignant disorders in their group of patients.[20] They have reported no significant differences between potentially malignant disorders and cancer. If more studies were available we would be able to assess if this can detect the early changes of malignancy in potentially malignant disorders. There are a limited number of studies assessing multiple sites within the same patient.[5,16] These two studies have not indicated whether the different sites sampled had any observable clinical differences or if there were any histopathological differences between the sampled sites. If variations are observed then the use autofluorescence spectroscopy as an adjunct to decide on the site of biopsy for diagnosis can be justified.

CONCLUSION

Autofluorescence spectroscopy, an optical diagnostic tool, offers the potential of assessing the optical properties of the tissues

in an non-invasive way. This systematic review proves the fact that autofluorescence spectroscopy has been studied quite extensively in the diagnosis of oral cancer. The fluorophores that have been mainly studied are porphyrin, NADH and collagen. There are numerous studies based on porphyrin as a fluorophore and have reported good sensitivity and specificity. However with respect to collagen and NADH the sensitivity obtained is less than that of porphyrin There are no clinical trials comparing all the three popularly used fluorophores viz porphyrin, collagen and NADH in the same patient and the diagnostic accuracy. Studies which have taken two or more fluorophores have reported their results in terms of spectral intensity ratios specific for each of the fluorophore, and not in terms of the individual intensity ratios for the same molecule in cancerous and normal states. Such a clinical trial will establish the diagnostic accuracy of each fluorophore. There is only one study based on lifetime of fluorophore for porphyrin. More attention can be given to life time studies than steady state studies. Life time studies give information on the structure and microenvironment of the molecule. Further there is a need studies to assess the pre-operative and post operative states of cancer which can prove its prognostic significance. Also it can be used to monitor post therapy states as this procedure can be carried out non-invasively. Further if the suggested approaches of sampling multiple sites in oral cancer is adopted then this diagnostic modality can be used as adjunct to decide on the ideal site for biopsy. In conclusion the use of autofluorescence spectroscopy as a non-invasive diagnostic modality in diagnosis of oral cancer has been established in many clinical trials. Studies on autofluorescence spectroscopy with histopathological correlation is lacking. Future research on bridging such gaps will improve the diagnostic efficacy of autofluorescence spectroscopy, which can be recommended in routine oral cancer screening to ensure early diagnosis and treatment for better prognosis in oral cancer patients.

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