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Ultra-sensitive high performance liquid chromatography–laser-induced fluorescence based proteomics for clinical applications☆

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ABSTRACT

An ultra-sensitive high performance liquid chromatography–laser induced fluorescence (HPLC–LIF) based technique has been developed by our group at Manipal, for screening, early detection, and staging for various cancers, using protein profiling of clinical samples like, body fluids, cellular specimens, and biopsy-tissue. More than 300 protein profiles of different clinical samples (serum, saliva, cellular samples and tissue homogenates) from volunteers (normal, and different pre-malignant/malignant conditions) were recorded using this set-up. The protein profiles were analyzed using principal component analysis (PCA) to achieve objective detection and classification of malignant, premalignant and healthy conditions with high sensitivity and specificity. The HPLC–LIF protein profiling combined with PCA, as a routine method for screening, diagnosis, and staging of cervical cancer and oral cancer, is discussed in this paper.

Biological significance
In recent years, proteomics techniques have advanced tremendously in life sciences and medical sciences for the detection and identification of proteins in body fluids, tissue homogenates and cellular samples to understand biochemical mechanisms leading to different diseases. Some of the methods include techniques like high performance liquid chromatography, 2D-gel electrophoresis, MALDI–TOF-MS, SELDI–TOF-MS, CE–MS and LC–MS techniques. We have developed an ultra-sensitive high performance liquid chromatography–laser induced fluorescence (HPLC–LIF) based technique, for screening, early detection, and staging for various cancers, using protein profiling of clinical samples like, body fluids, cellular specimens, and biopsy-tissue. More than 300 protein profiles of different clinical samples (serum, saliva, cellular samples and tissue homogenates) from healthy and volunteers with different malignant conditions were recorded by using this set-up. The protein profile data were analyzed using principal component analysis (PCA) for objective classification and detection of malignant, premalignant and healthy conditions. The method is extremely sensitive to detect proteins with limit of detection of the order of femto-moles. The HPLC–LIF combined with PCA as a potential proteomic method for the diagnosis of oral cancer and cervical cancer has been discussed in this paper.
This article is part of a Special Issue entitled: Proteomics in India.

1. Introduction

It is estimated that, in the next few decades, cancer will be the major cause of morbidity and mortality all over the world. It is estimated that by 2030, there will be 20.3 million new cases of cancer and 13.2 million deaths annually due to cancer related diseases, which is a large increase compared with the estimates for 2008 [1,2]. There is a demographic transition, and increase in the cancer incidence and mortality will have an enormous effect on social and economic development [1,2], especially for the developing countries, where facilities for screening, early detection, and therapy are limited to only the urban population, while more than 80% of the population reside in rural areas. The burden of cancer is increasing in economically developing countries as a result of changing life style [3]. Cervical cancer is the second leading cause of cancer mortality in women in developing countries and seventh in developed countries. One-third of the world's oral cancer cases are reported in India [4]. According to WHO the estimated cancer deaths in India are projected to increase to 7 lakhs by 2015 with one-third of these occurring in cervical cancer and oral cancer [2–4]. Research in early detection of these conditions through screening for protein

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markers in clinical samples by proteomic techniques is of recent origin [5–13]. Several groups are working at present in this area using different techniques like 2-D gel electrophoresis, MALDI–TOF mass spectrometry, HPLC–MS, SELDI–MS, Bio-bar codes, and HPLC–LIF. Each of these methods has got its own advantages and disadvantages as has been pointed out earlier [14].

We have developed an ultra-sensitive high performance liquid chromatography–laser induced fluorescence (HPLC–LIF) detection technique [5,15–24] as a protein-profiling approach for analyzing protein profiles of micro-quantities of clinical samples, such as serum [16,19], saliva [20], cellular samples [17] and tissue homogenates [18]. The method is highly objective, capable of diagnosing whether the subject under investigation has normal, pre-malignant, or malignant condition. The system records the protein profile of a clinical sample, with an efficient HPLC protein separation step combined with ultra-sensitive LIF detection, providing a chromatographic plot of fluorescence intensity versus time of elution. The system can detect sub-femto-mole levels of proteins using microgram amounts of a clinical sample and can give more or less complete profiles of the large number of proteins present at ultra-trace levels [15] in these samples.

2. Materials and methods

2.1. Sample collection and processing protocol

Blood, cellular samples from the cervix, and cervical tissue samples were collected at the OBG Department, Kasturba Hospital, Manipal. For oral cancer, body fluids (blood and saliva) were collected by the Department of Oral Medicine, MCODS, Manipal, and Mangalore. Samples were collected from healthy volunteers and pre-malignant and malignant cervical and oral cancer patients, with their informed consent. The protocol described below was followed for sample collection and processing. Ethical clearance from the Institutional Ethics Committee (IEC), Manipal University, Manipal was obtained for the study. Sample information details are given in Table 1.

2.1.1. Serum

Blood samples were stored at room temperature for about 30 min, centrifuged at 3000 rpm for 5 min and the supernatant (serum) was collected. The serum samples were diluted 100 to 500 times with ultrapure water (0.45 μm, 47 mm diameter, nylon membrane filter). Twenty microliters of the diluted sample was injected into the HPLC system for protein profiling.

2.1.2. Saliva

Saliva protein profiles from 100 (37 normal, 40 pre-malignant and 23 malignant) subjects were recorded. Whole saliva was collected from the subjects after clinical examination. Subject's mouth was cleaned with distilled water prior to sample collection. Volunteers were asked to generate saliva and spit into a wide-mouthed sterile sample collection bottle, in about 5–10 min. Approximately 3–5 ml of whole saliva was collected from each subject. Bottles were immediately transported to the lab in ice buckets. Samples were transferred immediately to Eppendorf tubes and centrifuged twice at 5000 rpm for 10 min to remove any debris, and to eliminate exfoliated cells from the sample. Saliva samples were diluted 20–50 times with the pre-filtered HPLC grade water. Twenty microliters of freshly diluted sample was injected into the HPLC system.

2.1.3. Cellular samples

Cellular samples (exfoliated cells of the cervix, normal and malignant) were collected by gentle scraping of the cervix with a wooden spatula and transferred to normal saline. The cells were washed several times with normal saline and then centrifuged (3000 rpm for 5 min, normal saline) to collect the cell pellets. The pellets were then mixed with Tris EDTA buffer. Twenty microliters of buffer solution was added for every 1 mg wet weight of the pellet. Cells were lysed using a sonicator (Sonics Vibra-Cell model: VC 130 PB). Lysed cells were again centrifuged and the supernatant was collected for recording protein profiles.

2.1.4. Tissues

Tissue samples were transported to the laboratory within 1 h of collection. The samples were washed several times with normal saline (0.9%) to remove traces of blood, if any. Then the samples were weighed and minced with Tris EDTA buffer, 10% the wet weight of the sample. The tissues were then homogenized using a homogenizer. The homogenate was centrifuged at 5000 rpm for 20 min and the supernatant was collected through a syringe filter (0.45 μm, 3 mm diameter, nylon filter). Twenty microliters of the collected sample was injected into the HPLC–LIF system for protein profiling. Homogenized tissue samples were used for protein profiling, to eliminate possible errors due to inhomogeneity of samples (tissue), "field cancerization" etc.

2.2. Instrumentation

The high performance liquid chromatography–laser induced fluorescence (HPLC–LIF) set-up is discussed in detail elsewhere [15]. In short, it consists of an HP1100 gradient HPLC system, with Rheodyne 7725 manual (20 μl loop) injector and Vydac 219 TP52 biphenyl narrow bore column. The eluent from the column is fed into a quartz capillary flow cell (75 μm ID, 360 μm OD), where excitation of proteins is done with 257 nm radiation from a frequency-doubled Ar^{+} laser (Coherent Innova 90C). Protein fluorescence is collected from the side perpendicular to the laser beam and the chromatographic profile is obtained by recording fluorescence in the 340–350 nm range as a function of time-of-flow, using a JY DH10 double monochromator, Hamamatsu R453 photomultiplier, EG&G 651 chopper (20 Hz), EG&G 5113 preamplifier, and EG&G 7265 lock-in amplifier.

All HPLC–LIF recordings were done under the following protocol: laser power 5 mW; monochromator set to 340 nm, with 14 nm band pass; PMT at 850 V; sample elution-gradient run with A (water + 0.1% v/v trifluoroacetic acid, TFA) 70–40%, B (acetonitrile + 0.1% v/v TFA) 30–60%, for 0–60 min, followed by B 60–100% for 1 min, 100% B for 14 min. After each run, the column was regenerated with A for 15 min; flow rate for all runs was 200 μl/min.

2.3. Data analysis

Before analyzing the protein profiles by PCA, all the protein profiles were preprocessed to minimize the random variations, noise and background signals. Multipoint background subtraction was performed to reduce the background which varies only slowly over the entire run. The Fourier smoothing technique was used to minimize random noise due to any high-frequency fluctuations in the laser power and photomultiplier dark current. The retention times of individual proteins may vary slightly from run to run because of possible small variations in day-to-day experimental conditions (e.g., room temperature, errors in sample injection speed, speed of the pump). This may cause small shifts in elution times of same proteins in different runs. To reduce this shift in the peak position, all the protein profiles were subjected to a calibration procedure by assigning the mean values for the protein peaks common in all the samples along the time scale. We observed that, this reduced shifts in peak positions between different runs to less than (±2 s). Finally, all the protein profiles were subjected to normalization with respect to a protein peak, the relative intensity of which remained more or less unaffected from run to run and which is common in all the protein profiles of that class of samples (see the peak in Fig. 1 with *). The pre-processed data were then analyzed by principal component analysis (PCA) using GRAMS/32 (Galactic Inc., USA) software for statistical classification and diagnosis. All pre-processing steps were also done with GRAMS/32.