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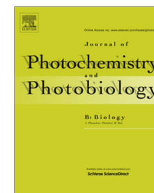

Fluorescence spectroscopic characterization of salivary metabolites of oral cancer patients

Manoharan Yuvaraj [a], Kanniyappan Udayakumar [a], Vadivel Jayanth [b], Aruna Prakasa Rao [a], Ganesan Bharanidharan [a], Dornadula Koteeswaran [b], Balu David Munusamy [c], Chilakapati Murali krishna [d], Singaravelu Ganesan [a,*]

[a] Department of Medical Physics, Anna University, Chennai 600 025, India
[b] Department of Oral Medicine and Radiology, Meenakshi Ammal Dental College and Hospital, Chennai 600 095, India
[c] Government Arignar Anna Memorial Cancer Hospital and Regional Cancer Center, Karapettai, Kanchipuram 631 501, India
[d] ACTREC, Tata Memorial Center, Navi Mumbai 410 210, India

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ABSTRACT

A pilot study has been carried out using human saliva in differentiating the normal subjects from that of oral squamous cell carcinoma (OSCC) patients, using the autofluorescence spectroscopy at 405 nm excitation. A markable difference in the spectral signatures between the saliva of normal subjects and that of oral cancer patients has been noticed. The possible reasons for the altered spectral signature may be due to the presence of endogenous porphyrin, NAD(P)H and FAD in the exfoliated cells from saliva. The elevated level of porphyrin in saliva of OSCC patients may be attributed to the disturbances in the amino acid degradation pathway and heme biosynthetic pathway, during the transformation of normal into malignant cells. The integrated area under the curve of fluorescence emission spectrum at 405 nm excitation and also fluorescence excitation spectrum for 625 nm emission were compared for the saliva of normal and oral cancer patients. The area under the curve for the emission spectrum provides 85.7% sensitivity and 93.3% specificity, where as the fluorescence excitation spectrum discriminates the same with 84.1% sensitivity and 93.2% specificity.

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1. Introduction

The incident rate of oral cancer was ranked, number one cancer for males and third most common cancer for females, when compared to other types of cancer in India [1] and it affects more than 481,000 new patients worldwide [2,3]. In spite of much treatment advancements, the death rate due to oral cancer was around 45% within five years from diagnosis [4]. One of the reasons for this high mortality rate was due to diagnosis only at the advanced stage. Although tissue biopsy and subsequent histopathological procedures have been considered as a golden standard method, oral cancer patients may not report to the physician at the early stage of the disease, as these patients may not have symptoms at early stage. Also the conventional oral cancer screening programmes such as visual inspection, use of toluidine blue, brush biopsy and fluorescence imaging have their own limitations and attention has been given to the mass screening of oral cancer patients at proteomics, genomics and metabolomics level.

Recently, diagnosis based on saliva, picked up much importance in the screening of various diseases [1,5–7] as its collection was very simple and easy, when compared to the collection of blood and also, any physiological and pathological changes in the body will be reflected in saliva. In addition, saliva has various functions like lubrication for speech, digestion of food and protection from microorganisms and it has many minerals, mucus, nucleic acids, enzymes, enzyme inhibitor, growth factor, etc. [5]. Based on these, it has been considered for monitoring diseases like dental caries, HBV, HIV, rubella, dengue, malignant diseases, hormonal analysis and analysis of drug abuse such as cocaine [6] and heroin. In the case of oral cancer, there were considerable changes in the DNA, oxidative protein in saliva, due to reactive nitrosamine (RNS) like nitric oxides (NO), nitrites (NO_2) and nitrates (NO_3) and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), oxygen (O_2^*) and hydroxyl (OH^*) free radicals [8].

For the last five years, the field of salivary research has been growing rapidly due to the advancement of new analytical techniques, that identify the physicochemical characteristics of saliva at metabolic, genomic and proteomic level. As protein was one of the important constituents of saliva, studies have been carried out, using surface enhanced laser desorption and ionization time

* Corresponding author. Tel./fax: +91 44 22358685.
E-mail addresses: sganesan@annauniv.edu, sganesan_61@yahoo.com (S. Ganesan).

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of flight [SELDI-TOF] coupled with mass spectrometry (MS), high performance liquid chromatography (HPLC-MS) to identify proteomic and enzymatic markers [9]. Similarly, the measurements of intracellular metabolites using electrophoresis coupled with mass spectroscopy have also been considered as a powerful tool to identify various oral salivary metabolic profiles of patients with oral cancer [5]. Besides the above techniques, laser induced fluorescence (LIF) coupled with high performance liquid chromatography (HPLC) [10] or capillary electrophoresis (CE) [11] have also been considered for analyzing the saliva. Inspite of these advancements on the analytical techniques for the characterization of saliva of cancer patients, research is still in the primitive stage to develop a simple, sensitive and rapid technique to characterize the saliva.

From the literature cited above, it is evident that any human system with altered metabolic and pathological conditions may have changes in the distribution and the photochemical characteristics of some of the key amino acids, proteins, enzymes and endogenous porphyrin etc. The elevated level of porphyrin in tissues may be due to altered heme biosynthetic pathway and/or amino acid degradative pathway, the excess amount of porphyrin on tissues may create oxidative stress, and may be released out from the malignant cells through pinocytosis [12]. Hence, biofluids of oral cancer patients may have elevated level of Porphyrin. Previously Meng et al. had reported that there was an elevation of protoporphyrin-IX in blood plasma from the tumor-implanted mouse model [13]. Madhuri et al. also reported, the cross validation of different groups such as liver diseases, OSCC and normal blood plasma using porphyrin as a marker [14]. However to the best of our knowledge no research has been carried out so far on discriminating normal subjects from oral cancer patients, based on the characterization of salivary porphyrin. In this context, a pilot study has been carried out to check the feasibility for discriminating the saliva of normal subjects and oral cancer patients based on spectroscopic characterization of porphyrin. In this relevance autofluorescence spectroscopy at 405 nm excitation using spectral and time domain studies have been considered. Statistical analyses were made and found that spectral signatures of the oral cancer patients and normal subjects were significantly different.

2. Materials and methods

2.1. Study group

For the present study, 63 patients with oral malignancy of age group of 25–80 (mean age group of 59 ± 11) and 60 normal subjects with age group of 25–57 (mean age of 38 ± 12), were considered for the native fluorescence spectroscopic analysis of saliva. The control group was with non-drinking and non smoking habits and also free from the history of malignancy, immunodeficiency, auto immune disorder, and hepatitis (or) HIV infection. The study group consisted of both male and female. The oral cancer patients were histopathologically confirmed at Government Arignar Anna Cancer Hospital, Kanchipuram, Tamilnadu, INDIA, as oral squamous cell carcinoma (OSCC). Protoporphyrin disodium salt (PpIX) Product no. 258385, Sigma Aldrich, St. Louis, USA, in phosphate buffered saline of pH-7.4 was used as standard.

2.2. Collection of saliva

Whole saliva was collected from both normal subjects and cancer patients under resting conditions. One hour prior to the collection of saliva the patients were advised not to take any food, drinking, smoking or using any oral hygiene products [11]. The patients were asked to gargle the oral cavity with clean water. After 15 min the secreted saliva was collected on a sterile container. The samples thus collected were kept in ice pack for analysis. However, the entire spectroscopic analyses were made within 3 h after sample collection.

2.3. Fluorescence spectroscopic characterization

The autofluorescence spectroscopic characterization of raw saliva were carried out using spectroflurometer of model Fluoromax-2, SPEX, Edison, New Jersey, USA. The excitation source, 150 W Ozone free Xenon arc lamp coupled to the monochromator, delivers light to the sample at a desired wavelength and the fluorescence emission from the sample was collected by an emission monochromator connected to a photomultiplier tube (R928P; Hamamatzu, Shizuoka-Ken, Japan). The gratings of the excitation and emission monochromators had a groove density of 1200 grooves mm^{-1} and were blazed at 330 nm and 500 nm, respectively. Excitation and emission slit width were fixed as 5 nm. The acquisition interval and the integration time were maintained as 1 nm and 0.1 s respectively.

The steady state fluorescence emission spectra were measured in the emission range 425–750 nm at 405 nm excitation. The fluorescence excitation spectra for emission at 625 nm for the excitation range 250–600 nm were also measured. To map all the possible fluorophores, the excitation emission matrix (EEM) was also carried out, for 23 normal and 33 cancer patients. The EEM was recorded by fixing excitation range 250–540 nm and the emission in the range 270–750 nm, with a constant wavelength interval of 5 nm at room temperature.

2.4. Fluorescence excited state kinetics

Lifetime measurements were made using Time Correlated Single Photon Counting (TCSPC) System on Fluorolog-3 (HORIBA Jobin Yvon, INC, Edison, NJ) by exciting the sample using 460 nm Nano LED (Pulse Width: >1 ns), with a fast response red sensitive PMT(R928P, Hamamatsu Photonics, Shizuoka-Ken, Japan) detector. The fluorescence emission was collected at 90° from the path of the exciting light source. The electrical signal was amplified by a pulse amplifier (Model No. TB-02, Horiba) fed to the single channel constant fraction timing discriminator (Model No. 6915, Philips Scientific, Mahawah, NJ). The first detected photon was used as a start signal by a time-to-amplitude converter (TAC), and the excitation pulse triggered the stop signal. The multichannel analyzer (MCA) recorded the repetitive start-stop signals from the TAC and generated a histogram of photons as a function of time-calibrated channels (55.7 ps/channel) until the peak signal reached 10,000 counts for 625 nm emission. The instrument response function was obtained using the Rayleigh scatter of Ludox-40 (40 weight percentage suspension in water; Sigma–Aldrich) at 460 nm excitation. Decay analysis software (DAS6 v6.0, Horiba) was used to extract the lifetime components. The goodness of fit was judged by chi-square values, Durbin-Watson parameters, as well as visual observations of fitted line, residuals, and autocorrelation functions. The average lifetime (τ_{avg}) with amplitude (α_i) and lifetime τ_i, i = 1, 2, 3 was calculated using the following equation.

$$\tau_{avg} = \frac{\sum_{i=1}^{3}\alpha_i\tau_i^2}{\sum_{i=1}^{3}\alpha_i\tau_i} \quad (1)$$

2.5. Statistical analysis

Statistical analyses of the fluorescence emission and fluorescence excitation spectral data were carried out to check the significance. Receiver Operator Characteristics (ROCs) analysis was carried out for the area under the curve (AUC) of the fluorescence

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