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Protein profile analysis of cellular samples from the cervix for the objective diagnosis of cervical cancer using HPLC-LIF

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ABSTRACT

Protein profiles of cytologic samples from the cervix were studied using High Performance Liquid Chromatographic (HPLC) separation combined with ultra-sensitive laser induced fluorescence (LIF) detection. HPLC-LIF protein profiles of samples from clinically normal subjects, individuals suffering from cervical cancer (different stages), and subjects who had other gynecological problems related to cervix, like erosion of cervix and Nabothian cyst, but no malignancy, were subjected to Principal Component Analysis (PCA). The application of HPLC-LIF protein profiling combined with PCA was found to be a highly efficient method for discrimination of different classes of samples with high sensitivity and specificity. Diagnostic accuracy and optimal threshold – decision criterion – for objective discrimination were estimated using sensitivity–specificity pairs and Youden's index (*J*) plots.

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1. Introduction

Pap smear screening test detects abnormal cells in cytological smears from the cervix, which can advance to malignancy. The reported sensitivity and specificity of Pap cytology to detect HSIL (high-grade squamous intraepithelial lesion) in over 15 retrospective and prospective randomized trials have been 55% and 98%, respectively [1]. In developed countries, Pap smear screening programs have reduced incidence of cervical cancer by up to 90% and has decreased cancer mortality substantially [2,3]. It has been reported that the majority of women who develop cervical cancer never had a Pap test or did not have a recent Pap test [4–6]. The relatively low sensitivity of Pap test is ascribed to effects of fatigue factor (examination of large number of samples in a limited time), errors in sampling, heterogeneous character of samples, and the subjective decision making which depends on experience of clinician/pathologist. In case of a positive Pap test, biopsies are taken after colposcopy from suspicious areas of the cervix for histopathology review. Biopsies are prone to errors like "past pointing" [7]. In two recent multi-centered studies, the sensitivity of colposcopy was shown to be about 50% only, for detecting CIN2+ [8,9]. In developing countries like India, facilities for regular screening in terms of number of clinics and qualified pathologists are available to a very limited extent. Many women are not subjected to routine screening; especially in the rural areas. Newer technologies have been developed, with the intention of improving the detection of cytological abnormalities, including liquid-based, thin layer cytology (ThinPrep, Autocyte) and computerized re-screening [10]. The available evidence indicates that use of liquid-based cytology gives modestly higher sensitivity for detecting any degree of cervical intraepithelial neoplasia (CIN), whereas specificity is lower than with conventional Pap smears [11].

The serum protein profiling method discussed in our earlier papers [12,13] do not suffer from the disadvantages of histopathology/colposcopy. Homogeneous samples only are used and the method depends only on instrumental measurements and statistical analysis of data and is thus highly objective. Our protein profiling method, applied to cellular samples from the cervix, can similarly eliminate the problems of histo-pathology mentioned earlier. The cells are homogenized for analysis, eliminating errors from inhomogeneous character of samples. Fatigue factor and inexperience of pathologist/clinician are also eliminated since the technique depends only on instrumental measurements and mathematical analysis of data. It should be mentioned here that cytological samples are widely used in detection of various forms of malignancy, for example, fine needle aspiration (breast, prostate, thyroid), brush biopsy (lung cancer), and identification of tumor cells in abdominal, pleural, and cerebrospinal fluids [14]. Our method of protein profile analysis can be adopted for all these samples.

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Our serum protein profiling method [13] is minimally invasive and easily carried out, without the need for histo-pathology. Serum samples are representative of the whole body conditions. The pre-malignant/malignant protein profiles will thus give in addition to the normal proteins, proteins representing the various changes occurring in induction of malignancy and highly enhanced cellular proliferation rates. These include various kinases, antigens and antibodies. However, proteins specific to dysplastic/malignant cells, like cell surface receptors and DNA/histone adducts of carcinogens, may be present only to a lesser extent in serum, since they will be derived from dysplastic/malignant cells leaking into the circulatory system. To get detailed information on such proteins it will be more appropriate to investigate the exfoliated cells themselves.

The HPLC-LIF method is highly sensitive and can detect the malignant condition even if one in a million cells only is malignant in the sample. The method is not subjective. A trained technician can carry out the runs and the mathematical analysis depends only on the recorded protein profile. Also there exists the possibility that abnormal cervical conditions, which may appear similar to malignancy by symptoms/histopathology, may be discriminated from malignancy by protein profile analysis. In view of the advantages of HPLC-LIF method combined with PCA in the analysis of protein profiles of the clinical samples, we have carried out a pilot study on protein profile analysis of cellular samples to develop a diagnostic tool in the detection of cervical cancer and other abnormal cervical conditions. Both visual and statistical methods were used for the discrimination of different classes of samples and the results of these studies are presented and discussed in this paper. The present work mainly focused on testing of the protein profile analysis of cellular samples using HPLC-LIF technique for a highly objective diagnosis of cervical cancer.

2. Materials and methods

2.1. Sample collection, storage and handling

Normal cellular samples (exfoliated cells of the cervix) were collected from healthy volunteers. Cellular samples of malignant/abnormal cervical condition subjects were collected at the Department of Obstetrics and Gynecology, Kasturba Medical College, Manipal. Samples were collected by gently scraping the exfoliated cells from the cervix with a wooden spatula and put them in normal saline. All samples were used with informed consent. Ethical clearance (KHEC No:31/2005) was obtained from the Kasturba Medical College Ethical committee for the present study. A total of 36 samples were collected for analysis (Table 1). The normal samples (1–13) were taken from healthy volunteers during their general screening for cervical cancer and were free of all abnormal conditions associated with the reproductive system based on pathology and visual analysis of cervix by the physician. The malignant subjects (37–72 years) were at different stages of cancer. Eleven were from stage IIIB and four, from stage II. Sample numbers 14–28 were from malignant subjects. Samples 29–36 were from volunteers who had gynecological conditions other than malignancy. The various disease conditions of cervix irrespective of whether they belong to inflammatory or non inflammatory conditions were categorized in to a totally different group, namely 'disease'. Since, there exist the possibility that, abnormal cervical conditions may happen to be similar to the malignant conditions, it is very important from the diagnosis point of view that a disease condition should be judged as malignant only if it is malignant and any other abnormal condition of the cervix should be discriminated from malignancy based on the change in the protein signatures. So we have included three study groups namely normal, malignant and disease conditions of the cervix for our study. The collected samples were immediately transported to the laboratory in normal saline. If storage was necessary, samples were stored at −80 °C in the deep freezer. They were passively thawed to room temperature just before processing and analysis.

Table 1
Sample details.

S. no.	Clinical conditions	Age
1–2		40, 35
3	Asthma	29
4–13		25–61
14–16	Stage IIIB	45–60
17	Stage IIB	44
18–21	Stage IIIB	55–72
22	Stage IIB	56
23	Stage IIIB	55
24	Stage IIIB	54
25	Stage IIB	37
26	Stage IIIB	37
27	Stage IIIB	60
28	Stage IIB	37
29	Inflammation of cervix	50
30	Trichomonas infection of cervix	53
31	Vagina unhealthy, cervix cervix unhealthy	30
32	cervix erosion	38
33	Erosion of cervix	29
34	Nabothian cyst	35
35	Inflammation of cervix	58
36	Nabothian cyst	35

2.2. HPLC runs—protocol

The cells were washed with normal saline several times and pelleted by spinning (3000 rpm for 5 min, normal saline) in a micro-centrifuge (Costar mini centrifuge, 10MVSS). The supernatant was discarded and the cell pellet was mixed with Tris EDTA buffer. For every 1 mg wet weight of the cellular pellet 20 μl of buffer solution was added. The cells were lysed by using a sonicator (Sonic vibra cell model:VC 130PB). Lysed cells were again centrifuged and the supernatant was collected.

HPLC-LIF set up is shown in Fig. 1. The HPLC system consists of an HP 1100 gradient system, Rheodyne 7725 Injection port and Biphenyl Reversed Phase narrow bore column (diphenyl, 2.1 mm × 250 mm, 5 μm, 300 Å). Chromatograms were recorded by measuring the fluorescence with a monochromator (Jobin Yvon DH10 SPEX, NJ, USA), Chopper (EG&G model 651), Photomultiplier (Hamamatsu R 453, NJ, USA), and Lock-in Amplifier (EG&G model 7265) system interfaced to a computer. Protein fluorescence was

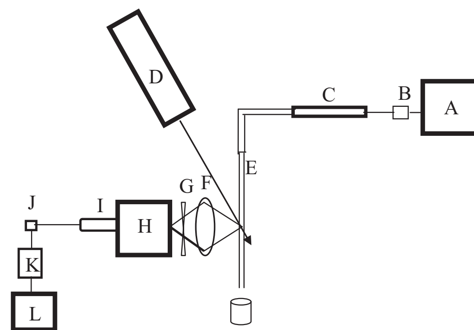

Fig. 1. Block diagram of the HPLC-LIF set up. A: HPLC, B: injector, C: biphenyl reversed phase column, D: frequency doubled argon ion laser (257 nm), E: quartz capillary, F: lens, G: Chopper, H: monochromator, I: PMT, J: preamplifier, K: Lock-in Amplifier, and L: computer.

excited by irradiation of the HPLC effluent with 257.5 nm from a frequency doubled Ar^+ (Innova 90C FreD, Coherent, CA, USA) laser. The experimental conditions were: laser power 15 mW, Chopper frequency 20 Hz, monochromator wavelength 340 nm, slits width: 2 mm, PMT voltage: 850 V, Lock-in Amplifier time constant: 2 s and Lock-in Amplifier gain: 6 dB.

Water (HPLC grade) with 0.1% (v/v) TFA and HPLC grade Acetonitrile [Merck] with 0.1% TFA (v/v) were used for gradient runs. A blank gradient was run before each sample to confirm the stability of the column and absence of contamination. 50 μl of sample was then injected into the narrow bore biphenyl column fitted with a 20 μl loop. The sample was eluted with water–Acetonitrile gradient. The gradient starts with 70% H_2O (0.1% TFA) + 30% Acetonitrile (0.1% TFA) and changes to 40% water + 60% Acetonitrile (0.1% TFA) in 60 min. The rate of elution was kept at 0.2 ml/min.

2.3. Data analysis

To derive the maximum information from the protein profiles by mathematical/statistical analysis, several data pre-processing techniques were adopted. The aim of the pre-processing is to reduce random variations, noise and unwanted background to a minimum level. While the chromatographic peaks are very sharp, the background varies only slowly over the entire run. This was reduced by multipoint background substraction. In addition to this the recorded chromatogram plots always contain noise due to the fluctations in the laser power, photomultiplier dark current and random noise. We used Fourier smoothing technique to remove the high frequency noise which can cause random errors. Because of possible small variations in day-to-day experimental conditions (eg. Room temperature, errors in sample preparation, speed of the pump), the retention times of individual proteins may vary slightly from run to run causing some shift in the elution time of same proteins in different runs. All the chromatograms (protein profiles) were subjected to a calibration procedure by taking the protein peaks common in all the samples along the time scale. This calibration reduced shifts in peak positions between different runs to a minimum (>±2 s). Finally, to facilitate intercomparison, all the chromatograms were subjected to normalisation with respect to a protein peak (2120 s peak), the relative intensity of which remained more or less unaffected from run to run. The pre-processed data were then analyzed by Principal Component Analysis (PCA) for classification and diagnosis. For statistical classification of individual samples we have used GRAMS/32 (Galactic Inc., USA) software. All pre-processing steps were also done with this software.

In our method of Principal Component Analysis (PCA), the mean of all samples in the data set is first formed. The average or "mean" is calculated from all the chromatograms and this is subtracted from every individual chromatogram to get the variation chromatograms. The variation chromatograms are subjected to PCA, thereafter, factors and scores are determined. The scores for a given sample correspond to the contribution of each principal component to the variation of that sample from the mean. If the variation from the mean is very small then the scores will be very small.

To get a more accurate evaluation, all the samples were tested against a normal and malignant standard set and Match/No Match test was performed using three parameters-scores, spectral residual, and Mahalanobis distance. The spectral residual is given by

$$\text{spectral residual} = \sum_{k=1}^{p} (\text{original spectrum}_k - \text{predicted spectrum from the factors}_k)^2$$

Spectral residuals can be calculated for different factors. The Mahalanobis distance is normally expressed in units of standard deviation. It is given by

$$D^2 = (S_{test})M^{-1}(S_{test})'$$

where S_{test} is the vector of the scores and sum of squared residuals for a given test sample, and M given by $M = ((S'S)/(n-1))$, where S contains the corresponding parameters for the calibration set of n standards. Any sample which lies outside of a desired range of standard deviation from the mean can be considered to be out of the group. To check the validity of the current method as a diagnostic aid, statistical evaluation of sensitivity–specificity pairs and Youden's index (J) have been calculated.

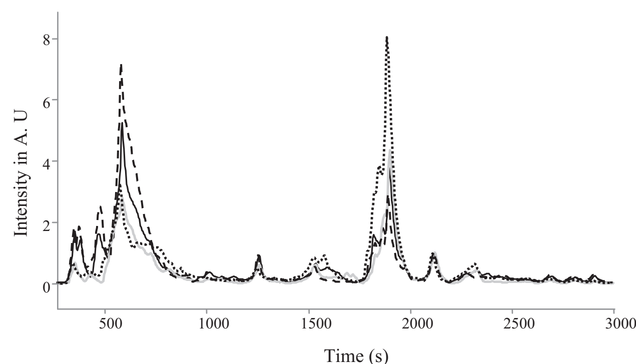

Fig. 2. Mean protein profile of cellular samples. Solid dark lines—normal; dark broken lines—disease; solid grey lines—stage II and dotted grey lines—stage III.

3. Results and discussion

Fig. 2 shows the mean of the protein profiles of samples of normal, malignant and disease conditions of the cervix. The complex nature of cellular protein composition will become much more apparent when we look at the patterns of the chromatograms in an expanded scale. We had mentioned that the sensitivity of our system, that is, signal-to-noise ratio, is sufficient to achieve femto mole detection limits [15]. The expanded scale of mean protein profiles of normal, malignant and disease conditions of the cervix other than malignancy are shown in Fig. 3. (The noise level is still so negligible that another ten fold expansion can still be done, if necessary.) What is immediately obvious from Fig. 3 is that there are hundreds of different proteins in these samples, some of them having probably very close structure/molecular weight, so that they overlap in their retention times. It is clear from Fig. 3 that the protein coming at ∼350 and ∼500 s is down regulated in malignant conditions. It is also interesting to observe that their relative concentrations are not changing much in other disease conditions of the cervix. The peak at 1250 s region shows a similar trend but to a lesser extent. The doublet seen around ∼1600 s and a weak peak at ∼1750 in normal samples are absent in other disease conditions and in stage II or III. The peaks in the region 1500–1600 s and the peaks between 2000 and 2500 s show higher concentrations in the malignant conditions.

We have observed that, for the cellular samples all normal samples showed a similar pattern, at least for the major peaks, though there were noticeable differences also. Since all the samples were thoroughly washed before use, it is highly unlikely that there are contaminations from extraneous sources like blood. It is also observed that many of the protein peaks observed in normal samples are also present in samples from subjects with various disease conditions, including malignancy. This happens because any sample from subjects with disease conditions will also contain a

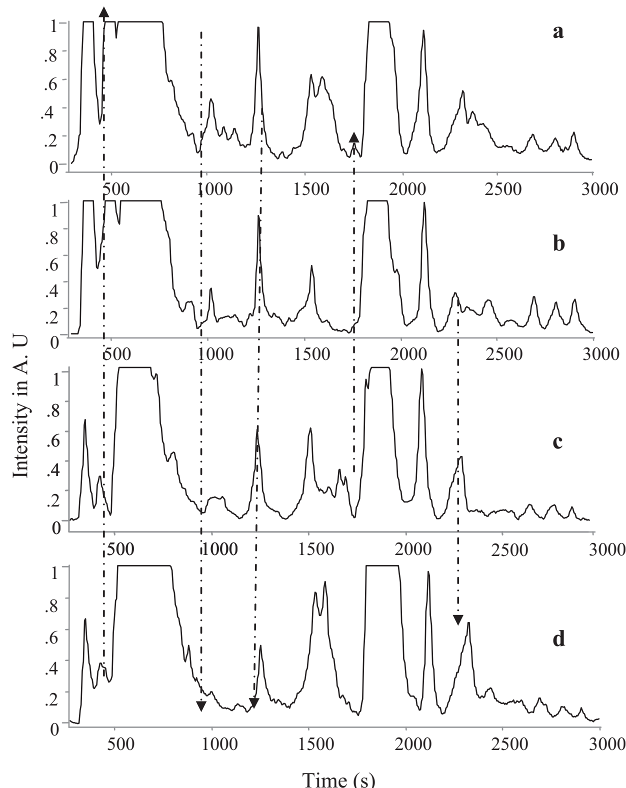

Fig. 3. Expanded scale of Fig. 2. (a) Normal, (b) disease condition of the cervix, (c) malignant stage II, and (d) malignant stage III in expanded scale.

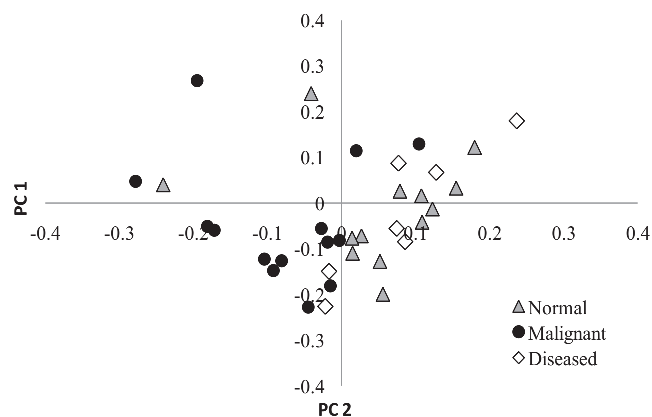

Fig. 4. Plot of sample number versus scores of factor 1.

large amount of normal cells. Such "anomalies" as seen in the cellular protein profiles could probably be one of the reasons for the high false negative/false positive results in Pap test, since a pathologist examining the sample may happen to see the abnormal cell in a normal sample and conversely only some of the large number of normal cells in the abnormal sample. This source of error is avoided in the protein profiling, since the entire sample is homogenized and used for analysis.

It is thus evident from Figs. 2 and 3, that while for serum [13], even a visual examination showed consistent differences between normal and malignant samples, no such visual discrimination is possible for cellular samples. This, as mentioned earlier, may arise from the possible presence of large amounts of normal cells also in the sample giving an inhomogeneous sample and the possibility of non-specific microbial contamination [14,16]. In spite of this drawback, as shown below, very good discrimination is possible by pattern analysis of the protein profiles using PCA.

3.1. Principal Component Analysis

PCA was first carried out using all the 36 chromatograms. To start with, PCA was done by taking 11 factors, as it has been observed that these factors contribute more than 95% of the variance of total data set. Fig. 4 shows the discrimination of the samples of normal, malignant and other disease conditions of the cervix using scores of factors 1 (PC1) and 2 (PC2). The samples from subjects with disease conditions other than malignancy are not discriminated from normal condition in these plots, although, good discrimination has been observed between majority of normal and malignant samples.

Our results with serum have shown that the technique of matching multiple parameters derived from PCA with standard calibration sets of each class, is a better method for classification of different types of samples (for example we could discriminate stages II and III in that analysis). It will be seen that here also this technique gives better discrimination and this is discussed below.

3.2. Discrimination of normal, malignant and other disease conditions

To get better discrimination between the different classes of samples (particularly between disease conditions other than malignancy from normal and malignant classes) Match/No Match test was carried out by forming calibration sets of certified samples of normal and malignant class. The scores, squared residuals and Mahalanobis distances [17] were taken here for "Match/No Match".

As the sample size under each category was not very large, a total of 10 out of 13 normal samples and 12 out of 15 malignant samples were taken for the preparation of calibration sets. All the 36 samples were then matched against each calibration set. It is worth mentioning that each member of the calibration set itself has been rotated out of the set and tested against the rest, for Match/No Match. Hence the samples used for calibration can be also considered as belonging to the test group since they have been independently tested to find to which class they belong or do not belong. The sample is classified as "PASS" or "FAIL", based on whether it matches all three parameters or not, with a given calibration set.

Table 2 shows the results of Match/No Match test with the normal and malignant calibration sets. From Table 2 it can be seen that the Mahalanobis distance and spectral residual of sample

Table 2
PCA results for the cellular samples compared against the standard normal and malignant calibration set.

Sample number	Limit tests	M-distance	Spec residual
For normal standard set			
1–4	PASS (PPP#)	0.71–1.005	1.17–43.50
5	FAIL (FPF#)	120.09	1457.96
6–7	PASS (PPP#)	0.72–0.98	2.105–27.27
8	FAIL (FPF#)	83.97	1038.34
9–13	PASS (PPP#)	0.75–1.00	0.851–19.94
14–28	FAIL (FPF#)	4.31–528.96	75.89–6376.45
29–36	FAIL (FPF#)	4.26–61.26	64.52–742.32
For malignant standard set			
1–13	FAIL (F?F#)	5.99–67.11	79.02–546.04
14–20	PASS (PPP#)	0.60–1.12	0.22
21	FAIL (FFF#)	34.64	391.62
22	PASS (PPP#)	0.71	8.71
23	FAIL (FFF#)	99.27	1139.56
24–28	PASS (PPP#)	0.71–1.12	3.11–9.69
29–32	FAIL (FFF#)	8.21–36.07	99.54–407.30
33	PASS (PPP#)	1.92	27.90
34–36	FAIL (F?F#)	2.92–181.93	45.14–2079.13

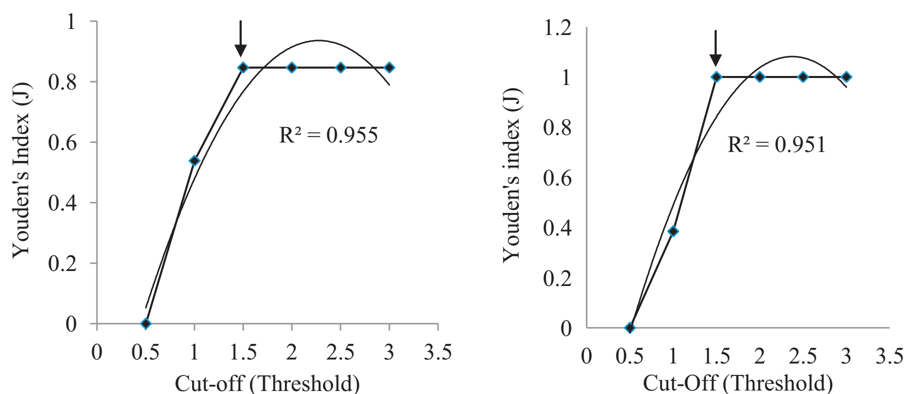

Fig. 5. Youden's index plots for (a) normal calibration set and (b) malignant calibration set.

numbers 5 and 8 are high and show "No Match" with normal calibration set. All the malignant samples and those samples taken from patients with non-malignant disease conditions associated with cervix showed "FAIL". That is, any disease condition – malignant or otherwise – is correctly classified as not normal. Table 2 also shows the sample prediction for all samples using a standard set of malignant samples. Malignant samples were randomly chosen from pathologically confirmed specimens. All the 13 samples belonging to normal cellular samples show "FAIL" giving a specificity of 100%. The sample numbers 5 and 8 from normal samples which did not match with normal set have shown no match with the malignant set also indicating that they are not malignant. One major advantage of the protein profiling technique is that these subjects can be repeatedly examined for better diagnosis, without colposcopy or another biopsy. Out of 15 malignant samples two samples were not matching with the malignant standard set giving a sensitivity of 87%. Only one sample (sample No. 33), with non-malignant disease condition of the cervix showed match result giving specificity of 95%. It is thus clear that disease conditions other than malignancy are also very well discriminated from malignant condition, once again reducing the need for colposcopy and biopsy in suspicious cases.

3.3. Diagnostic accuracy

Statistical evaluation is very important to know the performance of any kind of diagnostic test. It gives parameters like sensitivity and specificity pairs and Youden's index., through which one can judge diagnostic accuracy which is greatly affected by induced errors of pathological methods such as visual judgment, experience of the clinician/pathologist, and heterogeneity of sample [18–21]. We have carried out statistical evaluation of the Match/No Match test to check its feasibility as a diagnostic aid.

The diagnostic tests can be regarded as continuous measurements, since they can be screened in a range of different threshold values or cut-off operating points. To decide the value of an ideal threshold or cut-off point which discriminates best between disease and non-disease states, it is the usual practice to choose a point that has got high values for sensitivity and specificity. But, this may not be sufficient to evaluate the performance of a diagnostic test. It is well known that sensitivity and specificity have opposite trends in any diagnostic test. Attempts to increase one may result in decrease of the other. In such cases it is difficult to decide the threshold, and Youden's index (J) [22] can be used to choose an appropriate cut-off:

$$J = \text{sensitivity} + \text{specificity} - 1$$

Table 3
Sensitivity and specificity of normal and malignant samples compared against standard normal and malignant calibration set.

	Sensitivity	Specificity
With normal standard set	100%	86%
With malignant standard set	88%	100%

Results from the Match/No-Match tests are used to estimate the sensitivity (True Positive Fraction—TPF) and [1 − specificity] (True Negative Fraction—TNF), pairs and Youden's index. Operator can plot J for different operating points (threshold) and ideal operating point (threshold) can be selected as that for which J is maximum. In this case, sensitivity will be maximum and "1 − specificity" will be minimum. Youden's index gives an idea about combined measures (i.e. specificity and sensitivity). Fig. 5(a) and (b) shows plots of Youden's index and cut-off for normal and malignant calibration set test, respectively. In the figures one can see that for M-distance = 1.5 cut-off, Youden's index value is maximum (marked with arrows). The second order non-linear fit to the Youden's curve, regressions were found to be approx 0.956 and 0.952 for normal and malignant calibration sets, respectively. Since it is a combined measure of sensitivity and specificity one has to choose the threshold with high Youden's index, i.e. one has to choose at least 1.5 cut-off for the above test, which has J value 0.8462 and 1 for the Match–No Match test with normal and malignant calibration sets. Youden's index plots shows that the threshold 1.5 M-distance is sufficient to discriminate the different classes.

Even though the visual analysis of the protein profile of the cellular samples do not indicate disease condition as clearly as serum samples, the PCA analysis of the samples show high sensitivity and specificity (Table 3). One important result of the present study is the ability of the Match/No Match technique to discriminate normal and malignant conditions of the cervix from other disease conditions. This can greatly reduce the need of repeated colposcopic examinations and biopsies in suspect cases.

4. Conclusion

The pilot study of protein profile analysis of cellular samples from normal, malignant and disease conditions of cervix using HPLC-LIF technique has shown to be a promising method for objective diagnosis of cervical cancer. The PCA results show that the sensitivity and specificity of the present diagnostic method are 87% and 95%, respectively. Match/No Match test of samples with normal/malignant standard set of chromatograms can be used for the objective discrimination of normal, malignant and other disease conditions of the cervix. The protein profile analysis of cellular samples using HPLC-LIF presented here can be extended

as complimentary method to conventional cervical cancer screening techniques after its validation with large number of samples including premalignant conditions.

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