CORRELATION OF SPECTROSCOPIC CHANGES IN TISSUE, SALIVA AND SALIVARY DNA CHARACTERISATION OF ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL CANCER - AN IN VIVO STUDY

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ABBREVIATIONS

OSCC	Oral squamous cell carcinoma
OPMD	Oral Potentially malignant disorder
OSF	Oral Submucous fibrosis
LPA	Leukoplakia
PVL	Proliferative Verrucous leukoplakia
NADH	Nicotinamide Adenine Dinucleotide
CPS	Cycles per second
a.u	Arbitrary units
IL	Interleukins
HNSCC	Head and Neck Squamous cell carcinoma
COE	Conventional oral examination
TGF- B	Transforming growth factor
LOX	Lysyl oxidase
COX	Cyclooxygenase
MMP	Matrix Metalloproteinase
TIMP	Tissue inhibitor of matrix metalloproteinase
LTQ	lysine tyrosylquinone
HPV	Human Papilloma virus
PCA	Principal Component analysis
LDA	Linear Discriminant Analysis

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ABSTRACT

TITLE: "Correlation of spectroscopic changes in tissue, saliva and salivary DNA in oral potentially malignant disorders and oral cancer – an in vivo study"

Aim

To correlate the fluorescent spectroscopic characteristics of tissue and saliva in oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma(OSCC) and spectroscopic characterisation of salivary DNA using ethidium bromide dye in oral potentially malignant disorder and oral squamous cell carcinoma.

Objectives

1. To assess the auto fluorescence spectrum of tissue in OPMD and OSCC

2. To assess the auto fluorescence spectrum of saliva in OPMD and OSCC

3. To assess the auto fluorescence spectrum of salivary DNA in OPMD and OSCC

4. To compare and correlate the spectrum of tissue with saliva of OPMD and OSCC

Materials and methods

A total of 80 patients with average age of 20 to 60 in both the gender were selected and divided into 3 groups. Group A included clinically and histopathologically proven cases of oral cancer, Group B1 – clinically and histopathologically diagnosed cases of leukoplakia and group B2 clinically diagnosed cases of oral submucous fibrosis and group C –healthy controls. The tissue fluorescence spectrum was analysed using handheld fibro-optic probe attached to fluorolog spectrophotometer at 280nm. Whole unstimulated saliva was collected and

fluorescence spectrum was obtained using fluorolog spectrometer at 280nm and salivary DNA fluorescence spectrum obtained after adding ethidium bromide using fluorolog spectrophotometer at 480nm wavelength.

Results

The fluorescent spectrum of tissue, saliva and salivary DNA was recorded and subjected to one way ANOVA and multivariate linear discriminate analysis. It was observed from the ANOVA test that there was statistically significant difference among the groups. The discriminant analysis for the fluorescence emission spectrum of tissue shows predictive accuracy of 85% between control group and group A, 95% between control group and group B1 100% between the control group and group B2. The discriminant analysis of salivary fluorescent spectrum shows predictive accuracy of 95% between control group and group A, 75% between control and group B1 and 80% between control and group B2. The discriminant analysis of fluorescent emission of salivary DNA shows predictive accuracy of 90% between control and group A, 95% between control group and group B1 and 65% observed between control group and group B2.

Conclusion

The results of the study revealed that oral potentially malignant disorders and oral carcinoma can be diagnosed using auto fluorescence spectroscopy. In this study statistically significant difference observed between OSCC and control group based on the protein fluorescence in tissue and saliva, DNA fluorescence in saliva. Both saliva and tissue are efficient in diagnosing oral carcinoma at early molecular changes using fluorescence spectroscopy.

Key Words: fluorescence spectroscopy, Saliva, DNA, Ethidium bromide

INTRODUCTION

Oral cancer is the 6th most common cancers in the world[1]. High incidence of lip and oral cancer is observed in Southern Asia India and srilanka and leading cause of mortality in both the genders in India.[2]. 50% of oral cancers are diagnosed at advanced stage with 5 year survival rate ranging from 20-40% depending upon the site of the tumour.[3] Among the oral cancer,92-95 % reported were squamous cell carcinoma (OSCC).[4] There is increased risk of developing oral cancer as the age advances and majority of cases occurs in people aged 50 and above. Oral cancer cases also reported in age groups below 40.[1] The widespread variations in the global incidence of this disease is due to the increased usage of tobacco related products in Asian countries[13].

Mostly OSCC develops from oral potentially malignant disorder (OPMD) like leukoplakia (LPA) oral submucous fibrosis (OSF) and erythroplakia.[5] The survival and quality of life of these high risk people is depending upon the detection of OPMD and OSCC at an earlier stage. The diagnostic delay is either due to patient or physician delay. The reasons attributed for professional delay are the need for scalpel biopsy to confirm the diagnosis and the lack of effective adjunctive methods for non-invasive cancer diagnosis.[6]Early diagnosis of OPMD and OSCC is the corner stone to provide prompt treatment and better prognosis.

Conventional oral examination (COE) is the standard method of revealing OPMDs and OSCC, confirming the clinical suspicion by biopsy which can reveal architectural changes at the cellular and tissue level.[7] The numerous screening

tests or diagnostic aids available to diagnose oral cancer which include conventional oral examination, toluidine blue staining, oral brush biopsy, and scalpel biopsy with histopathology.[8] Optical diagnosis, DNA marker analysis, biomarkers in bio fluids are recent techniques for diagnosing OPMD and OSCC. The gold standard diagnostic test for OSCC and OPMD is histopathological test, but this technique requires specific training and is invasive, time-consuming, and expensive.[5]

The development of oral cancer is associated with phenotypic and genotypic alterations which includes activation of oncogenes and inactivation of tumour suppressor genes leading to tumourogenesis. These alterations will produce many biochemical alterations which can be detected in the affected cells much before the clinically evident change.[9,10] Early detection of malignant changes can be made using these biochemical alterations. The measurable indicators are called as biomarkers which may be expressed in the malignant tissue or in the bio fluids. The biomarkers for OSCC have been identified in body fluids and are considered convenient for earlier diagnosis. Due to the abundance of biomarkers in saliva , it is considered to be non-invasive, less complex, cost-effective medium than blood for the identification of biomarkers.[11]

Optical diagnosis includes autofluorescence, diffuse reflectance and Raman spectroscopy. The principles of optical imaging can be used both in-vivo to screen the tissues directly, on the excised tissue in-vitro and can also be used on the exfoliated cells in bio fluids like saliva, serum and urine. Among the various optical diagnostic methods, fluorescence is one of the most sensitive tools in

probing subtle changes in tissue environment by changes in its energy, wavelength, polarization, and direction. Optical diagnostic techniques studies about the biochemical changes in the malignant tissues and correlate them with various clinical stages.[12]

When light interacts with biological tissue, there is absorption of light and re-emission of light of varying colors which is called fluorescence and it can be detected by sensitive spectrometers.[13] Autofluorscence of tissues and saliva is produced by endogenous flourophores. These fluorophores are from tissue matrix molecules and intracellular molecules like collagen, elastin, keratin, tryptophan, NADH etc. OPMDs and malignancy can cause changes in concentration of these fluorophores along with light scattering and absorption properties of tissues.[14,15]

OSCC and OPMDs can be diagnosed with the help of salivary DNA using fluorescent spectroscopy. Initially isolation and detection of DNA present in saliva was done using electrophoretic technique and it was an accurate method, but tedious and time consuming. DNA present in the biofluids was not detectable by fluorescence spectroscopy due to its low quantum yield, by adding a fluorescent dye like ethidium bromide, DNA characterisation can be done using fluorescent spectroscopy. [16]

This study will focus on autofluorescence characteristics of tissue and saliva of OPMDs and OSCC using fluorescent spectroscopy due to endogenous flurophore. The spectroscopic characteristic of salivary DNA using ethidium bromide in OPMD and OSCC and Comparison of characteristics of salivary protein and DNA using fluorescent spectroscopy.

AIM AND OBJECTIVES

AIM:

To correlate the fluorescent spectroscopic characteristics of tissue and saliva in oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC). To assess the spectroscopic characterisation of salivary DNA using ethidium bromide dye in oral potentially malignant disorder and oral squamous cell carcinoma.

OBJECTIVES

1. To assess the auto fluorescence spectrum of tissue in OPMD and OSCC.

2. To assess the auto fluorescence spectrum of saliva in OPMDs and OSCC.

3. To assess the fluorescence spectrum of salivary DNA in OPMD and OSCC.

4. To compare and correlate the spectrum of tissue with saliva of OPMD and OSCC.

REVIEW OF LITERATURE

Oral Potentially Malignant Disorders

It has been known for over a century that oral cancer can develop from already existing mucosal lesions in the oral cavity.[17] The precancer concept begins in the year 1805 and the term was coined by **Victor Babes** in the year 1875. In 1870 sir **James Paget** was first to describe about the increased risk of malignant transformation in a tongue lesion[18] and **Schwimmer** also reported similar condition in 1877.[4] In May 2005, WHO recommended the name Potentially malignant disorders and they defined it as that not all lesions and conditions described under this term may transform to malignancy, rather that there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation. Potentially malignant disorders of oral mucosa are also risk indicators of future malignancies elsewhere in the oral cavity.[19]

ORAL SUBMUCOUS FIBROSIS

Susrutha named a condition called vidari which resembles like OSF in 600 BC. He described about the pain on taking food and depigmentation of oral mucosa and restricted mouth opening. [20]

Schwartz in 1952 reported a similar features like blanching, stiffness of oral mucosa with difficulty in mouth opening in migrated Indians in East Africa and Kenya and coined the word Atrophia Idiopathica tropica mucosae oris.[20]

Joshi in 1953 described the blanching and stiffness of palate and faucial pillars in India and he coined the word oral submucous fibrosis.[21,22]

In 1954 **Su** reported three cases of fibrosis in oral cavity in Taiwan and named it as idiopathic scleroderma of mouth. [23]

Epidemiology

In 1968 **Pindborg J.J** *et al* conducted epidemiological study of OSF in 4 states of India and reported the prevalence rate ranges from 0-0.4%.[24]

Ranganathan *et al* in 2004 conducted a survey in Chennai and reported male to female ratio as 9.1: 1 and average age of 32.4 + 10.4 years. The youngest and oldest ages of occurrence in this study was 16 and 76 years in males and 24 and 57 years in females.[25]

Etiology

Sirsat and **Khanolkar**(1962) suggested the allergic reaction is the cause for OSF and the allergen responsible for this reaction were suggested as chillies by **Hammer** *et al* in 1974.[26,27]

Wahi[28] *et al*, 1966 suggested nutritional deficiency is the etiology for OSF.

P.C.Gupta *et al*[29] (1980) in their 10year follow up study noticed that OSF occurs in those who are having pan chewing habit along with arecanut or plain

arecanut. They suggested that arecanut is main etiologic agent in OSF and also stated that once it develops there is no regression and nor proper treatment.

Trivedy C *et al* (1999) [30] in their study observed that arecanut chewing can lead to release of copper from their products which gets deposited in the oral tissues. They also found that the up-regulation oflysyloxidase activity in OSF patients. From these findings they hypothesized that cellularevents lead to cross linking of collagen and elastin, making them less degradable. The up-regulation of lysyloxidase in OSF may be an important factor in the pathogenesis.

Malignant Transformation Potential

Paymaster JC[31] in 1956 observed the development of oral squamous cell carcinoma from one third of the OSF patients in his study.

Pindborg *et al* [32] 1984 demonstrated a malignant transformation rate of 4.5% in 15 year follow up study and gave 5 criteria for precancerous nature

1. High occurrence of submucous fibrosis in oral cancer patients

2. A higher incidence of oral cancer in patients with submucous fibrosis

3. Histologic diagnosis of oral cancer without any clinical suspicion among submucous fibrosis cases

4. High frequency of epithehal dysplasia

5. Higher prevalence of leukoplakia among submucous fibrosis cases.

Murti PR *et al* (1985) [33] proposed malignant transformation rate in OSF as 7.6% in his17 year follow up observational study. The average age at the time of malignant transformation was 64.6 years with a range of 48-81 years.

Clinical Signs and Symptoms

The onset of the disease is insidious over a period of 2 to 5 years. The prodromal symptoms include burning sensation, vesicle formation, ulcerations or recurrent generalized inflammation of the oral mucosa, excessive salivation, defective gustatory sensation, and dryness of the oral cavity. As the disease advances blanching occurs in oral mucosa which appears slightly opaque and fibrotic bands appears leading to stiffness of oral mucosa and restricted mouth opening and restricted tongue movements. The disease progresses slowly.[34]

The laboratory findings include decreased hemoglobin level, decreased iron level, decreased protein level, increased erythrocyte sedimentation rate and decreased vitamin B complex level.

Classification Systems

Various classification systems are proposed for OSF based on clinical and histological stages and grades such as **Desa J.V** (1957) **Wahi P.N.** and **Kapur V.L.** *et al.*(1966) **Pindborg J.J** (1989) **Bailoor D.N.** (1993) **Maher R.** *et al.* (1996) **Haider S.M.** *et al.* (2000) **Ranganathan K.** *et al.* (2001) **Pindborg J.J**. and **Sirsat S.M**. (1966) **Utsonumiya H.** *et al.* (2005) **Khanna J.N**. and **Andrade N.N**. (1995) [35,36]

Haider *et al* (2011)[37] classification is based on severity of the disease with functional staging and objective measurement of inter-incisal opening.

Clinical Staging

Stage 1: Facial bands only

Stage 2: Facial and buccal bands

Stage 3: Facial and labial bands

Functional Staging

Stage A: Mouth opening more than 20 mm

Stage B: Mouth opening 10 to 20mm

Stage C: Mouth opening less than 10mm.

Pathogenesis of Oral Submucous Fibrosis

The pathogenesis of OSF has been described with different protocols and it is multifactorial in origin with arecanut as the main etiological agent. Arecanut contains alkaloids and flavonoids and traces of copper. Arecoline, arecaidine, guvacine, guvacoline are the alkaloids which stimulate fibroblasts and increases collagen synthesis and the principal causative factor is arecoline and arecaidine. When arecanut is combined with slaked lime, it hydrolyses the arecoline into arecaidine which increases the fibroblast proliferation and collagen deposition.[38]

The flavonoids of arecanut affects the collagen metabolism by increasing the activity of lysyl-oxidase (LOX) activity which in turn increases the crosslinking of collagen to form an insoluble collagen. The LOX is necessary

enzyme for final processing of collagen fibers. The cross-linking activity gives tensile strength and mechanical properties to the collagen fibers and makes them resistant to proteolysis.

In OSF the equilibrium between Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases (MMPs and TIMPs) is disturbed which results in excessive deposition of extracellular matrix leading to fibrosis.[38,39]

The lysyl-oxidase enzyme is also up-regulated by copper. The LOX is relayed on copper for its functional activity. LOX contains a co-factor lysine tyrosylquinone (LTQ), a covalently bound carbonyl prosthetic group. The LTQ is essential for the development of cross-links in the collagen fibers for the reaction mechanism of LOX. Copper plays a structural role in stabilizing the LTQ. In the process of cross-linking, copper re-oxidizes the reduced enzyme facilitating the completion of the catalytic cycle.

The copper levels in saliva has been increased after chewing arecanut.[38,40] **Vertika Rai** *et al* in their study evaluated that long standing oxidative stress can also lead to the damage in proteins, DNA which further helps in the progression of the disease. [41]

LEUKOPLAKIA

In 1978 **Kramer** reported that leukokeratosis and smokers patch has malignant potential.[42]Leukoplakia was described as "A white patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not

associated with any physical or chemical causative agent except the use of tobacco" in the First International Conference on Oral leukoplakia at Malmo, Sweden in 1984.[43] In 1996 **Axell *et al*** declared leukoplakia as a white patch measuring 5mm or more which cannot be scrapped off and cannot attributed to any other diagnostic disease. In 1997 WHO changed leukoplakia as "a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion.[44] In 2007, **Warnakulasuriya *et al*** defined as "Leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" and stated thatleukoplakia is a clinical term and the lesion has no specific histology. It may show atrophy or hyperplasia (acanthosis) and may or may not demonstrate epithelial dysplasia.[19]

Incidence and Prevalence

Gupta *et al* (1992) in their 10 year prospective study in India found out that prevalence of oral leukoplakia is 0.2% to 4.9% of the population present over 15 years of age[45]. Based upon the epidemiologic studies the prevalence of oral leukoplakia was between 1 and 7%in men and 0.17 and 1.5% in women [46]. The Malignant transformation rate of Oral leukoplakia varies from 0.13to 34% reported by **Warnakulasuriya S** and **Ariyawardana.A.**[47]The crude annual oral cancer incidence rate attributable to leukoplakia would be between 6.2 and 29.1 cases per 100 000 people.[48]

Site Specificity

Axéll *et al*. (1996) reported that leukoplakia was commonly present in buccal mucosa (76%), alveolar sulcus (19%), and tongue (5%).[44] **Martorell-Calatayud** *et al*. found that leukoplakia usually located on the floor of the mouth and in the ventro-lateral region of the tongue with a increased rate of 43% of malignant transformation.[49]

Etiopathogensis

The etiology of leukoplakia is multifactorial and tobacco is the most common risk factor either in smoke or smokeless form. **Brzak** *et al*. conducted study including 12,508 patients between 1998 and 2007 stated that highest frequency of leukoplakia in smokers.[50]

Roed-Petersen *et al*(1972) in their study found that bidi smoking and age is the important etiologic factor [51]. Various types of nitrosamine-producing *Candida* species have been isolated from clinically nonhomogeneous leukoplakias with histological dysplasia. In a recent study, compared to the normal epithelium,a 2-to-3-fold higher incidence of HPV infection and 4-to-5 fold greater incidence of squamous cell carcinoma were detected in oral precancerous lesions.[49]

Clinical Types

Two distinct clinical types homogenous and non-homogenous types. Homogeneous type is uniformly flat, thin and appear as shallow cracks of the surface keratin and the risk of malignant transformation is low.

Non- homogenous type have increased risk of malignant transformation and it includes

- Speckled – mixed white and red appearance

- Nodular - multiple polypoid outgrowths, rounded red or white excrescences

- Verrucous - wrinkled or corrugated surface appearance.

Another clinical entity known as proliferative verrucous leukoplakia (PVL) presents with multiple, simultaneous leukoplakia with a warty appearance and the disease is multifocal frequently covering a wide area. A provisional diagnosis of leukoplakia is made when a predominantly white lesion at clinical examination cannot be clearly diagnosed as any other disease or disorder of the oral mucosa where biopsy is mandatory. A definitive diagnosis is made when any aetiological cause other than tobacco /arecanut use has been excluded and histopathology has not confirmed any other specific disorder.[19]

Oral Leukoplakia(OLEP) classification system [52]

L (size of the leukoplakia)

L1 - Size of single or multiple leukoplakias together ≤2 cm

L2 - Size of single or multiple leukoplakias together 2 to 4 cm

L3 - Size of single or multiple leukoplakias together ≥4 cm

Lx - Size not specified

P (pathology)

P0 - No epithelial dysplasia (includes ``no or perhaps mild epithelialdysplasia'')

P1 - Distinct epithelial dysplasia (includes "mild to moderate" and "moderate to possibly severe" epithelial dysplasia)

Px - Absence or presence of epithelial dysplasia not specified in thepathology report

OLEP staging system

Stage I - L1P0

Stage II - L2P0

Stage III - L3P0 or L1L2P1

Stage IV - L3P1

ORAL CARCINOMA

Cancer is the second most common cause of death after heart diseases in developed countries, and the third cause of mortality following heart diseases in developing countries.

According to GLOBOCAN 2018 the estimated incidence of oral cancer in India is 119 992 with an estimated rate of 13.9 in males and 4.3 in females and death rate is 72616.[53] It is the 12th most common cancer in women and the 6th in men. In the oral cavity, frequently occurring tumour is of epithelial origin namely squamous cell carcinoma about 95%.[4] The five-year survival rates for oral cancers are around 50%.

Etiology and Risk Factors

Several risk factors or possible causative agents has been described for OSCC which include tobacco and alcohol, human papilloma virus (HPV), syphilis, oro-dental factors, dietary deficiencies and chronic candidiasis. These factors have been shown to be significantly associated with OSCC.

Tobacco and Alcohol

Tobacco and alcohol is major risk factors. Tobacco in any available form like smoke form, smokeless or snuff causes the initiation and promotion of oral cancer[54]. Tobacco smoke contains polycyclic aromatic hydrocarbons, N-nitrosoamines and Aszarenes which have the carcinogenic potential. Heavy alcohol consumption is a relevant risk factor fortumors of the upper aerodigestive tract. Alcohol and tobacco have synergistic effects. Alcohol and its metabolite acetaldehyde have several effects on the exposed cells including induction of cytochrome P- 4502E1 (CYP2E1), formation of ROS and cell cycle deregulation.[55]

Viruses

Epstein-barr virus and human papilloma virus has been in implicated in the oral carcinoma. HPV-16 is commonly identified and has been found in 90–95% of all HPV positive OSCC cases, followed by HPV-18, HPV-31, and HPV-33. The role of carcinogenesis is not clear.[54]

Candida infection

Candida has been suggested in initiation of OSCC. Clinical studies have reported that nodular leukoplakia infected with Candida has a tendency for higher rate of dysplasia and malignant transformation. *Candida* might induce OSCC by directly producing carcinogenic compounds like nitrosamines. These carcinogens will bind to DNA to form adducts with bases, phosphate residues, and/or hydrogen bonding sites that could cause miscoding or irregularities with DNA replication. Point mutations thus induced may activate specific oncogenes and initiate the development of OSCC.[56]

Other Risk Factors

Dholam KP, Chouksey GC (2016) in their study stated the poor oral hygiene, stress, dental trauma, low BMI, family history of cancer, exposure to environmental carcinogens have increased risk for oral squamous cell carcinoma.[57]

Carcinogenesis

Carcinogenesis in the oral cavity is a progressive disease as the normal epithelium varies through stages from dysplasia to finally forming invasive squamous cell carcinoma. Both genetic and epigenetic have been implicated in the pathogenesis towards development of OSCC.

The molecular pathogenesis of OSCC reflects an accumulation of genetic changes that occur over a period of years. These genetic alterations include activating mutations or amplification of oncogenes that promote cell survival and

proliferation, as well as inactivation of tumor suppressor genes involved in the inhibition of cell proliferation. Proto-oncogenes associated with HNSCC include ras (rat sarcoma), cyclin-D1, myc, erb-b (erythroblastosis), bcl-1, bcl-2 (B-cell lymphoma), int-2, CK8, and CK19. TSGs involved in HNSCC are *P53*, *Rb*(retinoblastoma), and *p16INK4A* and *TGF- R-II* (gene for transforming growth factor type II receptor).[9,58] The most common genetic alteration OSCC is the accumulation of somatic mutations in the TP53 gene. The loss of chromosomal region 9p21 and chromosome region 17p leading to progression of the disease.[9,55]

Clinical Presentation

Early lesions may appear as red oral mucosa failing to heal within two weeks, or as a persistent lump with spontaneous bleeding or ulceration, Lesions may appear flat, raised, exophytic or ulcerated without any initial symptoms. Over a period of time patients may complain of difficulties chewing, limited tongue movement or an abnormal sensation secondary to swelling. After the cancer growth, more symptoms occur and include bleeding, paresthesia, mobile teeth and induration and fixation of soft tissues.[59]

TNM classification of OSCC- AJCC[60]

Primary tumour (T)

Tx – Primary tumour cannot be assessed

Tis – Carcinoma in situ

T1- Tumour $\leq$ 2cm with depth of invasion $\leq$ 5mm

T2 - Tumour $\leq$ 2cm with depth of invasion > 5mm orTumour >2cm and $\leq$4 cm with depth of invasion $\leq$ 10mm

T3 - Tumour >2cm and ≤4 cm with depth of invasion > 10mm or Tumour>4cm with depth of invasion ≤ 10mm

T4 – Moderately advanced or very advanced local disease

T4a - Moderately advanced local disease Tumour >4cm with depth of invasion > 10mm or Tumour invades adjacent structures only

T4b – Very advanced local disease Tumour invades masticator space, pterygoid plates or skull base

Regional lymph node – N

Nx – Regional lymph nodes cannot be assessed

N0 – No regional lymph node metastasis

N1- Metastasis in a single ipsilateral lymph node, 3cm or smaller inits greatest dimension

N2a - Metastasis in a single ipsilateral lymph node, larger than 3cm but not larger than 6cm in greatest dimension

N2b - Metastasis in multiple ipsilateral lymph nodes, none largerthan 6cm in greatest dimension

N2c - Metastasis in bilateral or contralateral lymph nodes, nonelarger than 6cm in greatest dimension

N3 – Metastasis in a lymph node larger than 6cm in its greatest dimension

M – Distant metastasis

M0 – No distant metastasis

M1 – Distant metastasis

Mx – Distant metastasis cannot be assessed

AJCC Prognostic Stage Groups

TisN0M0 – 0

T1N0M0 – I

T2M0N0 – II

T3N0M0 – III

T1/T2/T3N1M0 – III

T4aN0/N1M0 – IVA

T1/T2/T3/T4aN2M0 – IVA

Any TN3M0 – IVB

T4banyNM0 – IVB

AnyTanyNM1 – IVC

FLUORESCENCE SPECTROSCOPY

History of Fluorescence

N. Monardes(1565) [61] first reported the fluoresecencc by an Emission of light by an infusion of wood Lignum Nephriticum.

V. Cascariolo (1602) [61]first reported the Emission of light by Bolognese stone called as phosphorescence.

David Brewster (1833)[61] described that when a beam of white light passes through an alcohol solution of leaves a red beam could be observed as the emitting light due to the presence of chlorophyll

Edmond Becquerel (1842)[61] reports the emission of light from calcium sulphate upon excitation in the Ultra violet region of the light spectrum. He also observed that the emitted light has a wavelength longer than the incident exciting light. In 1858 he built an instrument called phosphoroscope which enabled him to measure the decay time of phosphorescence.

Sir John Frederick William Herschel (1845)[61] first observed fluorescence from a quinine solution in sunlight

G.G.Stokes[62] (1853) dispersed the sunlight using a prism and noted that only light in the Ultraviolet region was able to excite the quinine solution and the other regions had no effect on quinine. He proved that every molecule has a specific excitation wavelength. He further stated and explained the change in wavelength of the exciting and emitting light and this change is named after him as "stokes shift". He introduced the term fluoroscencce. He committed that fluorescence can be used as an analytical tool to detect and discriminate organic substances.

F. Goppelsro¨der (1867)[62] did First fluorometric analysis of determination of Al (III) by the fluorescence of its morin chelate.

A. Von Baeyer (1871)[62] described about the Synthesis of fluorescein.

Fluorescence spectroscopy is a technique for evaluating the physical and chemical properties of a substance by analysing the intensity and character of light emitted in the form of fluorescence.

Autofluorescence spectroscopy analysis the intensity and character of light emitted due to the presence of endogenous fluorophores like collagen, elastin, porphyrin, NADH, FAD, Tyrosine, Tryptopan. Fluorescence spectroscopy can detect these substances and provide characteristic spectra that reflect biochemical changes occurring within the tissue. The resultant spectra not only detect the light that is fluoresced but also are sensitive to the structures that absorb light eg. Haemoglobin. Dysplastic and malignant tissues as well as having different spectral characteristics, tend to have increased red fluorescence and decreased green fluorescence. Significant increase in the red/green fluorescence ratio is an accurate predictor of dysplasia and malignancy.[63]

Principle of Fluorescence

When an energy from light interacts with matter causes the light to be absorbed involving transfer of energy to the system (absorption of light) and followed by emitting light of lesser energy and greater wavelength than the incident light.

Fluorophores are the molecules within the cell which are optically sensitive. These fluorophores on interaction with light take some energy from the exciting light source and move to a higher energy level (S1). But once they reach the higher energy state they after a period of time in nanoseconds(referred to as life time) which is specific for each fluorophore relax to the lower energy level or the ground relaxed state (S0). When they reach the ground state they emit the energy in the form of light which is of lower energy and higher wavelength than the exciting light source. This entire process is completed in a short time span of

10-9seconds. The entire process can be explained by the Jablonski diagram as mentioned named after the person who explained the process in a graphical way.[62]

Jablonski Diagram
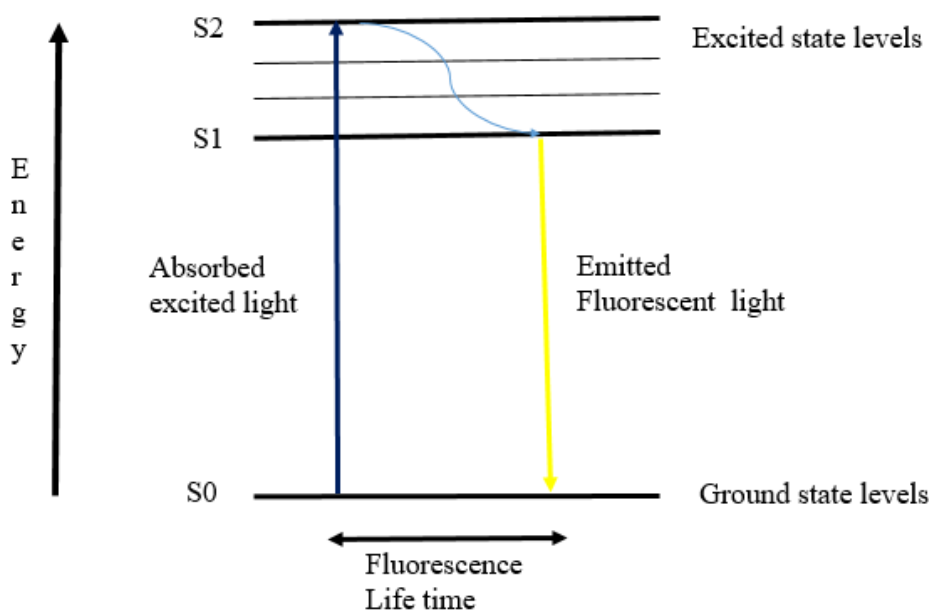
The wavelength of light photon causing the activation is excitation wavelength and emitted photon wave length is emission wavelength. These phenomenon of fluorescence is measured by spectroscopy. Fluorescence spectra produced are two types called emission spectra and excitation spectra. The intensity of the emission for a particular excitation wavelength can be plotted and is called as an emission spectrum. Conversely an excitation spectrum is a plot at a particular emission wavelength for a range of excitation wavelength. A fluorescence excitation-emission spectra is a two dimensional plot composed of intensities as a function of emission and excitation wavelength. The fluorescence intensity of a biologic molecule is function of its concentration, coefficient of

excitation wavelength and quantum yield at emission wavelength depends the concentration.[64]

Fluorophores are grouped into intrinsic and extrinsic fluorophores. Intrinsic fluorophores or endogenous fluorophores are naturally occurring include the aromatic amino acids, NADH, flavins, derivatives of pyridoxyl, and chlorophyll. Extrinsicfluorophores are added to the sample to provide fluorescence when none exists, or to change the spectral properties of the sample. Extrinsicfluorophores include dansyl, fluorescein, rhodamine, andethidium bromide and numerous other substances. The excitation and emission wavelength is specific for each fluorophore.[62] The specific emission and excitation wavelength forfluorophores are mentioned below.

Maximum excitation and emission wavelength of endogenous flurophores

S.No	Endogenous Fluorophore	Excitation maxima (nm)	Emission Maxima (nm)
1	Tryptophan	280	350
2	Tyrosine	275	300
3	Collagen	325,360	400,405
4	Elastin	290,325	340,400
5	Phenyl alanine	260	280
6	FAD	450	535
7	NADH	336	464
8	Porphyrin	450	630

Fluorescence in Diagnosis

Autofluorescence for the diagnosis of malignant lesions were emerged from photodynamic therapy for cancer treatment. In this therapy, the light-sensitive drug ("photosensitizer") is given either as a topical application or systemic drug, such as protoporphyrin IX (PpIX) to localise the tumour. The photosensitizer produces reactive oxygen species upon excitation with light of a certain wavelength, which damages vital cell organelles inducing death of cells in the direct environment. Since some of the sensitizers were believed to accumulate in malignant tissues, they could possibly serve for diagnostics as well. The use of exogenous fluorescence for tumour detection has been investigated for various organs. However, the use of exogenous fluorophores has some major drawbacks. A certain waiting time after application is necessary for the fluorophore to reach its optimal fluorescence intensity. Furthermore, the application of photosensitizers leaves the patient temporarily sensitive for light, which negatively affects his daily life. This makes the technique impractical, especially for use in regular screenings of high-risk patient groups. Finally, the specificity of the photosensitizers appeared to be less than expected.[65]

In 1911 **Stubel** identifies the diagnostic potential of tissue auto fluorescence. In 1970's **Profioand Doiron** discovered that cancer can be diagnosed using fluorescence spectroscopy. They had injected 250pg of hematoporphyrin derivative into patients with bronchogenic carcinoma. They had observed the fluorescence change using an endoscope with 200 W mercury vapour lamp through a fibre optic cable. This is the first usage of fluorescence in-vivo for diagnosis. [66]

The Biological Origins of Tissue Fluorescence

      Autofluorescence in tissues are produced by naturally occurring flurophores in cells after excitation at particular wavelength. The fluorophores will be in the tissue matrix or in cells which includes collagen, elastin, keratin and NADH etc. When light is incident on tissues two phenomenon take place other from fluorescence i.e. absorption and scattering. Absorption is primarily from the oxy- and deoxyhemoglobin within the tissues. Scattering results from the in homegenities of the refractive index of cell nuclei and cell organelles. The presence of disease changes the concentration of the fluorophores as well as the light scattering and absorption properties of the tissue, due to changes in blood concentration, nuclear size distribution, collagen content and epithelial thickness.[65]

Autofluorescencein Oral Oncology

Harris and Werkhaven [67] (1987) were credited with first using autofluorescence for imaging in oral cavity. They had observed the fluorescence emission after excitation at 630nm in healthy and malignant tissue which is the excitation wavelength of porphyrin.

Kolli *et al* [68](1995) is the first to do the In vivo study of oral cancer and measures the autofluoroscence excitation and emission spectra of neoplastic upper aerodigastric mucosa and normal mucosa and reported a significant difference between normal and malignancy.

Chen *et al* [64](1996) had measured autofluorescence spectra from malignant and normal excised tissue. They had used an excitation wavelength from 270 to 400 nm and measured the emission maxima at 10 nm intervals. It was found that there is a peak at 330 nm emission which was higher than normal tissue. But at 470 nm the emission was weaker than normal tissue.

Gillenwater *et al* [69](1998) recorded autofluorescence spectra of normal oral mucosa and premalignant or malignant lesions at 337,365, 410 nm excitation. From this study they achieved a sensitivity of 82% and specificity of 100% in distinguishing normal tissues from malignant tissues.

Ganesan *et al* [70](1998) studied native fluorescence spectroscopy of normal human oral and malignant epithelialcells under UV excitation. Observed a significant difference in the excitation spectra at 280nm between normal and malignant epithelial cells. They had observed that there is a decrease in the fluorescence intensity in malignant cells compared to normal cells. The observed difference may be due to an increase in tryptophan concentration which causes self-quenching of the fluorescence.

Majumder *et al* [71] (2003) studied human oral cancers and found sensitivity of 86% and specificity of 63% for distinguishing advanced tumours from visually uninvolved site.

Jayachandran *et al* [72](2009) showed that tissue autofluorescence and diffuse reflectance Spectroscopic analysis of premalignant and malignant lesion was a

Fast and noninvasive, diagnostic techniques tapping the potential to link the biochemical and morphologic properties of tissues for accurate diagnosis

Jayachandran *et al* [73](2009) studied autofluorescence intensity of pre and post treated Patients with OSF and reported variations in the intensity of fluorescenceat 385nm and 440nm intensity region corresponding to NADH. The average fluorescence spectrum of the post treated OSMF mucosa had a lesser intensity around 385 nm and a higher intensity around 440 nm than that of the pre-treated OSF mucosa, thereby mimicking the normal oral mucosa.

Betz *et al* [74](2012)Studied fluorescence intensity of 30 patients using excitation at 375-440 nm and had found excellent demarcation by lower fluorescence intensity in 20 tumors. In their study 10 cases responded negatively giving false negative results. In their study flat lesions were better outlined than large exophytictumors. According to them the porphyrin like fluorescence was not a reliable indicator as porphyrins are microbially synthesized and limited to necrotic surfaces of the exophytic growths. Further porphyrin fluorescence was observed on the dorsal surface of tongue.

Saliva as a Biomarker

Saliva is an important complex fluid composed of secretions from the salivary glands. In the last couple of decades saliva has been widely used for diagnosis of various oral and systemic cancers. The main advantages are easy to collect and non-invasive and also easier to handle for diagnostic procedures as no special equipment is needed for saliva sample collection and it does not clot, thus

reducing the manipulations which may be required for biochemical analysis. It is an informative body fluid containing an array of analytes (Protein, mRNA and DNA) that can be used as biomarkers for translation and clinical applications[75].

Shen *et al* (2007) proved that saliva of oral squamous cell carcinoma patients contains peptides and proteins useful for OSCC detection. In his study he used liquid chromatography to separate the proteins and then subjected to mass spectroscopy. He revealed that salivary thioredoxin mRNA levels were up-regulated in OSCC using mass spectroscopy. They concluded by saying that 46 different peptides were found to be different in both the groups.[76]

Jayachandran *et al* (2016) studied Raman spectroscopy of saliva, urine and serum (a non-invasive inelastic light scattering technique in which the wavelength of the incident laserlight shifts depending on the vibrations of the molecules). The specific biochemical, structural and conformational changes occurring in tissues is reflected by their Raman spectra well before the clinical manifestations start thus aiding in diagnosis and speedy treatment Planning.[7]

Salivary DNA Analysis

The genetic aberration of cancer cells leads to altered gene expression patterns, which can be identified long before the resulting cancer phenotypes are manifested. These changes are unique in cancer in comparison with that of normal tissue of same origin, hence, this can be utilized as molecular biomarkers. Diagnosis based on characterisation of DNA and RNA is highly sensitive and effective method. Characterisation of salivary DNA by fluorescence spectroscopy

to differentiate disease is a recent advancement and an easy procedure. Because of low quantum yield DNA in saliva was not detectable by fluorescence spectroscopy, so a fluorescent dye ethidum bromide is added which binds with DNA and emits fluorescence and measured the fluorescence spectra at 480nm.

Yuvaraj *et al* (2014) measured the fluorescent spectrum of salivary DNA using ethidium bromide in oral squamous cell carcinoma and normal patients. They reported a significant difference between the groups and proved that disease discrimination can be done using fluorescent spectroscopy with 88.9% specificity and 94% sensitivity.[16]

MATERIALS AND METHODS

Study design: Prospective experimental in- vivo study

Study setting:

1. Department of Oral Medicine and Radiology,

Tamil Nadu Government Dental College and Hospital,

Chennai – 600003.

2. Department of Medical Physics,

Anna University,

Guindy, Chennai- 600025

Armamentarium:

Examination of the patient: (Photograph 1)

- ➢ Electrically operated dental chair
- ➢ Disposable mouth mask
- ➢ Patient's apron
- ➢ A pair of disposable latex examination gloves
- ➢ Stainless steel kidney trays
- ➢ Mouth mirror
- ➢ Stainless steel probe
- ➢ Tweezer
- ➢ Vernier calipers Metallic scale
- ➢ Biopsy kit
  - ✓ No 15 BP blade
  - ✓ Tissue holding forceps

- ✓ Alleys forceps

- ✓ Non toothed tissue holding forceps

- ✓ Suture needle- cutting round bodied needle

- ✓ Hemostat –curved and straight

- ✓ Needle holder -6" inch

- ✓ Mosquito forceps

- ✓ Surgical gloves

Armamentarium for Auto fluorescence

- ➢ Spectrofluorometer with Xenon lamp – Fluorolog -3(Photograph 2)

- ➢ Optical fiber probe(Photograph 3)

- ➢ Ethyl alcohol

- ➢ Sterile containers

- ➢ Cotton

- ➢ Disposable gloves

- ➢ Face mask

- ➢ Retractor

Study participants

Group	Subdivision	Criteria
A	n = 20	Oral squamous cell carcinoma
B	B1 n= 20	Oral leukoplakia
	B2 n = 20	Oral submucous fibrosis
C	n = 20	Healthy controls

Patient selection criteria

Inclusion Criteria:

- Age 20-60 years

- Both genders

- Willing to participate in the study

- Clinically diagnosed cases of Oral Submucous fibrosis

- Clinically and histopathologically proven cases of Oral leukoplakia

- Clinically and histopathologically proven cases of Oral Squamous cell Carcinoma

Inclusion criteria for group A (OSCC)

- Clinical parameters (Photograph 4a and b)

  - Ulcer, ulceroproliferative, raised lesion with induration with/without palpable lymph nodes

- Histopathological parameters

  - Presence of epithelial dysplasia

  - Presence of keratin

Inclusion criteria for group B1 (Leukoplakia)

- Clinical parameters (Photograph 5a and b)

  - Presence of non-scrapable white, leathery patch

- Histopathological parameters

  - Presence of mild, moderate epithelial dysplasia without invasion in basement membrane

Inclusion criteria for group B2 (OSF)

- Clinical parameters (Photograph 6a and b)

  o Stiff and blanched oral mucosa with palpable fibrous bands

  o Reduced mouth opening (Haider *etal)*

  o Included Stage I and stage II (Haider *etal*)

Inclusion criteria for group C (Control)

Healthy patients who are free of any habits and systemic disease.

Exclusion criteria:

- Not willing to participate in study

- Previously treated cases of Oral Sub mucous Fibrosis and Oral leukoplakia

- Recurrence of Oral Squamous cell Carcinoma after previous treatment by radiation/excision

- Immunocompromised patients

Methodology

The study was approved by the Institutional Review Board (IRB Ref.No:7/IRB/2017). A total of 80 participants were recruited from the outpatient Department of Oral Medicine and Radiology, TamilNadu Government Dental College and Hospital, Chennai. The study participants were clearly informed about the purpose of the study and written informed consent was obtained from all the participants. The examination of the patient was done according to study protocol and the findings were recorded in the case proforma designed for the study. The study participants were taken to the Department of

Medical Physics, Anna University, Chennai and the tissue and saliva auto fluorescence spectroscopic analysis was done. Spectral Data of all participants were acquired from the system and the data was entered into the Excel format and the statistical analysis were done using SPSS version 20. The details of the procedure are explained below.

Diagnosis

Diagnosis of potentially malignant and malignant lesions was made based on clinical and histopathological criteria. Incisional Biopsy was done in unconfirmed cases and subjected to histopathological study. Tobacco cessation counselling was given to all the participants with potentially malignant disorders and OSCC. Oral hygiene instructions were also given.

Saliva collection

Whole unstimulated saliva was collected from all the participants during the morning hours. All the participants were asked to refrain from eating, drinking any form of liquids or perform oral hygiene procedures for at least one hour prior to saliva collection. Prior to saliva collection, the participants were asked to rinse their mouth with 10ml distilled water. Unstimulated whole saliva is collected and the participants were asked to spit approximately 5ml into a 40 ml sterile container. (Photograph 7). The whole saliva was subjected to fluorescence spectroscopic analysis within two to three hours and signatures are noted in steady state and excited emission spectrum.

Fluorescence of salivary protein and DNA

For Salivary protein analysis 1ml of whole saliva was used. The whole saliva was taken in a cuvette and mounted in projection unit of the spectrometer. (Photograph 8a and b) The steady state fluorescence emission spectra were measured in the emission range 300–540 nm at 280 nm excitation. For salivary DNA fluoroscopic characterisation,100 μL of ethidium bromide dye (Photograph 9) is added to 1 ml of saliva. The steady state fluorescence emission spectra were measured in the emission range 500–750 nm at 480 nm excitation

Direct Tissue fluorescence

Direct tissue fluorescence analysis was done with fibro-optic probe. The optical Fiber probe was disinfected with ethyl alcohol and rinsed with distilled water before and after every use. All the participants were asked to rinse the mouth with normal saline for 1 min in order to minimise the influence of food and drinks. The probe is placed gently over the affected tissue. The spectroscopic characterisation of tissue were analysed. The steady state fluorescence emission spectra were measured in the emission range 300 - 540 nm at 280 nm excitation.

Spectrofluorometer

The main components of spectroscopic system are light source, excitation Spectrometer, reference photodiode, and a fiber- optic probe, sample compartment, coupling optics, emission spectrometer and detector. The autofluorescencce spectroscopic characterisation of saliva and tissue were carried out in spectofluorometer model Fluorolog-3(HORIBA JobinYvon, INC, Edison,NJ). The light source is provided by a monochromator with 450W ozone

free xenon lamp. The preferred excitation wavelength and the emission spectrum were determined by PC-controlled monochromator. The excitation light was directed to illuminate samples by one arm of a Y-type quartz fiber bundle, and the emission fluorescence was gathered by another arm of the fiber bundle and directed to the photo multiplier detector. The signal was then amplified and displayed on the computer monitor. In the sample compartment the excited light source is directed to the sample through the mirror. Fluorescence from the sample is then cumulated and directed to the emission spectrometer and which is connected to photomultiplier detector. (R928P; Hamamatzu, Shizuoka-Ken, Japan). Excitation and emission slit width were fixed as 5 nm. The acquisition was made, with 1 nm spectral interval and 0.1 s integration time. The Fluorolog-3 comprise a built-in computer that controls the spectrometer and other hardware. It communicated through a serial (RS232) port to the external computer. All the functions of Fluorolog-3 are maintained by the Datamax software which communicates between a PC-compatible computer and Fluorolog-3. The DataMax software allow you to specify the experimental parameters, acquire and display data, manage files, process data, command the hardware components, control the spectrometers and supply high voltage to the signal detector.

The DataMax software is a windows based program, with higher accessibility to all the powerful windows TM-environment functionality. In addition, DataMax is built on the solid foundation of GRAMS/386® (GRAMS/32®) which means we can have access to superior post processing functions

PHOTOGRAPHS

Photographs 1: Armamentarium for clinical examination

Photograph 2: Spectrophotometer- Fluorolog -3 (HORIBA JobinYvon, INC,

Edison, NJ)

Photograph 3: Fibro optic probe for in vivo analysis

Photograph 4: a. Squamous cell carcinoma of tongue, b. Squamous cell carcinoma of buccal mucosa

Photograph 5:

a. Leukoplakia of tongue   **b. Leukoplakia of right buccal mucosa**

Photograph 6:

a.Oralsubmucous fibrosis with blanching

and restricted mouth opening   **b. Blanching in right buccal mucosa**

Photograph 7: Saliva collection done with spit method.

Photograph 8:

a. Saliva in cuvette,   **b. Cuvette mounted in the projection**

Unit of spectrometer
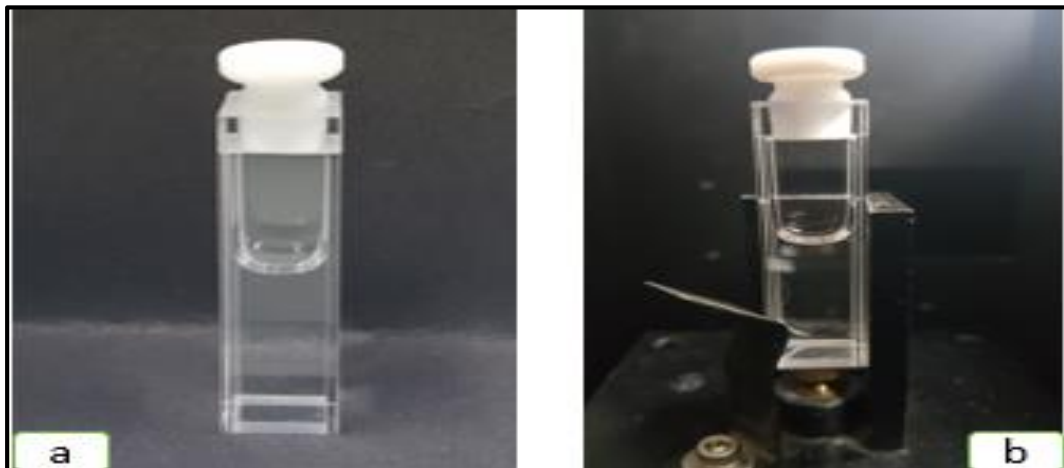
Photograph 9: Ethidium bromide dye for DNA analysis

STATISTICAL ANALYSIS

The statistical analysis was done using IBM SPSS (IBM Corp. Released 2011.IBM Statistics for windows, version 20.0. Armonk, NY: IBM Corp.). The demographic variables such as age, gender were calculated. Descriptive statistics was performed for all the four groups. One way ANOVA was done to compare the average fluorescence emission intensity of tissue protein, salivary protein and salivary DNA among the OSCC, OSF and oral leukoplakia and healthy control groups. Post hoc test of Bonferroni was done to find which group is significant from the other group for the Average Fluorescence Emission intensity. The P value is set as 0.005. Discriminant analysis was done to assess the predictive accuracy of the control and malignancy.

RESULTS

A total of 80 patients were enrolled in the study and they were categorized into Group A – oral squamous cell carcinoma (20), Group B1- oral leukoplakia (20) Group B2- oral Submucous fibrosis (20) and group C – Healthy controls (20). The mean age for group A was 48 years, group B1 was 41 years, group B2 was 36 years and group C was 32 years (Table 1 and Figure 1). Group Aincluded 13 males and 7 females, Group B1 includes 20 males and group B2 included 18 males and 2 females and in group C 13 males and 7 females were included (Table 2 and Figure 2).

Excitation Emission Spectrum

The fluorescence emission spectra of tissue and salivary protein were measured from 300 to 540nm in 1mm increments at 280nm excitation. The salivary DNA fluorescence emission spectra was measured from 500 to 750 nm in 1 mm increments at 480 nm excitation.

Comparison of auto fluorescence spectrum of tissue at 280nm

The tissue were excited at 280nm and emission spectrum of all groups were recorded from 300 to 540nm and the averaged fluorescence emission intensity of group A, group B1, group B2 and control group is shown in (Figure 3). From the graph it was observed that shape of the spectrum for all groups were similar with peak intensity was at a range of 350nm to 358 nm. The group A showed a minimum fluorescence emission intensity then control group and other groups and group B1 and group B2 showed higher fluorescence

intensity then group A and Group C with group B2 has the maximum intensity. The peak is because of the protein tryptophan. Among the four groups the group A and Group B2 showed a shift in the peak and it is attributed to the changes in tryptophan molecules in pathological state. (Figure 4) The descriptive statistics of tissue fluorescence is shown in table 3.

Table 4 shows a statistically significant difference among the groups with P value of 0.000. Post hoc Bonferroni test was done to find which group is significant from the other group for the Average Fluorescence Emission intensity. Table 5 describes that the Group A showed a statistically significant difference with the control group, group B1 and group B2 with P values of 0.03, 0.00, and 0.00 respectively. Significant difference was not observed between the group B1 and group B2 with the P value of 1.000.

Table 6 shows that discriminant function of tissue auto fluorescence for control and group A and it revealed that OSCC cases are discriminated with predictive accuracy of 85%. In this analysis, it is also obtained that 85% of the original grouped cases and 85% of the cross-validated grouped cases were correctly classified as shown in the scatter plotter (Figure 5)

The scattered plot (Figure 6) of the discriminant score and the value of discriminant function for Control group and Group B1 and 95% of predictive accuracy was observed. The classification Table 7 shows the 95.0% of original grouped cases correctly classified and also 95.0% of cross-validated grouped cases correctly classified.

Table 8 demonstrates the discriminant function of tissue auto fluorescence for control group and Group B2 and it revealed that OSF cases are discriminated with predictive accuracy of 100%. In this analysis, it is also obtained that 100% of the original grouped cases and 100% of the cross-validated grouped cases were correctly classified as shown in the scatter plot (figure 7).

Comparison of auto fluorescence spectrum of saliva at 280nm

The saliva collected from all the groups were excited at wavelength of 280nm and emission spectrum were recorded from 300nm to 540nm. Table 9 shows the descriptive data of salivary proteins among the four groups. The normalised average fluorescence intensity of saliva of group A, Group B1, Group B2 and control group showed a peak at 353 nm and the shape of the spectra were similar in all four groups (Figure 8). There was a shift in peak to right noted in group A (Figure 9). The group A showed a minimum intensity than control and other groups and group B2 showed a maximum intensity than all groups.

Table 10 shows a statistically significant difference between the groups with P value of 0.000. Table 11 shows that group A showed statistically significant difference with group B1 and control group and their P values are 0.00 and 0.01 respectively. Group B2 showed no significant differences with any other groups.

The scatter plot (Figure 10) of the discriminant score that is the discriminant function for control group and group A ,which reveals the original cases were classified correctly with predictive accuracy of 95%. Cross validation

also done and the groups were classified correctly with predictive accuracy of 95% as shown to classification table 12.

The discriminant score for control group and group B1 are shown in scatter plot (Figure 11). Table 13 shows the classification results and the original cases and cross validated cases were correctly classified with 75% predictive accuracy.

The scatter plot (Figure 12) of the discriminant score for control group and group B2 and the original cases were classified correctly with 80% predictive accuracy and the cross validated groups were classified with predictive accuracy of 80% and the classification results are shown in the table 14.

Comparison of fluorescence spectrum of salivary DNA at 480nm

The fluorescence of Salivary DNA was obtained by adding the ethidium bromide dye which binds with DNA and showed a fluorescence emission. The saliva is excited at 480nm and the Fluorescence emission spectrum was recorded from 500 to 750 nm and the shape of the spectrum appears similar in all groups with peak intensity at around 605nm and the averaged fluorescence intensity was measured (Figure 13 and 14). The descriptive data is shown in table 15. The fluorescent intensity of normal group is lesser then the other three groups and the group B1 showed a maximum intensity.

One Way ANOVA test was done to compare the DNA fluorescence spectrum among the groups and showed a significant difference with P value of

0.00 as shown in table 16. Table 17 shows that the group A showed a significant difference with control group and group B1 and group B2 with P values of 0.00, 0.07 and 0.04 respectively. No statistically significant difference was observed between the control and group B2.

The scatter plot (Figure 15) of discriminant score for control group and group A. From this analysis it shows that the original cases and cross validated cases were classified correctly with 90% of predictive accuracy. The classification is given in table 18.

The discriminant function of control group and group B1 are showed in scatter plot (figure 16) and the classification is given in table 19. This analysis shows 95% of predictive accuracy as the original cases and cross validated cases were correctly classified.

The scatter plot (Figure 17) of discriminant score for control group and group B2 and the classification is shown in the table 20. From the analysis it reveals original and cross validated cases were correctly classified with predictive accuracy of 65%.

TABLES

Table 1: Distribution of age among the groups

GROUP	N	Mean	SD
Group A	20	48.1500	8.00181
Group B1	20	41.0000	9.53111
Group B2	20	35.9500	9.56680
Group C	20	31.6500	5.61272

Table 2: Gender Distribution among the groups

GROUP	Gender	N	%
Group A	Male	14	70.0
	Female	6	30.0
Group B1	Male	20	100.0
Group B2	Male	17	85.0
	Female	3	15.0
Group C	Male	13	65.0
	Female	7	35.0

Table 3: Descriptive statistics (Average Fluorescence Emission intensity in cycles per second of Tissue protein)

Variables	Groups	Mean	Standard deviation
Tissue Protein	Group A	2408273	884839.52
	Group B1	19734683	8397478.72
	Group B2	16768307	4605379.43
	Group C	9715252.40	5652630.2

Table 4: One way ANOVA test to compare the Average tissue fluorescence

Intensity among the groups

Group A Mean + SD	Group B1 Mean + SD	Group B2 Mean + SD	Group C Mean + SD	P Value
2408273± 884839.52	19734683± 8397478.7	16768307± 4605379.4	9715252.4± 5652630.2	0.000

Table 5: Post Hoc Bonferroni test to compare the tissues fluorescence among

the groups

Dependent Variable	Group	Group	Mean Difference	P value
Tissue protein	Control	Leukoplakia	-10019430.59	.002
		OSF	-7053054.59	.046
		OSCC	7306979.4	.035
	Leukoplakia	Control	10019430.59	.002
		OSF	2966376	1.000
		OSCC	17326410	.000
	OSF	Control	7053054.59	.046
		Leukoplakia	-2966376	1.000
		OSCC	14360034	.000
	OSCC	Control	-7306979.40	.035
		Leukoplakia	-17326410	.000
		OSF	-14360034	.000

Table 6: PCA –LDA for   tissue fluorescence between Group C and Group A

			Predicted Group Membership		Total
		VAR00001	1.00	2.00	
Original	Count	Group C	19	1	20
		Group A	2	18	20
	%	Group C	**90.0**	10.0	100.0
		Group A	20.0	**80.0**	100.0
Cross-validated[b]	Count	Group C	9	1	10
		Group A	2	8	10
	%	Group A	**90.0**	10.0	100.0
		Group C	20.0	**80.0**	100.0

a. 85.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 85.% of cross-validated grouped cases correctly classified.

Table 7: PCA – LDA for tissue fluorescence between Group C and Group B1

			Predicted Group Membership		Total
		VAR00001	1.00	2.00	
Original	Count	Group C	20	0	20
		Group B1	1	19	20
	%	Group C	100.0	.0	100.0
		Group B1	10.0	90.0	100.0
Cross-validated[b]	Count	Group C	20	0	20
		Group B1	1	19	20
	%	Group C	100.0	.0	100.0
		Group B1	10.0	90.0	100.0

a. 95.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 95.0% of cross-validated grouped cases correctly classified.

Table 8 : PCA – LDA for tissue fluorescence between Group C and Group B2

	VAR00001	Predicted Group Membership		Total
		1.00	2.00	
Original	Count			
	Group C	20	0	20
	Group B2	0	20	20
	%			
	Group C	100.0	.0	100.0
	Group B2	.0	100.0	100.0
Cross-validated[b]	Count			
	Group C	20	0	20
	Group B2	0	20	20
	%			
	Group C	100.0	.0	100.0
	Group B2	.0	100.0	100.0

a. 100.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c.100.0% of cross-validated grouped cases correctly classified.

Table 9: Descriptive statistics (Average Fluorescence Emission intensity in cycles per second of salivary protein)

Variables	Groups	Mean	Standard deviation
Salivary Protein	Group A	8507118	5016990.26
	Group B1	16686385.50	4059714.99
	Group B2	13471449.50	3633254.42
	Group C	14569086.30	3133654.8

Table 10:One way ANOVA test to compare the Average salivary fluorescence intensity among the groups.

Group A Mean + SD	Group B1 Mean + SD	Group B2 Mean + SD	Group C Mean + SD	P Value
8507118± 5016990.26	16686385.5± 4059714.9	13471449.5± 3633254.42	14569086.3± 3133654.8	0.000

Table 11. Post Hoc Bonferroni test to compare the Salivary Fluorescence among the Groups

Dependent Variable	Groups	vs Group	Mean Difference	P value
Salivary protein	Control	Leukoplakia	-2117299.2	1.000
		OSF	1097636.8	1.000
		OSCC	6061968.3	.011
	Leukoplakia	Control	2117299.2	1.000
		OSF	3214936	.493
		OSCC	8179267.5	.000
	OSF	Control	-1097636.8	1.000
		Leukoplakia	-3214936	.493
		OSCC	4964331.5	.054
	OSCC	Control	-6061968.3	.011
		Leukoplakia	-8179267.5	.000
		OSMF	-4964331.5	.054

Table 12. PCA – LDA for salivary fluorescence between Group C and Group A

	VAR00242		Predicted Group Membership		Total
			1.00	2.00	
Original[a]	Count	**Group C**	**20**	0	20
		Group A	1	**19**	20
	%	**Group C**	**100.0**	.0	100.0
		Group A	10.0	**90.0**	100.0
Cross-validated[b]	Count	**Group C**	20	0	20
		Group A	1	19	20
	%	**Group C**	**100.0**	.0	100.0
		Group A	10.0	**90.0**	100.0

a.**95.0%** of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c.**95.0%** of cross-validated grouped cases correctly classified.

Table 13:PCA – LDA for salivary fluorescence between Group C and Group B1

	VAR00242		Predicted Group Membership		Total
			1.00	2.00	
Original	Count	**Group C**	19	1	20
		Group B1	4	16	20
	%	**Group C**	**90.0**	10.0	100.0
		Group B1	40.0	**60.0**	100.0
Cross-validated[b]	Count	**Group C**	19	1	20
		Group B1	4	16	20
	%	**Group C**	**90.0**	10.0	100.0
		Group B1	40.0	**60.0**	100.0

a.75.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c.**75.0%** of cross-validated grouped cases correctly classified.

Table 14: PCA – LDA for salivary fluorescence between Group C and Group B2

		VAR00242	Predicted Group Membership		Total
			1.00	2.00	
Original	Count	**Group C**	18	2	20
		Group B2	2	18	20
	%	**Group C**	**80.0**	20.0	100.0
		Group B2	20.0	**80.0**	100.0
Cross-validated[b]	Count	**Group C**	18	2	20
		Group B2	2	18	20
	%	**Group C**	**80.0**	20.0	100.0
		Group B2	20.0	**80.0**	100.0

a.**80.0%** of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c.**80.0%** of cross-validated grouped cases correctly classified.

Table 15: Descriptive statistics (Average Fluorescence Emission intensity in cycles per second of Salivary DNA)

Variables	Groups	Mean	Standard deviation
Salivary DNA	Group A	3771135.50	616089.94
	Group B1	5369227.45	1203353.92
	Group B2	2472118.89	1210762.48
	Group C	1524847.56	874305.8

Table 16: One way ANOVA test to compare the Average salivary DNA fluorescence Intensity among the groups

Group A Mean + SD	Group B1 Mean + SD	Group B2 Mean + SD	Group C Mean + SD	P Value
3771135.5±	5369227.45± 1203353.92	2472118.8± 1210762.4	1524847.5± 874305.8	0.000

Table 17: Post HOC Bonferrioni test for multiple comparisons between the groups for salivary DNA

Dependent Variable	Group	Group	Mean Difference	P value
Salivary DNA	Control	Leukoplakia	-3844379.89	.000
		OSMF	-947271.33	.255
		OSCC	-2246287.94	.000
	Leukoplakia	Control	3844379.89	.000
		OSMF	2897108.55	.000
		OSCC	1598091.95	.007
	OSF	Control	947271.33	.255
		Leukoplakia	-2897108.55	.000
		OSCC	-1299016.6	.040
	OSCC	Control	2246287.94	.000
		Leukoplakia	-1598091.95	.007
		OSMF	1299016.6	.040

Table 18: PCA – LDA for salivary DNA fluorescence Group C and Group A

	VAR00001		Predicted Group Membership		Total
			1.00	2.00	
Original	Count	**Group C**	19	1	20
		Group A	1	19	20
	%	**Group C**	90.0	10.0	100.0
		Group A	10.0	90.0	100.0
Cross-validated[b]	Count	**Group C**	19	1	20
		Group A	1	19	20
	%	**Group C**	90.0	10.0	100.0
		Group A	10.0	90.0	100.0

a. 90.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 90.0% of cross-validated grouped cases correctly classified.

Table 19: PCA – LDA for salivary DNA fluorescence between Group C and Group B1

	VAR00001		Predicted Group Membership		Total
			1.00	2.00	
Original	Count	**Group C**	20	0	20
		Group B1	1	19	20
	%	**Group C**	100.0	.0	100.0
		Group B1	10.0	90.0	100.0
Cross-validated[b]	Count	**Group C**	20	0	20
		Group B1	1	19	20
	%	**Group C**	100.0	.0	100.0
		Group B1	10.0	90.0	100.0

a.95.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c.95.0% of cross-validated grouped cases correctly classified.

Table 20 : PCA – LDA for salivary DNA fluorescence between Group C and

Group B2

			Predicted Group Membership		Total
		VAR00001	1.00	2.00	
Original	Count	Group C	16	4	20
		Group B2	3	17	20
	%	Group C	60.0	40.0	100.0
		Group B2	30.0	70.0	100.0
Cross-validated[b]	Count	Group C	16	4	20
		Group B2	3	17	20
	%	Group C	60.0	40.0	100.0
		Group B2	30.0	70.0	100.0

a 65.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c 65.0% of cross-validated grouped cases correctly classified.

FIGURES

Figure 1: Age Distribution among the groups

Age Distribution

- OSCC: 48.15
- Leukoplakia: 41
- OSF: 35.95
- Controls: 31.65

■ OSCC   ■ Leukoplakia   ■ OSF   ■ Controls

Figure 2: Gender distribution among the groups

Percentage %

- Group A: Males 70, Females 30
- Group B1: Males 100, Females 0
- Group B2: Males 90, Females 10
- Group C: Males 65, Females 35
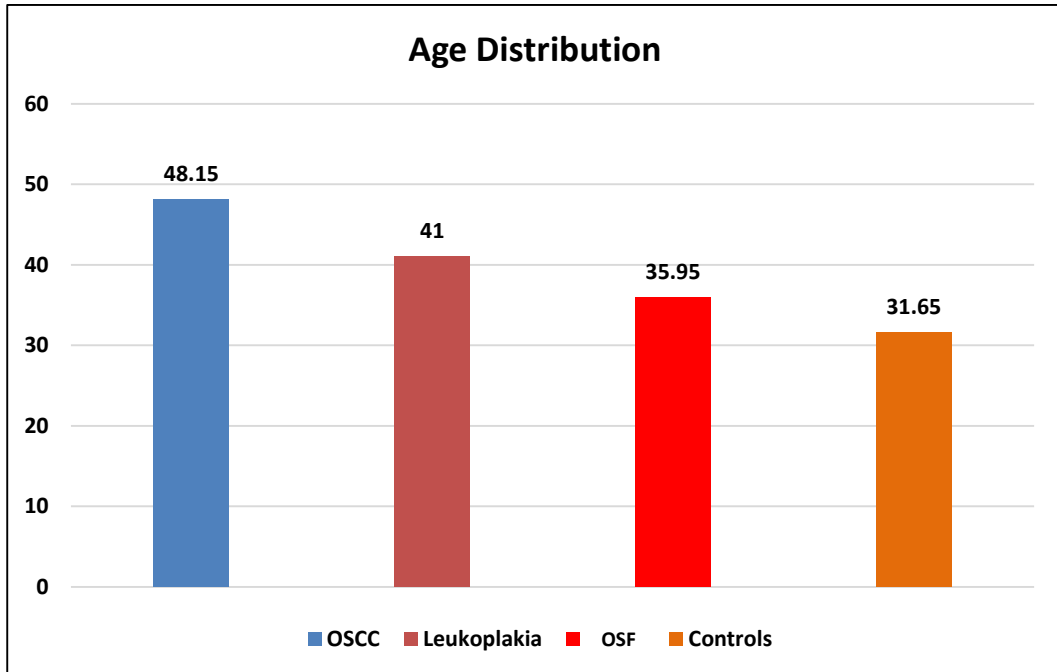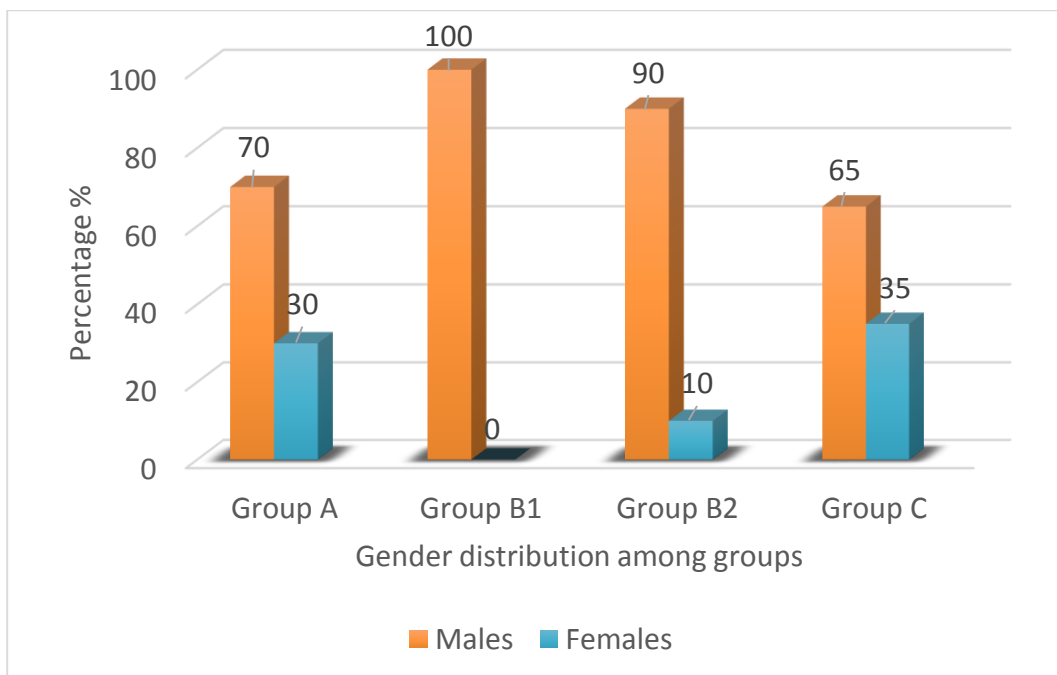
Gender distribution among groups

■ Males   ■ Females

Figure 3: Averaged fluorescence emission intensity of tissue at 280nm excitation
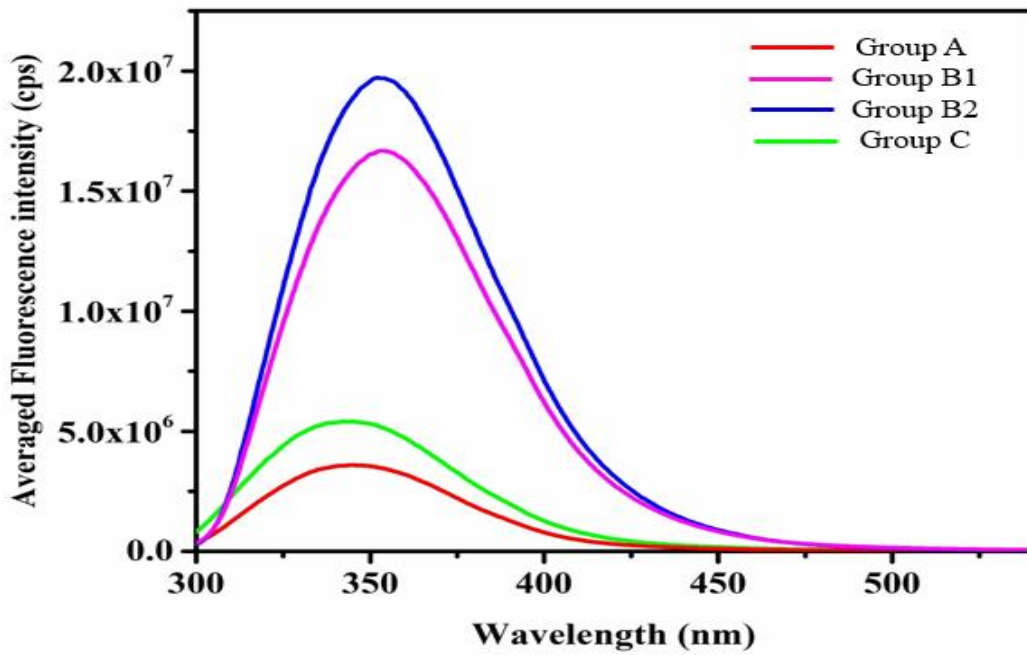

Figure 4: Normalised average fluorescence intensity of tissues

Figure 5: Scatter plot showing the discriminant function score of tissue fluorescence between control and Group: A

Figure 6: Scatter plot showing the discriminant function score of tissue fluorescence between control and Group B1

Figure 7: Scatter plot showing the discriminant function score of tissue fluorescence between control and Group B2

Figure 8: Average fluorescence emission intensity of saliva at 280nm excitation
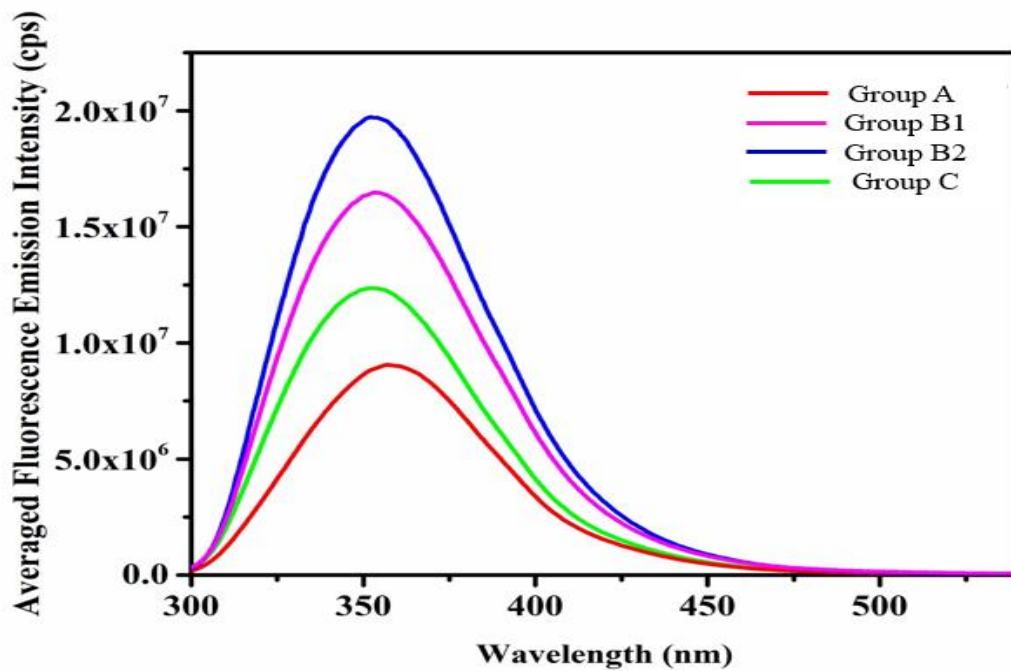
Figure 9: Normalised average fluorescence intensity of saliva

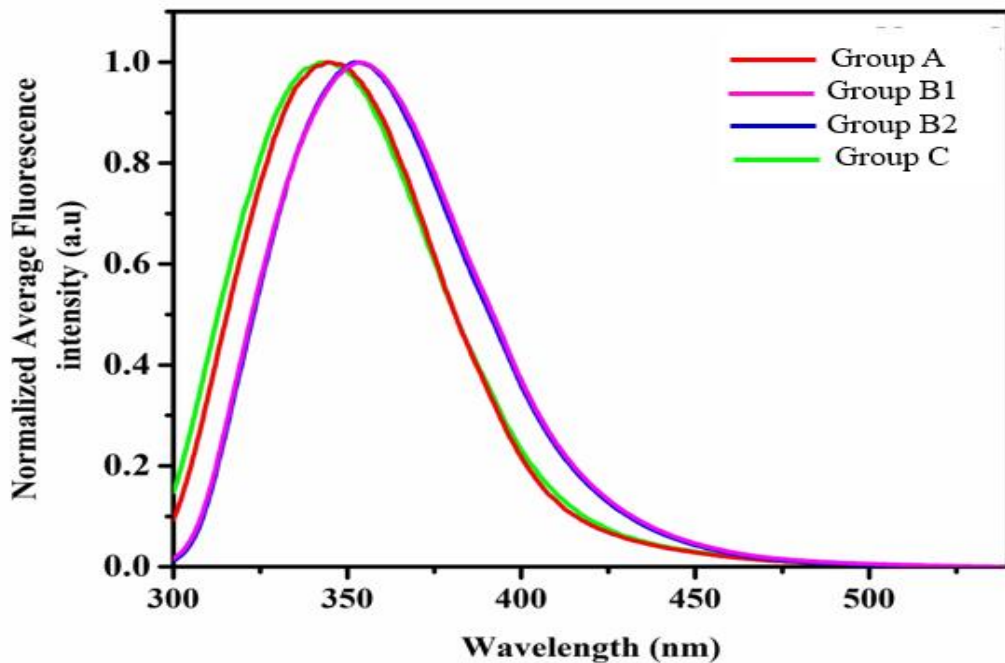
Figure 10: Scatter plot showing discriminant function score of salivary

fluorescence between control group and Group A

Figure 11:Scatter plot showing discriminant function score of salivary fluorescence between control group and Group B1

Figure 12: Scatter plot showing discriminant function score of salivary fluorescence between control group and Group A
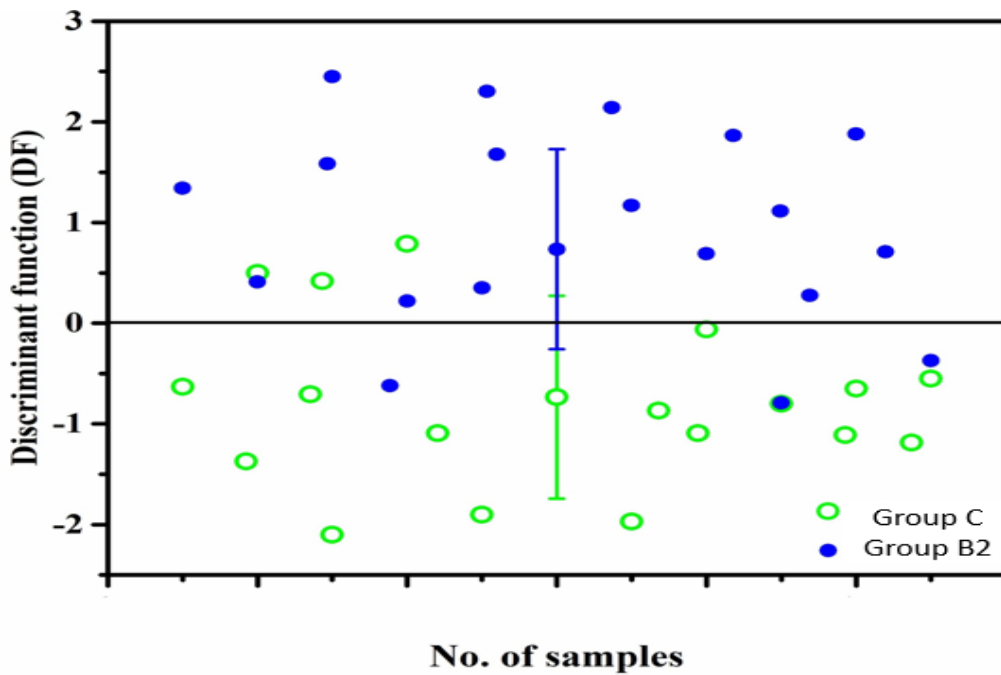
Figure 13: Averaged fluorescence emission intensity of salivary DNA at 480nm excitation
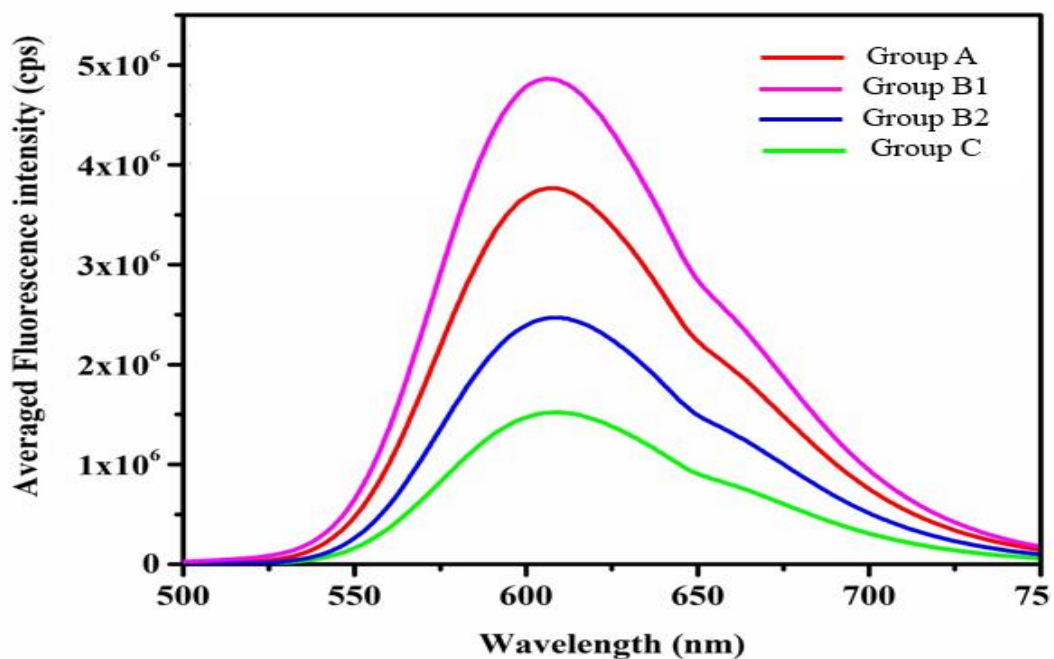
Figure 14: Normalised Fluorescence intensity of salivary DNA
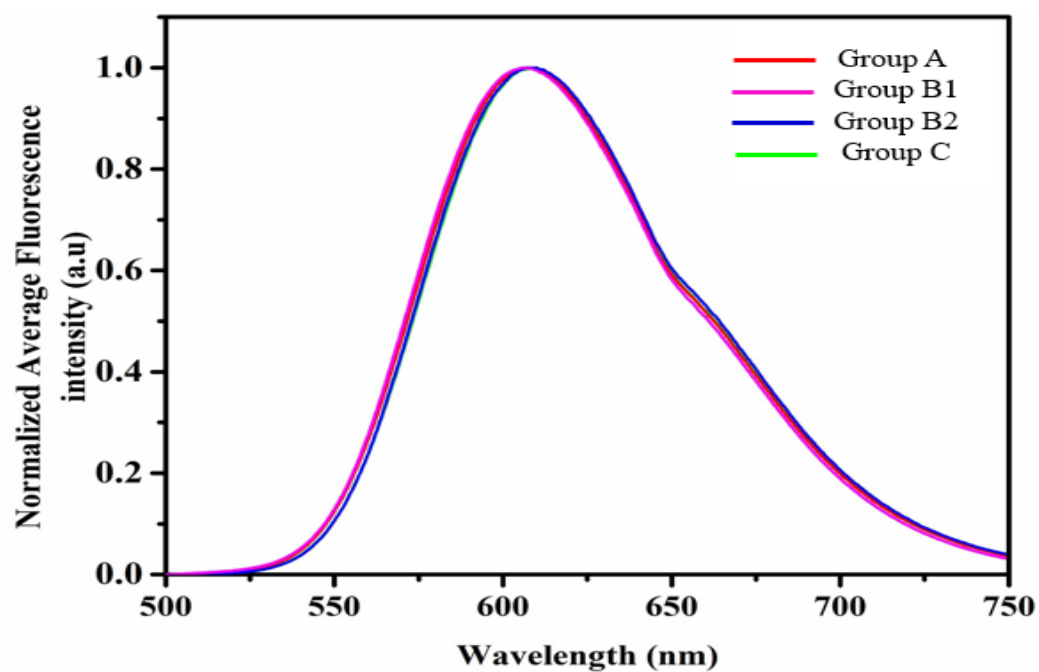
Figure 15: Scatter plot showing the discriminant function of salivary DNA fluorescence between control group and Group A
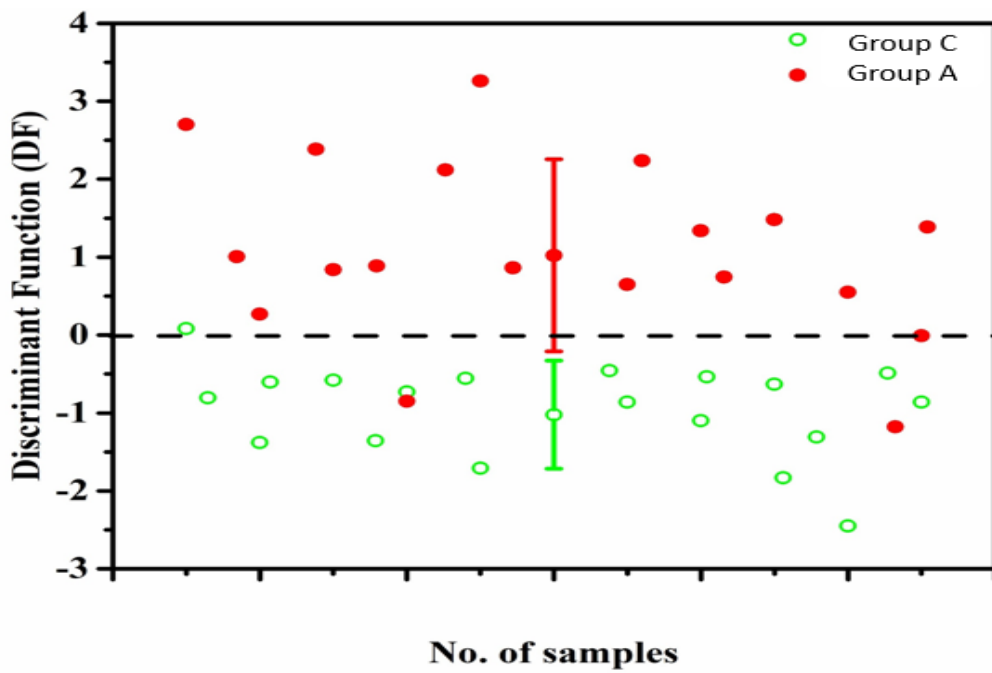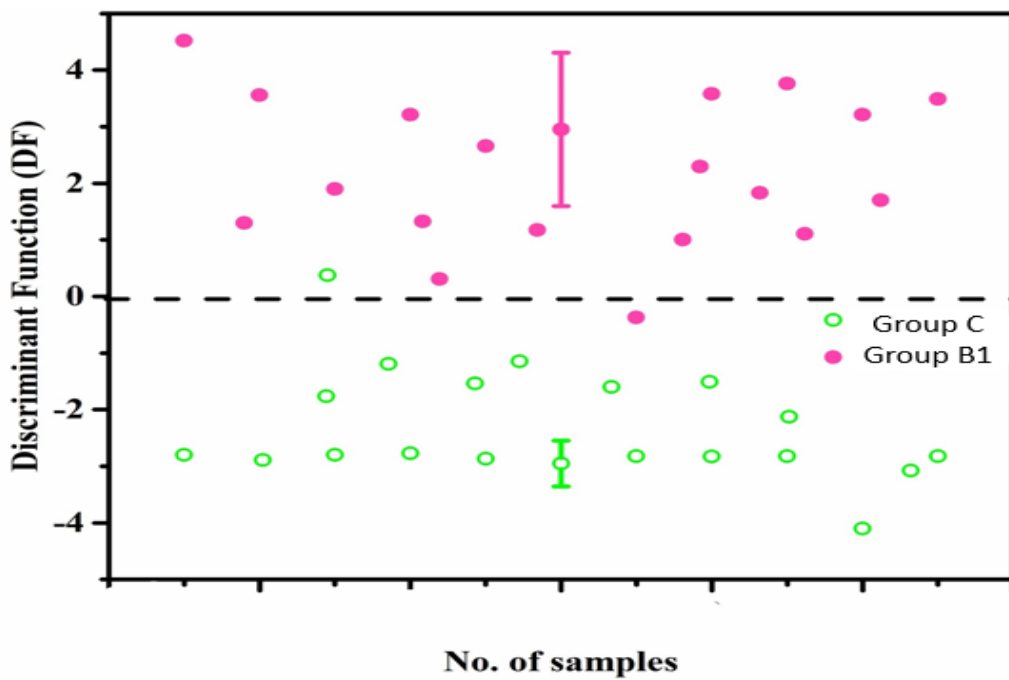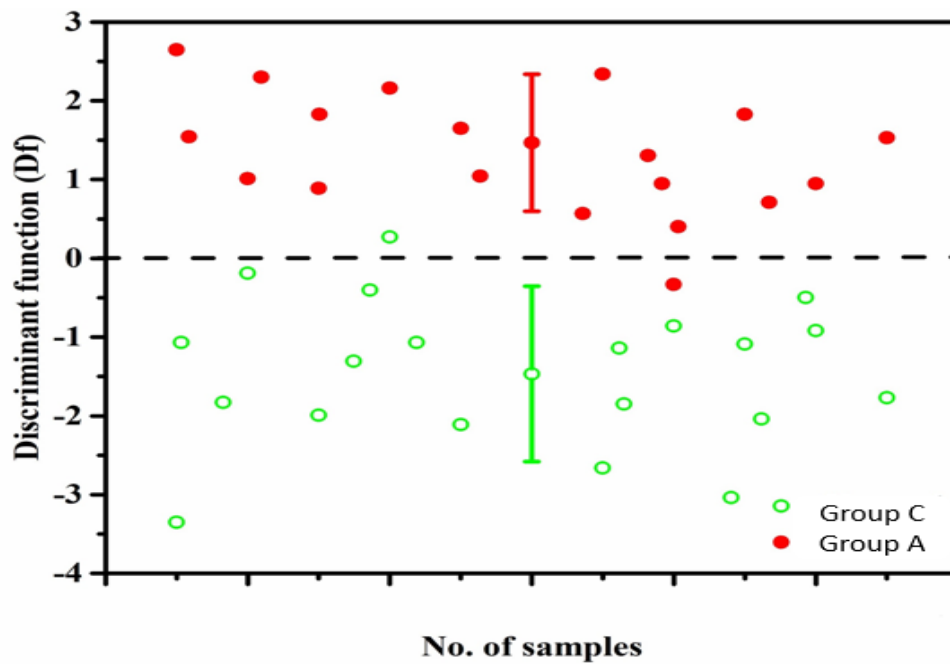

Figure 16: Scatter plot showing the discriminant function of salivary DNA fluorescence between control group and Group B1

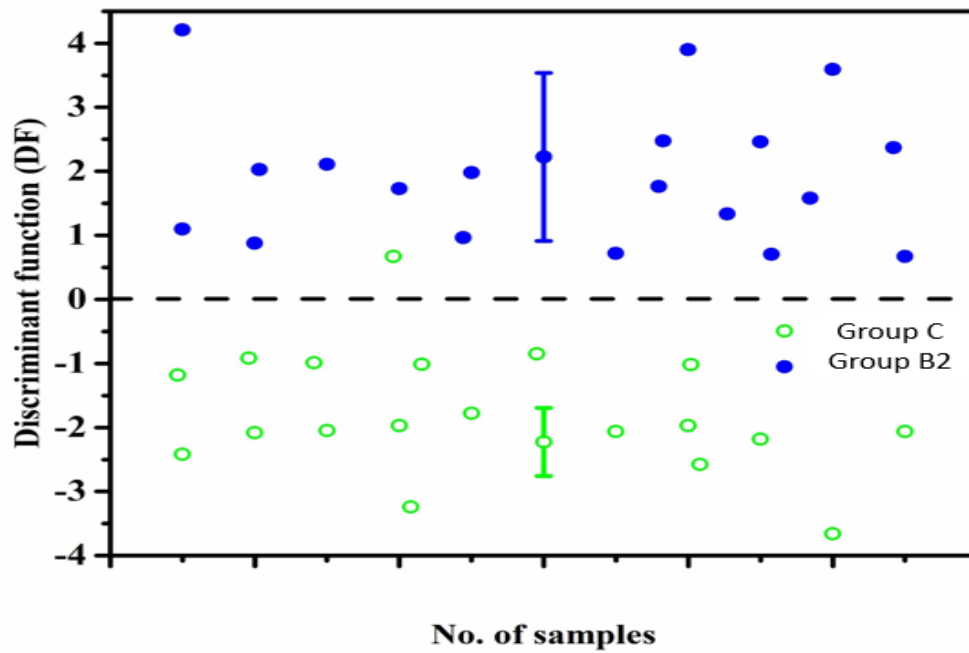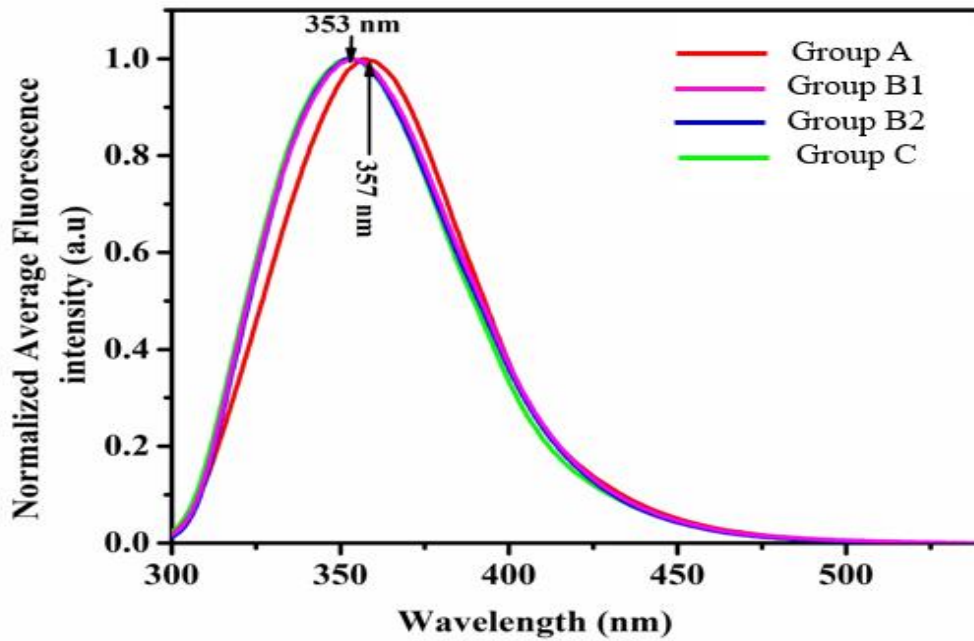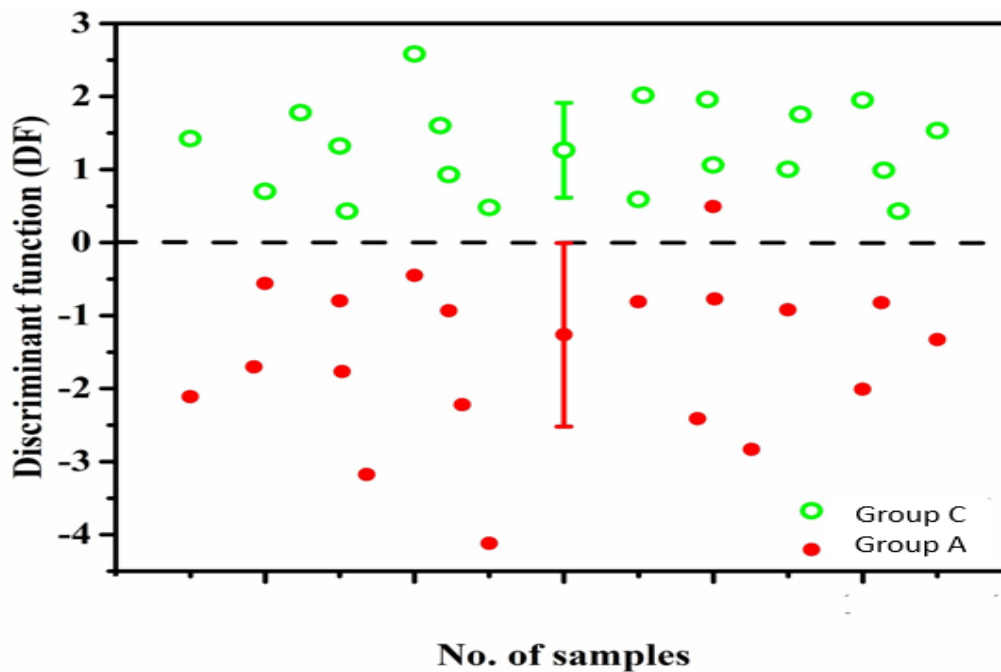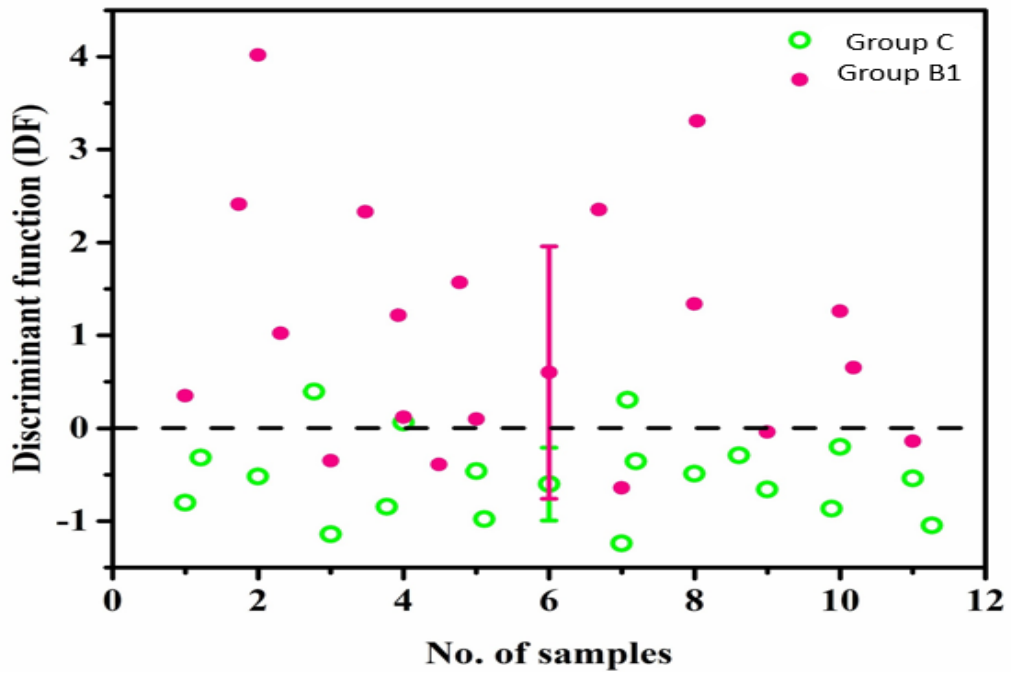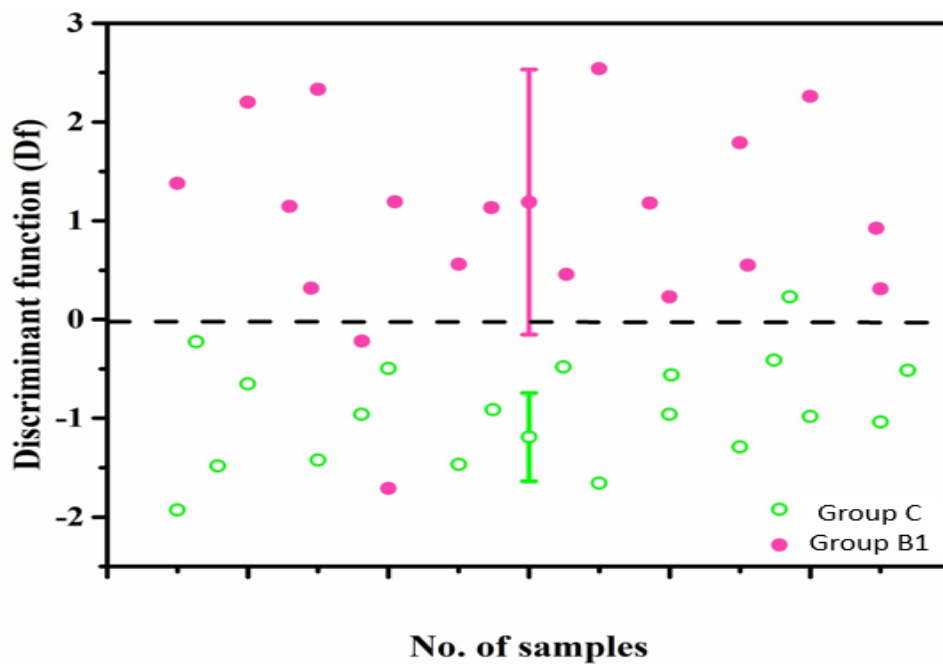**Figure 17: Scatter plot showing the discriminant function of salivary DNA fluorescence between control group and Group B2**
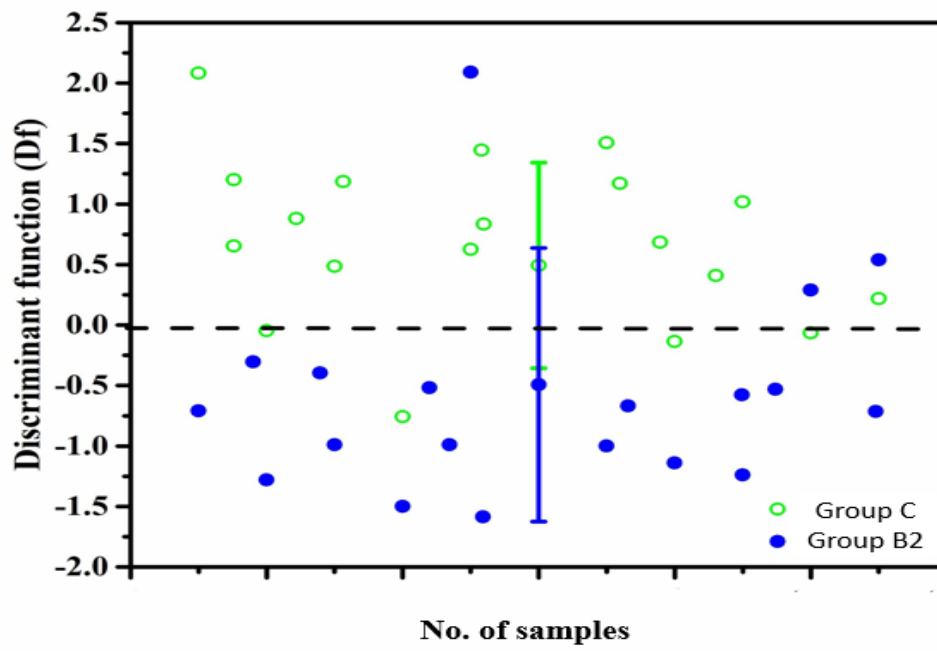
DISCUSSION

Optical spectroscopy is one of the upcoming methods with higher impact in the field of biomedical diagnostics which provide us with novel methods to diagnose the medical conditions at an early stage. Auto fluorescence spectroscopy has been broadly explored and used to diagnose the condition as it is highly sensitive to the biochemical changes of the tissue. It is easy, non-invasive, fast, and reliable and analyses the sample rapidly. It can be used both on tissues and saliva.

The results from the present study revealed that the malignant and premalignant conditions can be differentiated from normal tissue using fluorescent spectroscopy by assessing the tissue, saliva and salivary DNA.

This study includes a total of 80 participants and they were grouped into Group A- OSCC (20), Group B1- Leukoplakia (20), Group B2 - OSF (20) and Group C - control (20). All the study participants were explained about the study and after the informed consent participants were taken to the study centre Medical Physics Department, Anna university, guindy where the tissue and salivary fluorescence analysis was done.

Both the tissue and salivary samples were excited at 280nm wavelength and the emission spectrum was recorded from 300 to 540nm .The emission maxima was obtained around 350nm. The emission peak is because of the aromatic amino acid tryptophan.

Tryptophan is an essential aminoacid required for protein synthesis which gets inserted in proteins during the process of mRNA translation and it is also necessary for the synthesis of the essential cellular cofactor, nicotinamide adenine dinucleotide (NAD+). These reducing equivalents are important for the generation of ATP which is considered as the energy currency of the cell.[76] Tryptophan has a fluorescent property which can be used as diagnostic marker and prognostic marker. Minimum amount of flurophore is required to produce the fluorescence. The fluorescence intensity of tryptophan is highly sensitive to local environment and their quantum yields. In hydrophobic environment the intensity is at the maximum and in hydrophilic environment its intensity is less because of change in quantum yield. The emission maxima of proteins represents the average exposure of their tryptophan residues to the aqueous phase.[62,77]

The average tissue fluorescence intensity obtained for group A, group C, Group B1 and group B2 are 4.5×10^6 cycles per second (cps), 5×10^6 cps, 1.7×10^7 cps and 1.9×10^7 cps in an increasing order respectively. The group B2 and group B2 showed 2 to 3 folds increase in the spectral intensity than normal .Group A showed lesser spectral intensity than normal. The findings in our study are consistent with the study done by Jayachandran et al where he compared the tissue fluorescence intensity of normal, OPMDs and OSCC in which the OPMDs showed a greater intensity than control group.[72]

In OPMDs and OSCC, there is continuous proliferation of cells which requires tryptophan for protein biosynthesis. The levels of tryptophan and its metabolites were increased locally in OSCC than in normal which was observed

in the study done by Tankiewicz A *et al* They study also revealed that the tryptophan levels were reduced in saliva and serum.[78]

In group A(OSCC), the tryptophan levels are increased but the fluorescence intensity is reduced which may be due to the internal quenching and the alterations in the microenvironment.

Fluorescence quenching is the process where the intensity of the sample is reduced. The different mechanisms of quenching can be classified as dynamic quenching or static quenching between fluorophores and quenchers. Various molecular interactions like excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching can cause quenching. Quenching is caused by molecular oxygen, and heavy atoms like bromide and iodide. Tryptophan is quenched by acrylamide, succinimide, dichloroacetamide, dimethyl formamide and hydrogen peroxide.[62,79]

This changes may be attributable to the tryptophan position in the proteins, depth of the penetration of UV light, specific site selection various stages of disease, vascularity of the particular region, thickness of the epithelium.

In our study, the average fluorescent intensity of saliva obtained for group A, Group C, group B1 and Group B2 are 5.5×10^6 cps , 1.2×10^7 cps, 1.7×10^7 cps and 1.9×10^7 cps in an increasing order respectively. Group A had showed a lower intensity than other groups and the Group B2 had the maximum intensity and these findings were not in accordance with the stuys conducted by yuvaraj et al, [80]

where they found that the intensity of the premalignant conditions and OSCC are lesser than the control group. This may be due to the variability in the equipment, intensity of light source, staging of premalignant conditions, and conformational structure of protein.

Both salivary and tissue spectral characteristics of all the four groups are similar at 280nm with minimum intensity for group A and maximum intensity for group B2 . From the discriminant analysis study group A can be differentiated from control group with a predictive accuracy of 85% in tissues and 95% in saliva. The predictive accuracy to discriminate group B1 from control group are 95% for tissues and 75% for saliva. Group B2 and control group are correctly classified with predictive accuracy of 100%in tissues and 80% in saliva.

DNA based diagnosis is most accurate and sensitive method but because of its tedious procedure to separate and analyse the DNA, its usage is restricted. Saliva contains cell free nucleic acid, proteins and desquamated epithelial cells which are derived from the serum or from local secretions. These genotypic and phenotypic markers can be utilised to diagnose oral cancer.[10] The ethidium bromide dye is added with salivary sample to intercalate with DNA and excited at 480nm and emits fluorescence, based on this dye fluorescence the DNA can be assessed to differentiate the OSCC, and OPMDs from control group. In this study the salivary samples are excited at 480nm and emission is recorded from 500 to 750nm and the maximum peak obtained was around 600nm. The spectral intensity obtained for group C, Group B2, Group A, group B1 1.6×10^6 cps, 2.5×10^6, 3.7×10^6 and 4.9×10^6 in an increasing order respectively. Group A and Group B1

showed an increased intensity because of the continuous proliferation of cell which results increased amount of desquamated cells and DNA. From the discriminant analysis the control group can be differentiated from group A with predictive accuracy of 90%, from group B2 65% and from group B1 95% respectively.

Based on our study findings the auto fluorescence spectroscopy can be used to diagnose the molecular level changes before the clinical changes.

The variations in the fluorescence spectrum may be due to certain factors like age of the participants, gender of the participants, disease severity, site of placement of the fibrotic probe, vascularity of the tissue, pH variations, various types of leukoplakia, various stages of OSF and OSCC.

CONCLUSION

In this study a total of 80 participants which includes 20 OSCC, 20 OSF, 20 LPA and 20 controls. All the four groups were assessed for the fluorescence spectra of tissues and saliva based on the presence of endogenous fluorophore and DNA. The results were compared with fluorescence spectra of control group tissue and saliva. This study reveals that the OSCC and OPMDs can be differentiated from control group based on auto fluorescence with statistically significant difference between the groups. This will be helpful to diagnose early molecular changes of OPMDs and OSCC before the clinical and histopathological changes to appear. Hence Auto fluorescence spectroscopy could be used as an adjuvant non-invasive diagnostic method to diagnose malignancy at an early stage of the disease. Both saliva and tissue are efficient in diagnosing oral carcinoma at early molecular changes using fluorescence spectroscopy.

This is the first of its kind to assess the fluorescence spectroscopic characteristics of salivary DNA with ethidium bromide dye in OPMDs and OSCC. Statistically significant difference was observed in OSCC and OPMDs from control group. However further multi-centric studies is required with more study samples, various age groups, different genders, different stages of OSF, Leukoplakia and OSCC, various sites to validate our research findings.

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and Biomedical Spectroscopy (Elsevier Ltd, 2019). doi:10.1016/b978-0-323-48067-3.00012-3

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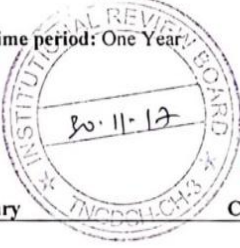
APPENDIX

TAMILNADU GOVERNMENT DENTAL COLLEGE AND HOSPITAL, CHENNAI-600003

AFFLIATED TO THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY,CHENNAI

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IRB Reference No: 7/IRB/2017

Project Title:
Correlation of spectroscopic changes in tissue and salivary DNA of potentially malignant disorders and oral cancer – An in vivo study.

Principal Investigator: Dr.R.Sangeetha

Review: New/Revised/Expedited

Date of review: 13.11.17

Date of previous review, if revised application: Not Applicable

Decision of the IRB: Approved

Recommended time period: One Year

Member Secretary   Chair Person

The Investigator / Investigating team is advised to adhere to the guide lines given below:

- Should be carried out under the direct supervision of the Guide
- Get detailed informed consent from the patients / participants and maintain confidentiality.
- Carry out the work without affecting regular work and without extra expenditure to the Institution or the Government.
- Inform the IRB in case of any change of study procedure, site, Investigator and Guide.
- Not deviate from the area of work for which applied for clearance.
- Inform the IRB immediately in case of any adverse events or serious adverse reactions. Should abide to the rules and regulations of the institution(s) .
- Complete the work within specific period and if any extension of time is required, should apply for permission again to do the work.
- Submit the summary of the work to the IRB : Students-every 3 months;
  Faculty-every 6 months.
- Should not claim any kind of funds from the institution for doing the work or on completion/ or for any kind of compensations.
- The members of the IRB have the right to monitor the work without prior intimation.
- The investigator and Guide should each declare that no plagiarism is involved in this whole study and enclose the undertaking in dissertation/ thesis.

PARTICIPANT INFORMATION SHEET

STUDY TITLE: "**CORRELATION OF SPECTROSCOPIC CHANGES IN TISSUE, SALIVA AND SALIVARY DNA CHARACTERISATION OF POTENTIALLY MALIGNANT DISORDERS AND ORAL CANCER – AN IN VIVO STUDY"**.

Name of the Research Institution: Tamil Nadu Government Dental College & Hospital, Chennai-03 and Department of Medical Physics, Anna University, Guindy, Chennai.

Aim of the study is to correlate the tissue and salivary fluorescent characters of potentially malignant disorder and oral cancer and the salivary DNA characterisation using the ethidium bromide by UV spectroscopy.

2. Procedures

    i.   Patient selection

    ii.   Obtaining thorough history and informed consent

    iii.   Complete Clinical examination (intra and extra oral examination) by using diagnostic instrument set

    iv.   Radiological examination by OPG in suspected cases of oral squamous cell carcinomas if needed.

    v.   Patients advised to refrain from oral hygiene procedures, eating and drinking I hour prior to sample collection. Patients will be provided with distilled water for gargling just before saliva collection

    vi.   5ml of saliva will be collected into sterile containers by drool method

    vii.   The in vivo probe will be placed in gentle contact with oral mucosa for spectroscopic analysis. All patients and volunteers rinsed their mouth

for 1 min with 0.9 %saline in order to minimize the influence of consumed food and bevarages.

viii. For Histopathological confirmation of oral squamous cell carcinoma incisional biopsy will be done.

ix. Test dose of 0.5ml lidocaine (local anaesthetic) will be administered to patients planned for biopsy to rule out anaphylactic reactions.

x. 1.5 cm of diseased and normal tissue will be taken for histopathologic examination followed by two to three sutures that will be removed after one week of healing.

xi. Pain killers and antibiotics will be prescribed for patients undergoing biopsy

3. Risk of participation and protection

i. Clean and sterile instruments will be used for the procedures.

ii. Risk of Radiation exposure for OPG evaluation in selected cases of oral squamous cell carcinoma – lead aprons and thyroid collars will be used for the patient.

iii. Biopsy for histopathologic confirmation of diagnosis.

4. Benefits

i. Patients with tobacco and tobacco related products use will be given counselling for cessation

ii. Patients diagnosed with potentially malignant disorders will be prevented from progressing to oral cancer by administration of prompt treatment

iii. Patients diagnosed as oral cancers will be referred to higher speciality treatment.

5. Confidentiality

The identity of the patients participating in the research will be kept confidential throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

6. Participant's rights

Taking part in the study is voluntary. You are free to decide whether to participate in the study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled. The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

7. Compensation: Nil

8. Contacts for queries related to the study:

Primary Investigator name : Dr. R.Sangeetha,

Contact details : Department of Oral Medicine and Radiology

Tamilnadu Government Dental College & Hospital, Chennai – 600003

Phone number: 8220980784

(For queries related to the rights as a study participant, please write to:

The Chairman,

The Ethical Committee,

Tamilnadu Government Dental College & Hospital, Chennai – 600003

ஆராய்ச்சி பற்றிய தகவல் படிவம்

ஆராய்ச்சி மேற்கொள்பவர்   வழிநடத்துபவர்

மருத்துவர்.இரா.சங்கீதா   மருத்துவர்.ச.ஜெயச்சந்திரன்

ஆராயச்சியின் தலைப்பு

"வாய் புற்று நோய் மற்றும் புற்றுநோய் முன்னோடி நோய்களில் திசு மற்றும் உமிழ்நீரில் உள்ள டி.என்.ஏ-வை ஒளிரும் நிறமாலை கொண்டு மதிப்பிடுதல்- ஓர் விவோ ஆய்வு"

செய்முறை

கீழ்கண்ட ஆய்வுகள்/ பரிசோதனைகள் உங்களுக்கு செய்யப்படும்.

* வாய் பரிசோதனை- உட்புறம், வெளிப்புறம்

* புகையிலை மற்றும் அதை சார்ந்த பொருட்களை உபயோகப்படுத்துபவராயின் அதைவிட்டு வெளிவர ஆலோசனை வழங்கப்படும்.

* வாய் புற்று நோய் இருப்பின் சிலருக்கு மட்டும் எலும்பின் ஊடுருவலை கண்டுபிடிப்பதற்காக ஊடுகதிர் படம் எடுக்கப்படும்.

* திசு ஆய்வுக்கு ஸ்பெக்ட்ரோஸ்கோபிக் பகுப்பாய்வுக்கான வாய்வழி சளிப்பால் மென்மையான தொடர்பில் வைக்கப்படும்.

* வழக்கமான இரத்தப் பரிசோதனை செய்யப்படும்.

* உங்கள் கையிலிருந்து பரிசோதனைக்காக 2மி.லி அளவு இரத்தம் எடுக்கப்படும்

* ஒவ்வாமை ஏற்படுகிறதா என்பதை தெரிந்து கொள்ள 0.5 மி.லி லிக்னோகெயின் என்னும் மரத்துப் போக செய்யும் மருந்து உங்களின் கையில் பரிசோதனைக்காக செலுத்தப்படும். பின்பு நோயுற்ற பகுதியில் இம்மருந்து வாய் சதை பரிசோதனைக்காக செலுத்தப்படும்.

* வாய் சதை பரிசோதனையின் போது லிக்னோகெயின் செலுத்திய பின் 1.5 செ.மீ அளவு நோயுற்ற சதையும், எடுக்கப்பட்ட பின், இரண்டு அல்லது மூன்று தையல் போடப்படும். ஒரு வாரத்துக்குள் புண் ஆறிய பின் தையல் பிரிக்கப்படும். சதை பரிசோதனைக்கு பின் வலி நிவாரண மருந்துகள் மற்றும் கிருமி கொல்லி மருந்துகள் கொடுக்கப்படும்.

* வாய் சதை பரிசோதனைக்கு முன், உங்களிடமிருந்து 5மி.லி அளவு உமிழ்நீர் எடுக்கப்பட்டு பரிசோதனை செய்யப்படும்.

* உமிழ்நீர் எடுக்கப்படுவதற்கு 1 மணி நேரம் முன் நீங்கள் சாப்பிடவோ அல்லது பல் துலக்குவதோ கூடாது. உமிழ்நீர் எடுப்பதற்கு முன்

தங்களது வாயை காய்ச்சி வடிகட்டிய நீரினால் கொப்பளித்தல் வேண்டும்.

- உமிழ்நீரை சேகரிக்க தொற்றற்ற கொள்கலன்கள் கொடுக்கப்படும்.

பங்கேற்பதினால் வரக்கூடிய பக்க விளைவுகள்

- பக்க விளைவுகள் ஏற்படாமல் தடுக்க உரிய முறைகள் பின்பற்றப்படும்.

- ஊடுகதிர் ஆர்த்தோபான்டமோகிராம் எடுக்கப்படும் பொழுது ஈய உலேக கவசம்; தைராய்டு காலர் பயன்படுத்தப்படும்.

- சிறந்த தரம் மற்றும் சுத்தமான கருவிகள் பயன்படுத்தப்படும்.

பங்கேற்பதினால் விளையும் நன்மைகள்

- நீங்கள் புகையிலை மற்றும் புகையிலை சார்ந்த பொருட்களிலிருந்து வெளிவர ஆலோசனை வழங்கப்படும்.

- வாய் புற்றுநோயின் முன்னோடி நோய்கள் இருப்பின், அவை புற்றுநோயாக மாறாவண்ணம் தகுந்த சிகிச்சை அளிக்கப்படும்.

- வாய் புற்றுநோய் இருப்பின், அதற்காக மேற்சிறப்பு சிகிச்சைகளுக்கு பரிந்துரைக்கப்படும்.

இரகசிய காப்பு

உங்களைப் பற்றிய குறிப்புகள் பிறர் அறியாவண்ணம் ஆராய்ச்சி முடியும் வரை இரகசியமாக பாதுகாக்கப்படும். அதை வெளிப்படுத்தும் நேரங்களில் எந்த தனிநபர் அடையாளங்களும் வெளிப்பட வாய்ப்பு கிடையாது.

தன்னார்வ பங்கேற்பு

இந்த ஆராய்ச்சியில் பங்கு பெறுவது தங்களின் தனிப்பட்ட முடிவு மற்றும் இந்த ஆராய்ச்சியிலிருந்து தாங்கள் எப்பொழுது வேண்டுமானாலும் விலகிக் கொள்ளலாம். தங்களின் இந்த திடீர் முடிவு தங்களுக்கோ அல்லது ஆராய்ச்சியாளருக்கோ எந்தவித பாதிப்பும் ஏற்படுத்தாது என்பதனை தெரிவித்துக் கொள்கிறோம்.

....
நோயாளியின் பெயர் நோயாளியின் கையொப்பம்

ஆராய்ச்சி தொடர்புடைய தகவல்களுக்கு பங்கேற்பாளரின் உரிமை தொடர்புடைய தகவல்களுக்கு:

மருத்துவர்.இரா.சங்கீதா மருத்துவர் பி.சரவணன்

தமிழ்நாடு அரசு பல் மருத்துவமனை தலைவர், நிறுவன நெறிமுறைகள் குழு, தமிழ்நாடு அரசு பல்

சென்னை-3. மருத்துவக் கல்லூரி மற்றும் மருத்துவமனை, சென்னை-3.

INFORMED CONSENT FORM

STUDY TITLE: "Correlation of spectroscopic changes in tissue, saliva and salivary DNA characterisation of potentially malignant disorder and oral cancer – an in vivo study"

Participant ID No:

"I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this study and understand that I have the right to withdraw from the study at any time without in any way it affecting my further medical care."

Date   Name of the participant   Signature/thumb impression

                                                                of the participant

[The literate witness selected by the participant must sign the informed consent form. The witness should not have any relationship with the research team; If the participant doesn't want to disclose his / her participation details to others, in view of respecting the wishes of the participant, he / she can be allowed to waive from the witness procedure (This is applicable to literate participant ONLY). This should be documented by the study staff by getting signature from the prospective participant]

_____

_____

"I have witnessed the accurate reading of the consent form to the potential participant and the individual has had opportunity to ask questions. I confirm that the individual has given consent freely"

Date   Name of the witness   Signature of the witness

Date   Name of the interviewer   Signature of the interviewer

ஆராய்ச்சி ஒப்புதல் படிவம்

ஆராயச்சியின் தலைப்பு
"வாய் புற்று நோய் மற்றும் புற்றுநோய் முன்னோடி நோய்களில் திசு
மற்றும் உமிழ்நீரில் உள்ள டி.என்.ஏ-வை ஒளிரும் நிறமாலை கொண்டு
மதிப்பிடுதல்- ஓர் விவோ ஆய்வு"

பெயர்   புறநோயாளி எண்
வயது/ பால்   ஆராய்ச்சி சேர்க்கை எண்
முகவரி

தொலைபேசி

    நான் .... வயது ....
என்னுடைய சுய நினைவுடனும் மற்றும் முழு சுதந்திரத்துடனும் இந்த
மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள ஒப்புதல்
அளிக்கிறேன்.

கீழ்காணப்படும் நிபந்தனைகளுக்கு நான் சம்மதிக்கிறேன்.

    நான் இந்த ஆராய்ச்சியின் நோக்கம் மற்றம் செயல்முறைகள் பற்றி
முழுமையாக தெரிவிக்கப்பட்டுள்ளேன்.

    என் உடல் நலம் பாதிக்கப்பட்டாலோ அல்லது எதிர்பாராத
வழக்கத்திற்கு மாறான நோய்குறிகள் தென்பட்டாலோ அதற்கு சிகிச்சை
பெற்றுக்கொள்வதற்கும் முழு உரிமை உள்ளதாக அறிகிறேன்.

    நான் ஏற்கனவே உட்கொண்ட மற்றும் உட்கொள்கின்ற
மருந்துகளிந் விபரங்களை ஆராய்ச்சியாளரிடம் தெரிவித்துள்ளேன்.

    என் மருத்துவ குறிப்பேடுகளை இந்த ஆராய்ச்சியில்
பயன்படுத்திக்கொள்ள சம்மதிக்கிறேன். இந்த ஆராய்ச்சி மையமும்
ஆராய்ச்சியாளரும் என்னுடைய விபரங்கள் அனைத்தையும் இரகசியமாக
வைப்பதாக அறிகிறேன்.

....
  நேயாளியின் பெயர்   கையொப்பம்   தேதி

....
  ஆராய்ச்சியாளர் பெயர்   கையொப்பம்   தேதி

CASE PROFOMA

"CORRELATION OF SPECTROCSOPIC CHANGES IN TISSUE, SALIVA AND SALIVARY DNA CHARACTERISATION OF POTENTIALLY MALIGNANT DISORDERS AND ORAL CANCER –AN IN VIVO STUDY"

Date: Serial no:

Name: PIN No:

Age/Sex:

Address: Phone no:

Occupation: Income:

Religion:

Centre: Department of Oral Medicine and Radiology,

Tamil Nadu Govt Dental College & Hospital, Chennai -3

Department of medical physics,

Anna University, Chennai– 3

Presenting complaint with duration:

Past medical history:

Past Surgical history:

Past dental history:

Personal history:

A) Diet:

B) Teeth cleaning habits:

- Cleaning aids used:

- Frequency   :

C) Smoking habit:

- Material used:

- Frequency   :

- Duration of the habit:

D) Chewing habit:

- Material used:

- Frequency   :

- Duration of the habit:

E) Other habits (alcohol, snuff):

Marital status:

Menstrual History:

Family history:

CLINICAL EXAMINATION

GENERAL EXAMINATION:

Extra oral Examination:

Facial Symmetry

Swelling

Lymph node examination

TMJ Examination

Mouth opening

Intraoral examination:

Teeth**:**

Decayed

Missing

Mobility

Filled teeth

Gingiva

Labial and buccal mucosa:

Hard palate:

Soft Palate:

Pillar of fauces and Tonsils:

Tongue:

Floor of the mouth:

Retromolartrigone:

Investigations:

1. Biochemical / Hematological Investigation :

2. Blood Pressure :

3. spectral characterisation of salivary DNA

4. spectral characterisation of tissue

RADIOGRAPHIC EVALUATION

OPG:

Provisional Diagnosis

TNM staging:

Leukoplakia staging:

OSMF staging

Histopathology report

NAME OF THE INVESTIGATOR:

SIGNATURE OF THE INVESTIGATOR:

<u>TRIPARTITE AGREEMENT</u>

This agreement hereinafter the "Agreement" is entered into on this day……………. between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai- 600 003, (herein after referred to as, 'the college') And **Dr. S. Jayachandran, M.D.S., Ph.D., MAMS., M.B.A., M.Sc FDS RCPS (Glasg)** aged 56 years working as **Professor and HOD** in Department of Oral medicine and Radiology at the college, (herein after referred to as the 'Principal Investigator') And **Dr. R.Sangeetha,** aged 34 years currently studying as final year **Postgraduate student** in the Department of Oral Medicine and Radiology, Tamil Nadu Government Dental College and Hospital, Chennai -3 (hereafter referred to as the 'PG and co- investigator') Whereas the PG student as part of her curriculum undertakes to research on for which purpose the Principal investigator shall act as Principal investigator and the College shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a Co-investigator. Whereas the parties, by this agreement have mutually agreed to the various issues including in particular the copyright and confidentiality issues that arise in this regard

Now this agreement witnessed as follows:

1. The parties agree that all the Research material and ownership therein shall become the vested right of the college, including in particular all the copyright in the literature including the study, research and all other related papers.

2. To the extent that the college has legal right to do go, shall grant to licence or assign the copyright so vested with it for medical and/or commercial usage of interested persons/entities subject to a reasonable terms/conditions including royalty as deemed by the college.

3. The Royalty so received by the college shall be shared equally by all the three parties.

4. The PG/Research student and PG/Principal Investigator shall under no circumstances deal with the copyright, Confidential information and know- how-generated during the course of Research/study in any manner whatsoever, while shall sole west with the college.

5. The PG student and Principal Investigator undertake not to divulge (or) cause to be divulged any of the confidential information or, know-how to anyone in any manner whatsoever and for any purpose without the express written consent of the college.

6. All expenses pertaining to the research shall be decided upon by the principal investigator/Co-investigator or borne sole by the PG student.(co-investigator)

7. The college shall provide all infrastructure and access facilities within and in other institutes to the extent possible. This includes patient interactions, introductory letters, recommendation letters and such other acts required in this regard.

8. The Principal Investigator shall suitably guide the Student Research right from selection of the Research Topic and Area till its completion. However the selection and conduct of research topic and area research by the Student Researcher under guidance from the Principal Investigator shall be subject to the prior approval, recommendations and comments of the Ethical Committee of the College constituted for this purpose.

9. It is agreed that as regards other aspects not covered under this agreement, but which pertain to the research undertaken by the PG student, under guidance from the Principal Investigator, the decision of the College shall be binding and final.

10. If any dispute arises as to the matters related or connected to this agreement herein, it shall be referred to arbitration in accordance with the provisions of the Arbitration and Conciliation Act, 1996.

In witness where of the parties herein above mentioned have on this the day month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its **Principal**   **PG Student**

Witnesses   **Student Guide**

1.

2.

MASTER CHART

Group A– Oral Squamous Cell Carcinoma

S.No	Age	Gender	Habit	Duration	site	Histopathology
1.	50	M	smoked tobacco	20 years	Left Buccal Mucosa	Moderately differentiated OSCC
2.	39	M	smoked tobacco	10 years	Right lateral border of tongue	Moderately differentiated OSCC
3.	60	F	Smokeless tobacco	15 years	Right Mandibular Alveolus	well differentiated OSCC
4.	49	M	Smokeless tobacco	8 years	Left lateral border of tongue	Moderately differentiated OSCC
5.	60	F	Smokeless tobacco	25 years	Right buccal vestibule	well differentiated OSCC
6.	55	M	Smokeless tobacco	12 years	left dorsal suface of tongue	poorly differentiated squamous cell carcinoma
7.	30	M	smokeless tobacco	5 years	Left Buccal Mucosa	Moderately differentiated squamous cell carcinoma
8.	48	M	Smokeless tobacco	15 years	lower lip	well differentiated OSCC
9.	40	M	smoked tobacco	10 years	Left lateral border of tongue	poorly differentiated squamous cell carcinoma
10.	49	M	smoked tobacco	25 years	Right lateral border of tongue	Moderately differentiated OSCC
11.	40	M	Smokeless tobacco	8 years	Right Retromolar region	Moderately differentiated OSCC
12.	50	M	both	22 years	Right lateral border of tongue	poorly differentiated squamous cell carcinoma
13.	45	F	Smokeless tobacco	6 years	RtBuccal mucosa	Moderately differentiated OSCC
14.	54	M	smoked tobacco	20 years	left alveolus	Moderately differentiated OSCC
15.	48	M	both	18 years	posterior one third of tongue	well differentiated OSCC
16.	53	F	Smokeless tobacco	15 years	Lt Retromolar pad	Moderately differentiated OSCC
17.	55	M	smoked tobacco	25 years	palate	poorly differentiated squamous cell carcinoma
18.	48	F	Smokeless tobacco	20 years	Lt Retromolar pad	Moderately differentiated OSCC
19.	35	M	both	10 years	RtBuccal mucosa	Moderately differentiated OSCC
20.	55	F	Smokeless tobacco	28 years	Lt Buccal vestibule	Moderately differentiated OSCC

Group B1 – Oral Leukoplakia

S.No	Age	Gender	Habit	Duration	site	Histopathology
1.	39	M	Smoked tobacco	4 years	Left Buccal Mucosa	dysplasia
2.	38	M	smoked tobacco	5 years	Lower Lip	no dysplasia
3.	58	M	Smoked tobacco	10 years	Left Buccal Mucosa	dysplasia
4.	35	M	Smoked tobacco	3 years	Right Buccal Mucosa	no dysplasia
5.	29	M	Smokeless tobacco	5 years	Right Buccal Mucosa	no dysplasia
6.	60	M	Smokeless tobacco	25 years	Right Buccal Mucosa	dysplasia
7.	46	M	Smoked tobacco	4 years	Left Buccal Mucosa	no dysplasia
8.	53	M	Smoked tobacco	12 years	Dorsal surface of the tongue	no dysplasia
9.	37	M	Smoked tobacco	5 years	Left Buccal Mucosa	no dysplasia
10.	23	M	Smoked tobacco	3 years	Right Buccal Mucosa	no dysplasia
11.	40	M	Smoked tobacco	9 years	Left Buccal Mucosa	No dysplasia
12.	35	M	both	10 years	Rt Retrocommisure	no dysplasia
13.	30	M	Smoked tobacco	5 years	Lt Retrocommisure	mild dysplasia
14.	40	M	Smoked tobacco	8 years	Lt Retrocommisure	no dysplasia
15.	33	M	both	5 years	Rt Retrocommisure	no dysplasia
16.	45	M	Smoked tobacco	20 years	Buccal mucosa	dysplasia
17.	50	M	Smokeless tobacco	25 years	Labial Vestibule	dysplasia
18.	39	M	Smoked tobacco	9 years	Lt Retrocommisure	no dysplasia
19.	42	M	Smoked tobacco	11 years	Rt alveolar ridge	no dysplasia
20.	48	M	Both	12 years	B/L Buccal mucosa	dysplasia

Group B2 – Oral Submucous Fibrosis

S.No	Age	Gender	Habit	Duration	Mouth Opening	Stage
1.	45	F	Smokeless tobacco	22 years	39 mm	I
2.	24	M	Smokeless tobacco	3 years	30mm	I
3.	30	M	Smokeless tobacco	8 years	28mm	I
4.	46	M	Smokeless tobacco	7 years	20mm	II
5.	35	M	both	5 years	25mm	I
6.	54	M	Smokeless tobacco	12 years	25 mm	I
7.	57	M	Smokeless tobacco	15 years	23mm	I
8.	27	M	Smokeless tobacco	5 years	33mm	I
9.	35	M	Smokeless tobacco	10 years	28mm	I
10.	25	M	Smokeless tobacco	7 years	30mm	I
11.	40	F	Smokeless tobacco	12 years	25mm	I
12.	33	F	Smokeless tobacco	5 years	18mm	II
13.	25	M	Smokeless tobacco	3 years	22 mm	II
14.	35	M	Both	6 years	33mm	I
15.	30	M	Smokeless tobacco	5 years	29mm	I
16.	42	M	Smokeless tobacco	12 years	17mm	II
17.	27	M	Both	2 years	30mm	I
18.	28	M	Smokeless tobacco	3 years	22mm	I
19.	39	M	Smokeless tobacco	8 years	19mm	II
20.	42	M	Both	10 years	10mm	II

Group B2 – Oral Submucous Fibrosis

Group C – Controls

S.NO	AGE	GENDER
1.	24	F
2.	24	F
3.	26	F
4.	36	F
5.	28	F
6.	39	M
7.	28	F
8.	26	F
9.	28	M
10.	33	M
11.	29	M
12.	38	M
13.	40	M
14.	29	M
15.	34	M
16.	32	M
17.	41	M
18.	40	M
19.	45	M
20	30	M