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## Research Article

# Raman Spectroscopic Analysis of Blood, Urine, Saliva and Tissue of Oral Potentially Malignant Disorders and Malignancy-A Diagnostic Study

### Abstract

**Background:** Oral cancers are mostly being preceded by oral potentially malignant disorders (OPMD). Early diagnosis of oral cancer or OPMDs speed up treatment and improve prognosis. Raman spectroscopy of blood, urine, saliva and tissue samples collected from OPMD and oral cancer patients were compared with similar samples from normal controls.

**Context and purpose:** Raman spectroscopy is a noninvasive inelastic light scattering technique in which the wavelength of the incident laser light 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. This helps in early diagnosis and speedy treatment planning.

**Results:** Raman spectroscopy gave an accuracy of 78%, 90.5%, 93.1%, and 97.4% respectively for blood, urine, saliva and tissue samples in discriminating oral pre malignancy and malignancy from normal control.

**Conclusion:** Results of the study validate that Raman spectroscopy has the potential to be a diagnostically useful tool for the in vitro detection of OPMDs and oral cancers at the molecular level.

**Brief Summary:** Early diagnosis of OPMD and oral cancer by analyzing Raman spectroscopic changes improves the prognosis. Total sample size was 205. Spectra recorded on a confocal Micro Raman System (LABRAM HR 800) were statistically analyzed using the computer software SPSS/PC +19 under one of the multivariate technique analysis - Principal Component analysis followed with the Linear Discriminant Analysis (PC-LDA). The statistical analysis using PC-LDA combination of normal vs malignant vs premalignant group accuracy are shown.

## Introduction

Oral cancer is ranked as the sixth most common cancers in the world [1]. Oral potentially malignant disorders (OPMDs) which are clinically evident precede most of the oral squamous cell carcinomas [2]. Most cancers of the oral cavity are oral squamous cell carcinomas (OSCC), and tobacco, alcohol and betel use are the main risk factors for these and many OPMDs [3,4]. The high risk group is older adult males who use tobacco and alcohol. It is expected that early diagnosis of OPMDs can reduce mortality [5,6]. Early diagnosis of OSCC can speed proceeding early intervention to treatment and can improve the prognosis [7]. Conventional oral examination (COE) is the standard method of revealing OPMDs and OSCC, confirming the clinical suspicion by biopsy. It is subject to interpretation of pathologists, and although it can detect cellular changes, it can only detect molecular changes if special techniques are employed. Currently available diagnostic technologies are histopathological examination, vital staining, biomarkers, DNA analysis, brush

biopsy and optical techniques [8]. Early diagnosis of OPMDs and oral cancers play an important role in reducing the mortality rate [5-7]. Such early diagnosis is made possible with optical spectroscopy which will contain information about histological and biochemical characteristics [8]. The study is done to assess the diagnostic utility of Raman spectroscopy in metabolic fingerprinting of biologic fluids and tissues in OPMDs and oral cancer. Raman Effect [9], is based on interaction of light with matter. Raman or inelastic scattering is produced by about 1 in 10<sup>6</sup> to 1 in 10<sup>8</sup> of incident photons. The scattered photon has a wavelength different from the incident photon. This wavelength shift is recorded in Raman spectroscopy, which produces a detailed biochemical 'fingerprint' of the sample characteristic for the constituent chemical bonds [10]. In neoplastic cells nuclear material, nuclear to cytoplasmic ratio and mitotic activity are increased. There is a progressive loss of cell maturation, abnormal chromatin distribution, decreased differentiation, cellular crowding and disorganization. Rapid angiogenesis with leaky vessels are present

due to increased metabolic activity. So neoplastic cells show specific changes in quantities and/or conformations of protein, nucleic acid, carbohydrate and lipid [11], which is reflected as change in spectral characteristics of these cells from their normal counterparts.

### Aim and objective

The study is to analyze Raman spectrum of blood, urine, saliva and tissue samples in oral premalignancy and malignancy and to analyze, correlate the diagnostic predictability.

### Materials and Methods

After approval from the institutional ethical committee and obtaining written consent from the study and control groups, samples were collected in the Department of Oral Medicine and Radiology, Tamilnadu Government Dental College and Hospitals, Chennai, with laboratory and technical support from Department of Medical Physics, Anna University, Chennai. Total sample size was 205 (male 152(74%), female 53(26%), of age group 18 to 80 years) which includes 94(46%) cases of premalignancy (53(26%) cases of oral sub mucous fibrosis and 41(20%) cases of leukoplakia), 63(31%) cases of oral cancer (oral squamous cell carcinoma) and 48(23%) cases of healthy controls. From these groups 158 samples of blood, 158 samples of urine, 158 samples of saliva (50(32%) oral cancer, 87(55%) OPMDs, 21(13%) control), and 89 tissue samples, 29(32%) oral cancer, 22(25%) premalignancy, 38(43%) control were collected.

### Inclusion criteria

Patients clinically diagnosed with oral leukoplakia (Warnakulasuriya 2007), oral submucous fibrosis (WHO Bulletin OMS. Vol 72 1994) and clinically and histopathologically (Anneroth et al.) diagnosed with oral squamous cell carcinoma were included in the study. Willing patients who visited the hospital for other treatments were enrolled as normal controls.

### Exclusion criteria

Subjects with history of systemic diseases, those under any medications and those with regional or distant metastasis or a history of recurrences of any of the lesions/ conditions under study were excluded from the study.

### Sample collection

Urine, saliva and blood samples (random) were collected at a standardized time of the day (9 am to 11 am) and transported in ice box to the laboratory. 3ml of unstimulated saliva is collected in a sterile container by drooling method. 3 ml of blood is collected under aseptic conditions from ante-cubital vein in sterile EDTA coated tube. Patient is asked to collect 5 ml of urine in a sterile plastic container. Tissue sample is obtained from the lesion site and stored in normal saline for analysis. The samples were refrigerated and analyzed the same day in laboratory. Tissue samples for biopsy and for Raman spectroscopic analysis were taken at the same appointment and were of similar size and range.

### Equipment

Spectra were recorded on a confocal Micro Raman System (LABRAM HR 800), equipped with Peltier cooled CCD detector,

with 600 gm/mm grating edge filter. Excitation was with a 785 nm diode laser (SDL 8530) with laser power of 100Mw

### Statistical analysis

The statistical analysis was done using the computer software SPSS/PC +19. Principal Component analysis followed with the Linear Discriminant Analysis (PC-LDA) of normal vs. malignant vs. premalignant group accuracy are shown.

### Results

From the results we observe intensity variations and peak shift among the three groups.

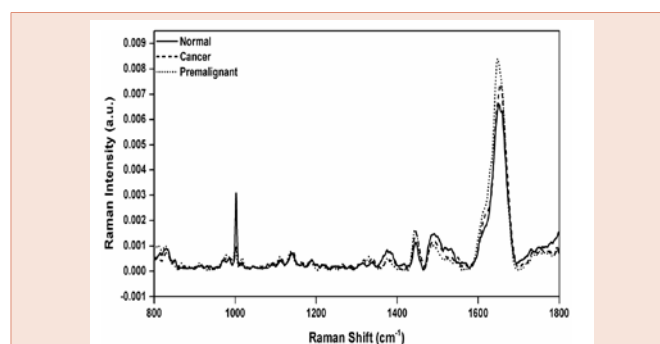
#### Blood samples (Figure 1)

**Finger print region:** 800 to 1800  $\text{cm}^{-1}$  was studied. In blood samples, Raman peaks were observed for Phenyl alanine (1002  $\text{cm}^{-1}$ ), carotene (1190  $\text{cm}^{-1}$ ), amide III (1247  $\text{cm}^{-1}$ ), lipid (1372  $\text{cm}^{-1}$ ), collagen (1449  $\text{cm}^{-1}$ ), purine (1485  $\text{cm}^{-1}$ ), amide I (1656  $\text{cm}^{-1}$ ).

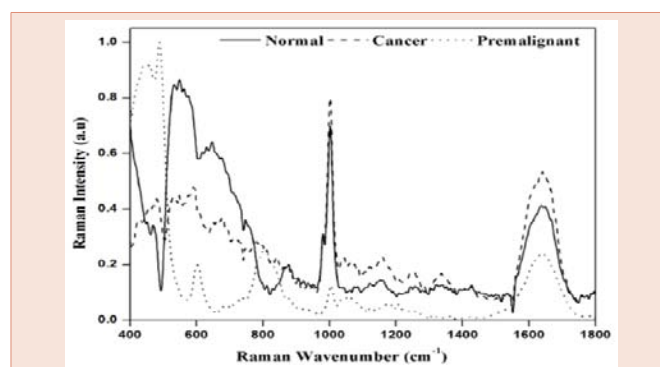
PC - LDA: normal vs. malignant vs. premalignant group: 78% accuracy.

#### Urine samples (Figure 2)

**Finger print region:** 400 to 1800  $\text{cm}^{-1}$  was studied. In urine samples Raman peaks were observed for uric acid (567  $\text{cm}^{-1}$ ), FAD (606  $\text{cm}^{-1}$ ), creatinine (692  $\text{cm}^{-1}$  in normal, 1336  $\text{cm}^{-1}$  & 1427  $\text{cm}^{-1}$ ).



**Figure 1:** Raman spectroscopic intensity variations and peak shift among blood samples of three groups.



**Figure 2:** Raman spectroscopic intensity variations and peak shift among urine samples of three groups.

1 in malignant), ethanol peak (890 $\text{cm}^{-1}$ ), urea (1002 $\text{cm}^{-1}$ ), glucose peak (1046 $\text{cm}^{-1}$ ), tryptophan (1417 $\text{cm}^{-1}$  in normal and 1547 $\text{cm}^{-1}$  in premalignant & malignant), indoxylsulphate (1615 $\text{cm}^{-1}$  in normal, 1351 $\text{cm}^{-1}$  in malignant).

PC – LDA: normal vs. malignant vs. premalignant group: 90.5% accuracy.

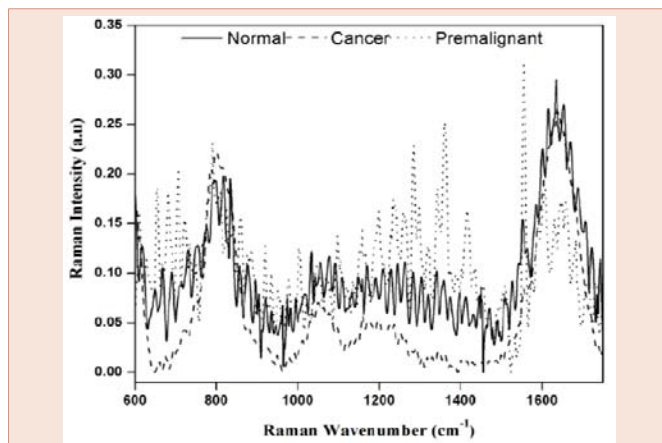
### Saliva samples (Figure 3)

**Finger print region:** 600 $\text{cm}^{-1}$  to 1000 $\text{cm}^{-1}$  was studied. In saliva samples Raman peaks were observed for pyrimidine (767, 1236, 1330, 1662 & 1688 $\text{cm}^{-1}$  in normal), amide (1652  $\text{cm}^{-1}$  normal> malignant > premalignant), mucin 1444 $\text{cm}^{-1}$ , hemocyanin (752 $\text{cm}^{-1}$ , sharper for normal saliva – broader for malignant and premalignant), carotenoids (1158 & 1525  $\text{cm}^{-1}$ , absent in normal)

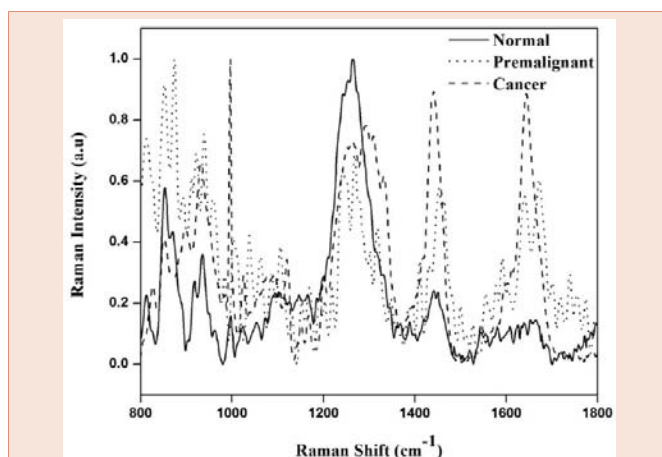
PC – LDA: normal vs. malignant vs. premalignant group: 91.3% accuracy

### Tissue samples (Figure 4)

**Finger print region:** 800 to 1800 $\text{cm}^{-1}$  was studied. Peaks were



**Figure 3:** Raman spectroscopic intensity variations and peak shift among saliva samples of three groups.



**Figure 4:** Raman spectroscopic intensity variations and peak shift among tissue samples of three groups.

observed at 850, 935, 1000, 1130, 1156, 1174, 1200-1350, 1314, 1340, 1364, 1443, 1547, 1572 & 1650  $\text{cm}^{-1}$ . Lipid peaks were more in normal than premalignant and malignant samples (normal > premalignant > malignant). Amide III & I peaks were more in malignant than premalignant and normal samples (malignant > premalignant > normal).

PC – LDA: normal vs. malignant vs. premalignant group: 97.4% accuracy.

### Discussion

Early diagnosis is made possible with Raman spectroscopy systems based on the principle that optical spectrum acquired from a tissue and biological fluids from blood, urine and saliva will contain information about its histological and biochemical characteristics [8]. The result obtained from this Raman study of blood plasma using PC-LDA analysis showed an accuracy of 78%. Raman peaks observed for phenylalanine in albumin possess C-C stretch at Raman shift 1002  $\text{cm}^{-1}$ , carotene at 1190  $\text{cm}^{-1}$ , amide III peaks of proteins at 1247  $\text{cm}^{-1}$ , lipid shows peak at 1372  $\text{cm}^{-1}$  and collagen peak at 1449  $\text{cm}^{-1}$ , purine base has peak at 1485  $\text{cm}^{-1}$  and amide I peaks of protein at 1656  $\text{cm}^{-1}$  respectively. Intensity variations and peak shift among the three groups are observed. As the blood plays a major role in the metabolic activity and carries the end product released after the metabolism of the cells, Raman spectroscopy has its unique nature by providing specific Raman bands of the biological molecules. Hence, this Raman study using blood plasma could be used in the near future in the clinical diagnosis of oral premalignant and malignant cases.

The result obtained from this Raman study of urine spectrum using PC-LDA analysis showed an accuracy of 90.5% was achieved between discriminating the normal, premalignant and malignant group. Vibration of urea peak at 1002  $\text{cm}^{-1}$ , at 890  $\text{cm}^{-1}$  of ethanol, at 692  $\text{cm}^{-1}$  of creatinine, at 1046  $\text{cm}^{-1}$  of glucose, at 1417  $\text{cm}^{-1}$  of tryptophan, at 1615  $\text{cm}^{-1}$  of Indoxylsulphate shows variation in the three groups. Urine is an important biological fluid that reflects the end products of metabolism that is excreted from the body. Hence, this Raman study of urine samples could be used as a noninvasive method in the near future in the clinical diagnosis of cancer in mass screening procedure. The study reveals a classification efficiency with an accuracy of 93.1% for saliva samples. The biological components pyrimidine, glycoproteins especially mucin, oxygenated hemocyanin and carotenoids showed difference in the three groups of saliva. Mucin matrices peak at 1444  $\text{cm}^{-1}$ , at 752  $\text{cm}^{-1}$  of oxygenated hemocyanin, at 1158 and 1525  $\text{cm}^{-1}$  of carotenoids in saliva show variation between the three groups. These results validate that Raman spectroscopy can be used to objectively discriminate and correctly classify saliva of normal, premalignant and malignant samples and may be used as a complimentary to the existing conventional methods of disease diagnosis.

The findings obtained in this study provide reliable evidence on Raman spectroscopic discrimination of different premalignant and malignant tissue samples from normal with an accuracy of 97.4%. Primary tissue Raman bands are observed at 850, 935, 1000, 1130, 1156, 1174, 1200-1350, 1314, 1340, 1364, 1443, 1547, 1572 and 1650  $\text{cm}^{-1}$  shows variation in the three groups. The complexity of tissue



structure and environment make the interpretation of tissue Raman spectra difficult. To achieve the maximum benefit from Raman based diagnostic systems, an understanding of the molecular, microscopic and macroscopic origin of observed tissue Raman signals is required. In vitro results have demonstrated contributions from proteins, lipids and nucleic acids which are altered under neoplastic transformations. The biochemical differences between normal and pathological conditions of oral tissue and biological fluid samples are also discussed from spectral differences of the different classes of spectra. A study on fluorescence emission spectrum for OSF mucosa analyzed the changes in the fluorescence intensity of the endogenous fluorophores [12]. Raman spectral mapping for oral cancer diagnosis has also been reported [13]. Molecular finger print of Keratin, a well-established tumor marker for oral squamous cell carcinoma (OSCC) has been compared and OSCCs detected by Raman microspectral matching [14]. Serum samples of post-surgical cases of oral squamous cell carcinoma shows the possibility of predicting recurrences, which needs to be validated on large scales [15].

## Conclusion

The findings show that Raman spectroscopy has the potential to be a diagnostically useful tool for the in vitro detection of diseases (e.g. premalignant, malignant) in the oral cavity at the molecular level. It is shown that by this method of Raman spectroscopy, blood, urine, saliva and tissue samples can be successfully used to discriminate and diagnose correctly the normal tissue from OPMDs and oral cancer.

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