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An overview on applications of optical spectroscopy in cervical cancers

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ABSTRACT

Despite advances in the treatment modalities, cervical cancers are one of the leading causes of cancer death among women. Pap smear and colposcopy are the existing screening methods and histopathology is the gold standard for diagnosis. However, these methods have been shown to be prone to reporting errors, which could be due to their subjective interpretation. Radiotherapy is the mainstay of treatment for the locally advanced stages of cervical cancers. The typical treatment regimen spans over 4 months, from the first fraction of radiation to clinical assessment of tumor response to radiotherapy. It is often noticed that due to intrinsic properties of tumors, patients with the same clinical stage and histological type respond differently to radiotherapy. Hence, there exists a need for the development of new methods for early diagnosis as well as for early prediction of tumor radioresponse. Optical spectroscopic methods have been shown to be potential alternatives for use in cancer diagnosis. In this review, we provide a brief background on the anatomy and histology of the uterine cervix and the etiology of cervical cancers; we briefly discuss the optical spectroscopic approach to cervical cancer diagnosis. A very brief discussion on radiation therapy and radiation resistance is also provided. We also share our experiences with the Raman spectroscopic methodologies in cervical cancer diagnosis as well as in the prediction of tumor radioresponse.

KEY WORDS: Cervix cancers, optical diagnosis, Raman spectroscopy, FTIR, fluorescence

The cervix is an extension of the body of the uterus. The upper end communicates with the uterine body via the internal os and the lower end opens into the vagina through the external os. Except for the lower part, the uterine cervix is lined by a single-layered, columnar epithelium with tubular glands. The lower region is covered by nonkeratinizing stratified squamous epithelium. The squamocolumnar junction - between the columnar secretory epithelium of the endocervical canal and the stratified squamous covering of the ectocervix - has the greatest clinical significance as it is most common site for epithelial abnormalities that may progress to malignancy.

Cervix cancers are the second most common cancers among women (15% of female cancers in developing countries) and the seventh in frequency overall. Estimates suggest that approximately 4,93,000 patients were diagnosed with cervical cancer and 2,74,000 died from the disease in the year 2002.[1] In developing countries, cervical cancer accounts 15% female cancers out of 83% new cases. Among this women below 65 years age group carry a risk of 1.5%. In developed countries, cervical cancer accounts for only 3.6% of all new cancers, with a cumulative risk of 0.8% for the same age-group. The major risk factor for cervical cancers is human papilloma virus (HPV) infection, especially with strains like HPV 16, 18, 31, 33, and 45, which are termed as high risk factor strains. Other known risk factors include: smoking, HIV infection, low socioeconomic status, chlamydia infection, oral contraceptives, and multiple pregnancies.[2]

Pap smear and colposcopy are widely-used screening methods for the detection of cervical cancers. Eighty percent of all cervical cancer deaths are reported in developing countries, where these screening tests are not routinely practiced.[3] This emphasizes the importance of effective screening methods and early clinical detection techniques in cervical cancer management. However, the existing screening techniques have also been shown to suffer from high false negative/positive results, which could be attributed to the subjective interpretations of these tests.[4,5] Histopathology, the gold standard of diagnosis, is a time-consuming process and has also been shown to be influenced by subjective interpretation.

Radiotherapy is the treatment of choice for the locally advanced stages of cervical cancers. The radiotherapy regimen, from the first fraction of treatment till clinical evaluation of tumor radioresponse, spans over 4 months. A typical radiotherapy regimen is as follows: a patient with stage IIB to stage IV disease is subjected to external-beam radiotherapy (EBRT) of 45 Gy in 20 fractions over a period of 4 weeks, using a linear accelerator. The patient is then allowed

to rest for 2 weeks for parametrial regression, after which two doses of remote afterloaded high dose rate intracavitary brachytherapy (HDR) of 8.5 Gy to point A is given once a week. After 4 weeks of rest the patient is subjected to clinical assessment by per vaginal and per rectal examination 'to evaluate' tumor radioresponse; that is, conventionally, tumor response to radiotherapy is evaluated after 4 months from patient is exposed to first fraction of radiation treatment. Radiation resistance is a well-known and a serious hurdle in radiotherapy of cancers. Intrinsic factors such as DNA aneuploidy, S-phase fraction and proliferation kinetics, tumor vascularity and hypoxia, and glutathione content are believed to influence the radiation resistance of a tumor.[6-8] Under the influence of these intrinsic factors, tumors of the same clinical stage and histological type often exhibit differential radioresponse. The tumor response to radiation therapy is graded based on the degree of shrinkage of the volume of the tumor, which is, as mentioned above, assessed at the end of the treatment. A 100% shrinkage in the volume indicates complete response, shrinkage in volume of 65% and above is considered as partial response, and shrinkage in volume of less than 65% is assumed to indicate no response.[9,10] Very few studies have been reported in the literature on prediction of tumor response using radiobiological, magnetic resonance, and NMR spectroscopy methods.[6,11-14] Radiobiological studies indicate that prediction of tumor response to radiation could be assessed by estimation of glutathione (GSH) levels.[6] Though these studies do demonstrate the feasibility of prediction of tumor radioresponse, so far there are no prescribed methods for use in clinical practice.

From the above discussion it is clear that conventional screening methods have high rates of reporting errors, and there is no established method to predict tumor radioresponse at an early stage of the treatment. Hence, there exists a need for the development of new methods: 1) for detecting malignancy at an early stage and 2) for predicting the tumor radioresponse at an early stage of the treatment so that treatment protocols can be individualized.

Optical spectroscopic techniques, fluorescence,[15-24] FTIR[25-30] and Raman,[31-36] which are sensitive to changes at the level of biomolecular composition of samples, have been gaining considerable importance in biomedical applications, including diagnosis of cancer. In case of autofluorescence, the sample is impinged by suitable radiation, causing excitation of intrinsic fluorophores. These molecules emit photons on their return to the ground state. FTIR and Raman are the two complementory vibrational spectroscopic techniques. Infrared (IR) spectroscopy is an absorption process, whereas Raman spectroscopy is an inelastic scattering phenomenon. Fluorescence and FTIR are more sensitive and are popular methods in optical diagnosis. In the case of Raman scattering, molecules are excited to a virtual state on interaction with photons. The vast majority of these molecules deexcite back to the initial vibrational energy level, which results in release of photons of the same energy and

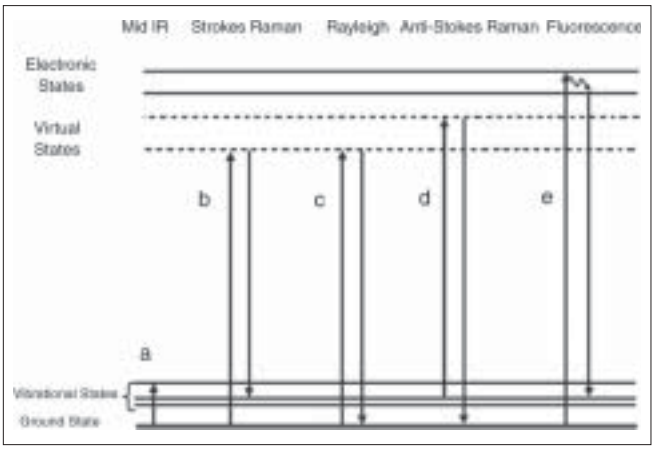

Figure 1: Diagrammatic depiction of different photophysical processes: a. Infrared absorption, b. Stokes Raman, c. Rayleigh scattering, d. Anti-stokes Raman, e. Fluorescence

wavelength - Rayleigh scattering. On the other hand a very tiny fraction of molecules deexcite to a different vibrational state. This phenomenon is known as 'Raman scattering.' A shift of scattering towards longer wavelengths is called the 'Stokes effect' and a shift towards shorter wavelengths leads to 'anti-Stokes.' Nevertheless Stokes lines are the more predominant and are commonly observed in Raman spectroscopy. The energy level transitions of various photophysical processes are depicted in Figure 1. FTIR and Raman spectroscopy provides rich information through 'molecular fingerprint.' Though, as described earlier, Raman scattering is an inherently weak process, Raman spectroscopy has certain distinct advantages over the other two popular methods, which include: 1) virtually no sample preparation (*vs* FTIR), 2) no strict dependence on wavelengths (*vs* fluorescence), 3) use of less harmful NIR radiations (*vs* fluorescence), 4) adaptability to *in vivo*/*in situ* measurements (*vs* FTIR), and 5) easy extraction of information from sharp spectral features (*vs* fluorescence and FTIR). However, as been stated earlier, a major drawback of Raman scattering is that it is an inherently weak process. To overcome this, several improvisations have been developed over conventional Raman spectroscopy; these include: resonance Raman, surface-enhanced Raman scattering (SERS), and Raman microspectroscopy.

OPTICAL SPECTROSCOPY - CERVICAL CANCER

Fluorescence spectroscopy

To the best of our knowledge, Alfano *et al*.[37] were the first to measure autofluorescence spectra of normal and malignant tissues. The group led by R.R. Kortum has been carrying out extensive investigations on fluorescence spectroscopy in normal and pathological conditions of the uterine cervix.[38-45,47-54] They have demonstrated the classification of normal and pathological cervical tissues at different excitation wavelengths as early as 1993.[38] The same group has reported that an increase in NADH fluorescence and a decrease in collagen fluorescence provided clinically significant differences

between normal and dysplastic tissues[45]; this corroborates the findings of Feld's group, which proposed that NAD(P)H and collagen could be used as quantitative fluorescence biomarkers for *in vivo* detection of dysplasia.[46] In a combined fluorescence and reflectance spectroscopy study they have shown that fluorescence emission spectra in 330-360 nm and 460-470 nm provided the best discrimination among normal, precancerous, and cancerous tissues compared to reflectance spectroscopy as well as a combination of both.[52] More recently they have developed a multispectral digital colposcope (MDC).[54] MDC is an unique multispectral imaging system that is a modification of the standard colposcope with a video camera adapter to measure reflectance and fluorescence images of the uterine cervix using an color video camera. Feld and his coworkers, the other major group in the field of laser-induced fluorescence (LIF), have demonstrated the potential of trimodal spectroscopy (intrinsic fluorescence, diffuse reflectance, and light scattering) for *in vivo* detection of cervical precancerous changes.[55] Recently, in another study, the accuracy of three integrated optical measurements - LIF, white light diffuse reflectance spectroscopy, and video imaging - in the detection of cervical disorders was investigated by Schomacker *et al*.[56] Several groups have investigated the potential of fluorescence spectroscopy in cervical cancer diagnosis. Gupta and his coworkers have demonstrated the efficacy of nitrogen LIF in cervical cancer diagnosis.[57] The group led by Kartha has demonstrated the potential of fluorescence spectroscopy at 325 nm excitation in discriminating between normal and malignant cervical tissues.[58] The novelty of their study was the comparison of gross tissue spectral features with the fluorescence emitted by individual fluorophores of the tissue homogenates, which was measured using high-performance liquid chromatography-LIF (HPLC-LIF) instrumentation. Recently, a correlative study between fluorescence spectra and detailed histopathological protocol of various parameters (such as sample thickness, type of epithelium, orientation, and location of the biopsy, etc.) demonstrated that fluorescence spectroscopy could classify disorders of the cervix with a high sensitivity and specificity.[59,60] Recent time-resolved confocal fluorescence experiments on monolayered cell cultures, using 365 nm excitation, demonstrated that time-resolved autofluorescence decays of free and bound NADH signals are the sensitive indicators of cellular metabolism.[61] Alvarez *et al*. reported that a combination of optical spectroscopy with colposcopy provided a clinically meaningful increase in the detection of CIN I, III in women referred for the evaluation of mildly abnormal cytology results.[62] Martin *et al*. characterized the spectroscopic changes that take place during neoplastic progression of cervical cancer using organotypic epithelial raft culture as an *in vitro* model of cervical tissue.[63] Werner *et al*. demonstrated that spectroscopy combined with cervical cytology is equally sensitive and twice as specific as HPV testing alone in identifying high-grade cervical neoplasia.[64]

FTIR spectroscopy

To the best of our knowledge Wong *et al*. were the first to record the IR spectra of normal connective tissue and the normal and malignant epithelial tissue of the cervix. They proposed that this technique could be used for mass screening.[65] A few other groups had demonstrated that IR spectroscopy could be used not only for the diagnosis of cervical cancers but also for characterizing molecular abnormalities during progression of the disease (CIN I-III).[66,67] In a comparative study, Fung *et al*. demonstrated that FTIR has a better false-negative rate and negative predictive value than standard Pap smears.[68] Cohenford *et al*. demonstrated the efficacy of the chemometric approach, using principal components analysis (PCA) for the discrimination of several conditions of the cervix.[69,70] The group led by Diem carried out extensive FTIR spectroscopy investigations on formalin-fixed paraffin-embedded cervical tissues, cell lines, and exfoliated cells.[71-77] They found that dysplastic samples have spectral features that are about halfway between that of normal and cancerous samples.[70,71] They demonstrated the implications of IR microspectroscopy in monitoring cell proliferation, drug response, etc., using He La cell lines[72-74] and also reported that IR spectroscopy is a highly sensitive technique for the detection and differentiation of cell types and for diagnosis.[75] They have demonstrated that a combination of FTIR microspectroscopy and multivariate spectral processing methods provide important insights into the fundamental spectral signatures of individual cells.[76] More recently, they have developed a database of IR spectral patterns for different tissue types as well as for different stages of cancer.[77] As described above, several studies have explored the feasibility of diagnosis of cervical cancers using exfoliated cell smears. As is well known, FTIR spectroscopy demands several steps of sample preparation and this can, in the end, induce artifacts if proper care is not taken.[78] The heterogeneity of real-life cell smears compounds the problem. McNaughton and his coworkers have investigated this aspect.[79,80] Though several factors such as bacterial contamination and the presence of leukocytes and thrombocytes can influence the spectral features, they opine that it is still possible to arrive at a diagnosis by means of careful data analysis. An independent study by Shaw *et al*. demonstrates the same.[81] Chang *et al*. found that the ratio of the areas of two spectral regions between 1130-1180 cm^{-1} and 1180-1260 cm^{-1} was an exceptionally useful factor in discriminating precancerous tissues from normal tissues of the uterine cervix.[82] Mark *et al*. reported that the grading of neoplasia based on FTIR microspectroscopy (FTIR-MSP) and probabilistic neural network (PNN) differentiates normal from premalignant tissue with a high level of accuracy.[83,84] Mordechai *et al*. demonstrated that in FTIR-MSP of formalin-fixed cervical tissues, glycogen levels could be a potential biomarker in the detection of cervical neoplasia.[85] However, this aspect is a matter of debate. In a very recent work, Walsh *et al*. reported that attenuated total reflection-Fourier transform IR (ATR-FTIR) coupled with PCA and subsequent linear discriminant analysis (LDA) facilitated the identification of phosphate and glycogen in the IR spectral region of 950-1200 cm^{-1} as potential biomarkers of abnormality.[86]

Raman spectroscopy

As mentioned earlier (introduction) section, FTIR and LIF are far more popular methods than Raman. This could be attributed to the higher sensitivity and simpler instrumentation of LIF and FTIR. Though recent advances in instrumentation (lasers and CCDs) make the recording of the Raman signal from biological samples relatively easy, the number of Raman-based studies are very few. Mahadevan *et al*. were the first to record the Raman spectrum of cervical tissues.[87] As early as 1998, they reported the potential of NIR Raman spectroscopy to distinguish cervical precancers from other conditions. They developed a compact fiberoptic probe to measure the *in vivo* Raman spectra of cervical tissue[88] and have demonstrated its potential in the detection of squamous dysplasia in normal and precancerous conditions.[89] Very recently, Lyng *et al*. demonstrated the potential of Raman microspectroscopy in the identification of biochemical changes in tissues during disease progression, using formalin-fixed paraffin-preserved (FFPP) tissues.[90]

RAMAN SPECTROSCOPY OF CERVICAL TISSUES: OUR APPROACH

Diagnostic applications

Some of the major issues pertaining to biomedical application are as follows:

1. Since the cervix is easily accessible, it is possible to develop noninvasive techniques using suitable fiberoptic probes. To carry out such studies, conventional Raman spectroscopy by NIR radiation is more suitable as the NIR radiation is relatively less harmful. Since conventional spectroscopy probes a large area (*vs* microspectroscopy), the spectra are more representative and, to a reasonable extent, the spectra recorded in *ex vivo* conditions can be extrapolated to *in vivo* conditions.
2. Methodologies should be relatively simple so that an untrained technician can use them. However, rigorous evaluation of models is a prerequisite before implementation in routine practice. Optical spectroscopic data are amenable to multivariate statistical tools such as artificial neural network (ANN), hierarchical cluster analysis (HCA), discriminant analysis (DA), principle components analysis (PCA), etc. Since there are several discriminating algorithms, it is necessary to explore and identify a suitable rapid, robust, and simple discriminating algorithm from the clinician's point of view.

In view of these considerations, we have developed a conventional Raman spectroscopic diagnostic model for discrimination of normal and malignant tissues of the uterine cervix using 15 normal and 24 malignant certified tissues.[91] We further evaluated the model by using 72 blinded samples, where the spectroscopists did not have prior information on the nature of the sample. A typical Raman setup assembled by us is shown in Figure 2.

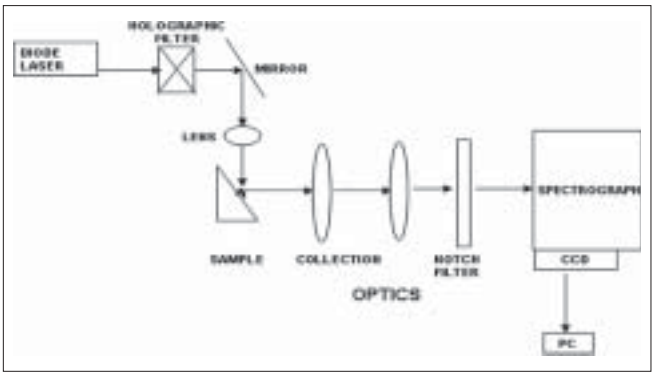

Figure 2: Schematic representation of Raman setup

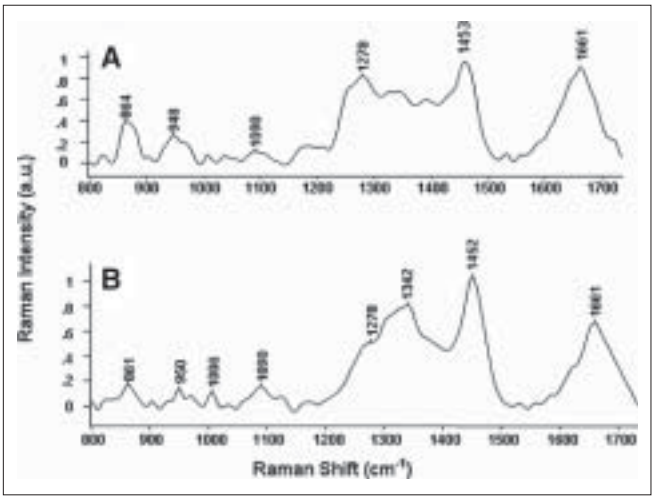

Figure 3: Typical mean spectra of (A) normal and (B) malignant

Mean Raman spectra of normal and malignant tissues is shown in Figure 3. The normal spectrum is characterized by broader Amide I and III and peaks at 1278, 864 and 948 cm^{-1}, which can be assigned to structural proteins such as collagen and elastin [Figure 3A]. The mean malignant spectrum shows sharper amide I, minor blue shift in δCH_2 band, and sharper features in the amide III region, and these bands can be assigned to biomolecules such as lipids, DNA, and noncollagenous proteins [Figure 3B].[92,93] These observations corroborate earlier studies.[87]

We have analyzed spectral data by PCA. Data analysis was carried out in both supervised and unsupervised modes. In the unsupervised approach, spectra from different tissue types were pooled and analyzed. This approach is necessary to verify if spectra can be classified on the lines of normal and malignant conditions. In our analysis, scores of factor 1 gave good classification among normal and malignant tissue spectra of the uterine cervix [Figure 4A]. Though unsupervised classification based on scores of factor is a widely used approach to obtain discrimination between tissue types, it is somewhat cumbersome and tedious for routine diagnosis from the clinical point of view. Therefore, we have developed a supervised approach where multiple discriminating parameters can be used to achieve an objective diagnosis.

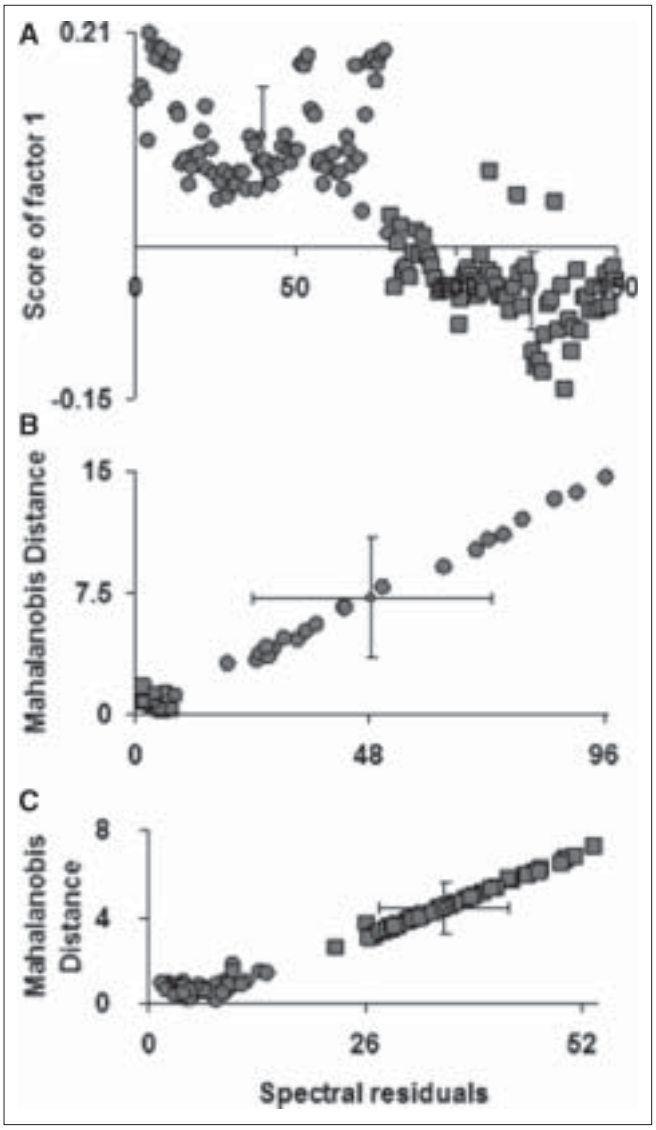

Figure 4: PCA of certified samples: • Normal, ▪ Malignant. (A) Unsupervised approach based on score of factor 1. (B) Verification of training set against malignant training set by supervised mode. (C) Evaluation of training set against normal training set by supervised mode

In this direction, we have built models for the normal and malignant conditions using 24 randomly picked normal and 28 malignant spectra. Exclusiveness and the representative property of these models were verified by rotating out spectra and comparing them against both the standard sets, using Mahalanobis distance and spectral residuals as discriminating parameters.[94-96] In this case, if the test spectrum and the standard set belongs to the same class, then the values of Mahalanobis distance and spectral residuals should be very low and vice versa. As an example, verification against the malignant standard set is shown in Figure 4B. As expected, all malignant spectra yielded lower values of Mahalanobis distance and spectral residual, whereas normal spectra gave higher values. Thus good discrimination among normal and malignant condition could be achieved, which suggests that

Table 1: Limit test approach of certified samples compared against normal training set

Sample number	Match	Limit tests
1	POSSIBLE	PASS (PP?#)
2	POSSIBLE	PASS (PP?#)
3	YES	PASS (PPP#)
4-68	YES	PASS (PPP#)
69	NO	FAIL (P?F#)
81	NO	FAIL (PFF#)
82	NO	FAIL (FFF#)
83-150	NO	FAIL (PFF#)

standard sets are exclusive and representative. Further, these standard sets are evaluated by test spectra (i.e., spectra that were not a part of the standard sets). Once again, all spectra are matched against both the standard sets. As an example, evaluation against the normal standard set is shown in Figure 4C. Good classification among the tissue types could be obtained. This approach to data analysis is less tedious as compared to the cumbersome PCA, but it requires training. In the next step we have implemented match/mismatch 'limit test' methodology, where pretreated spectra are matched against both the standard sets with fixed inclusion/exclusion criterion for all the three discriminating parameters: scores of factor, Mahalanobis distance, and spectral residuals. In this case, spectra are classified on the basis of both match and mismatch status, i.e., a normal spectrum should match with the normal standard set and fail or mismatch with the malignant standard set. Thus, the match/mismatch status of each spectrum against both the standard sets provides objective discrimination [Table 1]. This methodology was successfully implemented to diagnose oral,[97] breast,[98] stomach,[99] and colon[100] cancers. As shown in Table 1, malignant spectra (1-68) did not match, whereas all normal spectra (69-150) match against the normal standard set. We have obtained exactly opposite results against the malignant standard set (data not shown). Thus, we could differentiate both the tissue types objectively and unambiguously.

However, it is very essential for medical applications that any diagnostic methodology is verified and tested rigorously over a large sample size in order to establish its validity before it is routinely practiced. Hence, our discriminating methodology was further evaluated by 70 blinded samples (the nature of the sample was not revealed to the spectroscopist) using the 'limit test' methodology. To illustrate the advantages of the methodology, analysis of 20 blinded samples is shown here [Table 2]. All spectra of samples 1, 2, 4-11, 14, 15, and 17-20 match with only one standard set and do not match with another and they can be diagnosed, in a straightforward manner, as normal or malignant. In the case of sample 3, at least one spectrum matches with the malignant standard set; thus it is treated as malignant in the lines of histopathology. For samples 12, 13, and 16, most of the spectra match with the normal standard set. Thus, these tissues are treated as normal. The results obtained in the analysis show good correlation between Raman spectroscopy and histopathology.

Table 2 Limit test approach of blind samples

Sample number	Spectra analyzed	match/mismatch status against normal standard set	match/mismatch status against malignant standard set	Histopathology report	Raman spectroscopy report
1	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
2	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
3	a,b,c,d,e,f (6)	N,N,N,N,N,P	Y,Y,Y,Y,Y,N	Malignant	Malignant
4	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
5	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
6	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
7	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
8	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
9	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,P,Y	Malignant	Malignant
10	a,b,c,d,e,f (6)	N,N,N,N,N,N	P,Y,Y,P,P,P	Malignant	Malignant
11	a,b,c,d,e,f (6)	Y,Y,Y,Y,Y,Y	N,N,N,N,N,N	Normal	Normal
12	a,b,c,d,e,f,g,h,I, j (10)	Y,Y,Y,Y,N, Y,Y,Y,Y,Y	N,N,N,N,N, N,N,N,N,N	Normal	Normal
13	a,b,c,d,e,f, g, h (8)	Y,N,Y,N,Y, Y,Y,Y,	N,N,N,N,N,N,N,N	Normal	Normal
14	a,b,c,d,e,f, g, h,I, j (10)	Y,Y,Y,Y,Y, Y,Y,Y,Y,Y	N,N,N,N,N,N,N,N,N,N	Normal	Normal
15	a,b,c,d,e,f,g (7)	Y,Y,Y,Y,Y,Y,Y	N,N,N,N,N,N,N	Normal	Normal
16	a,b,c,d,e,f (6)	Y,Y,N,Y,Y,Y	N,N,N,N,N,N	Normal	Normal
17	a,b,c,d,e,f (6)	Y,Y,Y,Y,Y,Y	N,N,N,N,N,N	Normal	Normal
18	a,b,c,d,e,f (6)	N,N,N,N,N,N	P,P,P,P,P,P	Malignant	Malignant
19	a,b,c,d,e,f (6)	N,N,N,N,N,N	Y,Y,Y,Y,Y,Y	Malignant	Malignant
20	a,b,c,d,e,f, g, h (8)	Y,Y,Y,Y,Y,Y,Y,Y	N,N,N,N,N,N,N,N	Normal	Normal

N - NO (no match), Y - YES (match), P - Possible

PREDICTIVE ASSAYS FOR RESPONSE TO RADIOTHERAPY

We have also explored the feasibility of prediction of tumor radioresponse at an early stage of treatment.[101,102] In order to achieve this we have recorded Raman spectra of cervix cancer tissues that were collected before radiation therapy (referred to as malignant) and 24 h after the patient was treated with the second fraction of external-beam radiotherapy (referred to as 2-RT). Independent unsupervised analysis of malignant and 2-RT spectra was carried out. Unsupervised PCA of malignant tissue spectra failed to provide any classification [Figure 5A], whereas PCA of 2-RT spectra gave clear classification of responding (complete and partial response) and nonresponding conditions, based on the score of factor 1. A tendency of separation among responding conditions based on score of factor 2 could also be observed [Figure 5B].

We have also further explored the feasibility of classification among responding conditions. In order to achieve this, we have recorded the Raman spectra of tissues that were collected 24 h after the fifth fraction of EBRT (referred to as 5-RT).[102] PCA of 5-RT tissue spectra could discriminate between responding and nonresponding conditions based on the score of factor 1, and a tendency of separation was also observed between complete response and partial response based on the score of factor 2 [Figure 5C]. The clinical assessment of tumor response of a few cases is still awaited. Nevertheless, good correlation could be seen between Raman spectroscopy and available tumor response.

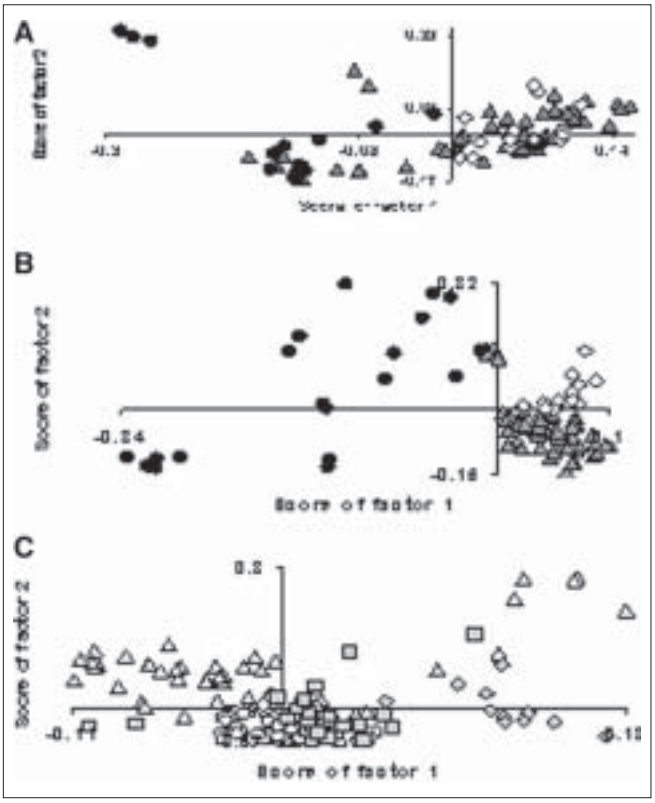

Figure 5: PCA of first derivative Raman spectra in 1250-1500 cm^{-1} region. (A) PCA of malignant tissues: ▲ complete response, • no response, and ◊ partial response. (B) PCA of 2-RT tissues: ▲ complete response, • no response, and ◊ partial response (C) PCA of 5-RT tissues: Δ complete response, ○ partial response, ♦ no response, and ■ response awaited

SUITABILITY OF FORMALIN-FIXED TISSUES IN OPTICAL DIAGNOSIS BY RAMAN SPECTROSCOPY

Fresh tissues in saline would be ideal for optical spectroscopy, but this often causes major constraints in sample procurement and handling. Because of the scarcity and other associated problems with fresh tissues and the availability of *ex vivo* samples, it would be very useful to evaluate the suitability of

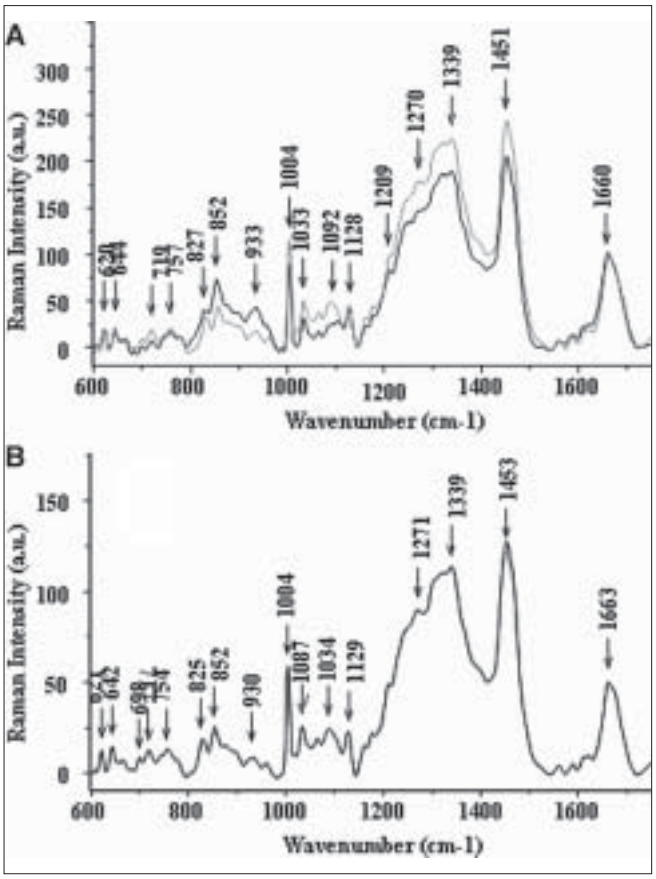

Figure 6: (A) Mean micro-Raman spectra of normal (solid line) and malignant (grey line) formalin-fixed cervix tissues. (B) Mean micro-Raman spectra of malignant (solid line) and 2-RT (grey line) formalin-fixed cervix tissues

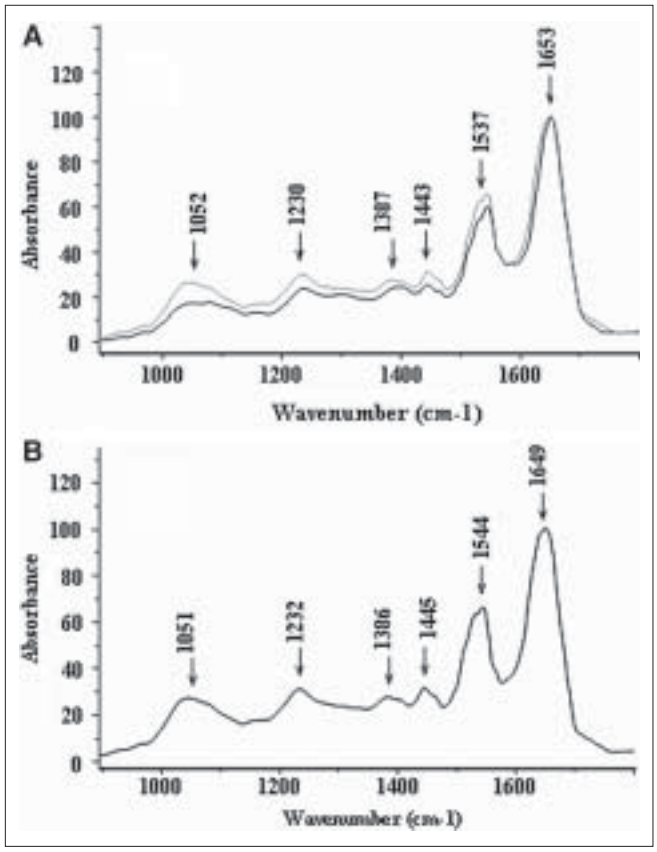

Figure 7: (A)Mean FTIR spectra of normal (solid line) and malignant (grey line) formalin-fixed cervix tissues. (B) Mean FTIR spectra of malignant (solid line) and 2-RT (grey line) formalin-fixed cervix tissues

fixed samples for optical pathology since they are more readily available and accessible for such studies. Paraffin-embedded tissues are the most widely used procedure for sample storage in histopathology. In order to evaluate the suitability of *ex vivo* handled tissues from the Raman spectroscopy point of view, we carried out pilot Raman and FTIR microspectroscopic study of formalin-fixed normal, malignant, and 2-RT tissues.[103] Raman and FTIR spectra of these tissues were recorded using commercial Raman microspectrometer (LabRam, Jobin-Yvon - Horiba, France) and FT-IR imaging system (SPOTLIGHT, Perkin-Elmer, France) coupled to a FT-IR spectrometer (Spectrum 300, Perkin-Elmer, France), respectively. Raman and FTIR spectra exhibit large differences for normal and malignant tissues and subtle differences are seen between malignant and 2-RT tissues. The major differences between the mean Raman spectra of malignant and normal tissue are the relatively stronger peaks of δCH_2 and amide III in the 1000-1200 cm^{-1} and 600-800 cm^{-1} regions and weak peaks in the 800-1000 cm^{-1} region [Figure 6A]. As mentioned above, the differences between the malignant and 2-RT spectra are very minute [Figure 6B]. Typical mean FTIR spectra of normal and malignant epithelia also exemplify significant differences as shown in Figure 7A, whereas differences among malignant and 2-RT spectra are very minute [Figure 7B]. Spectral data were analyzed by two-step PCA. In the first step of the cluster analysis, spectra from all tissue types (normal, malignant, and 2-RT) were pooled and analyzed. This resulted in two clusters corresponding to normal and malignant + 2-RT [Figure 8A, B]. Unsupervised analysis of malignant and 2-RT spectra was carried out as a second step. In this analysis, Raman spectra gave better classification among malignant and 2-RT compared to FTIR spectra [Figure 9A, B]. This indicates the feasibility of discriminating 2-RT tissues from malignant despite the minute biochemical differences. However, these results could not be pursued to explore the predictive capability as all 2-RT tissues were from the complete response condition.

CONCLUSIONS

This review presents a birds-eye view of the recent developments in optical spectroscopy in the diagnosis of cervical cancers. These techniques have been shown to provide noninvasive/less invasive means for the diagnosis of cervical caners. In the case of LIF, multi-digital colposcope is being evaluated in clinical conditions. FTIR spectroscopy demonstrates the ability to diagnose cervix cancer using exfoliated cells and formalin-fixed paraffin embedded tissues. However, for *in vivo* diagnosis, the application of this technique is very much limited due to the presence of strong water bands. But this is a very useful method

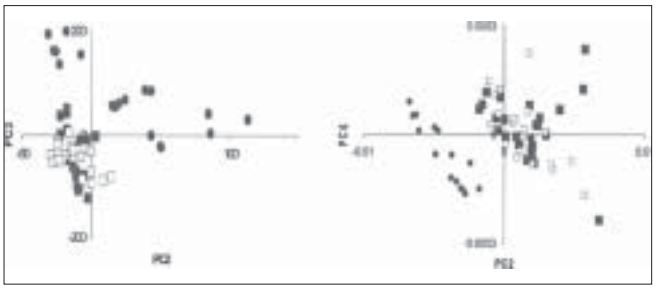

Figure 8: PCA of formalin-fixed spectra of cervix tissues. (A) PCA of 1st derivative micro-Raman spectra of normal (•), malignant (■), and 2-RT (□). (B) PCA of 2nd derivative FTIR spectra of normal (•), malignant (■), and 2-RT (□)

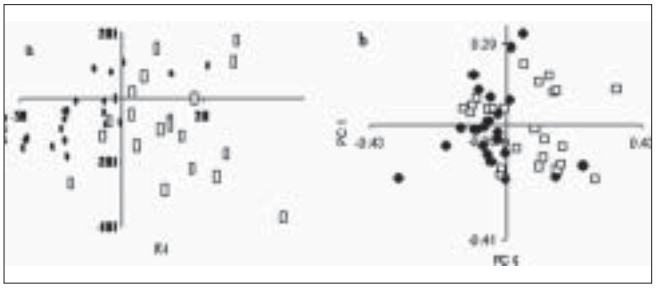

Figure 9: PCA of formalin-fixed cervix tissue spectra. (A) PCA 1st derivative micro-Raman spectra of • malignant and □ 2-RT. (B) PCA 2nd derivative FTIR spectra of • malignant and □ 2-RT

for mapping/imaging as well as for optical histopathology and optical diagnosis using *ex vivo* handled tissues. Few Raman spectroscopic studies that have been reported so far demonstrate its potential in cervical cancer diagnosis. Our Raman spectroscopy studies yielded encouraging results. Moreover, our methods, user-friendly 'limit test' methodology, can provide objective discrimination and could be practical in routine clinical practice.

Prospectively, by developing models encompassing different pathological conditions and rigorous validation of the models using multicentric blinded studies, optical diagnosis of cervical cancers can be realized. In the case of Raman spectroscopy, with the development of a suitable fiberoptic probe, implementation of the innovative technique of noninvasive *in vivo* Raman spectroscopic diagnosis in the clinic can become a reality.

So far, other than our own studies wherein the feasibility of Raman spectroscopic prediction of tumor radioresponse was demonstrated,[101,102] optical spectroscopy is limited to diagnostic or screening applications. Therefore another interesting area for application of optical spectroscopy methods is prediction of therapeutic response or prognosis.

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