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RAMAN SPECTROSCOPIC STUDY ON PREDICTION OF TREATMENT RESPONSE IN CERVICAL CANCERS

S. RUBINA*, M. S. VIDYASAGAR† and C. MURALI KRISHNA*,‡
*Chilakapati Laboratory, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre Kharghar 410210, India
†Department of Radiotherapy and Oncology Shirdi Sai Baba Cancer Hospital, Kasturba Medical College Manipal University, Manipal 576 104, India
‡mchilakapati@actrec.gov.in
‡pittu1043@gmail.com

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Concurrent chemoradiotherapy (CCRT) is the choice of treatment for locally advanced cervical cancers; however, tumors exhibit diverse response to treatment. Early prediction of tumor response leads to individualizing treatment regimen. Response evaluation criteria in solid tumors (RECIST), the current modality of tumor response assessment, is often subjective and carried out at the first visit after treatment, which is about four months. Hence, there is a need for better predictive tool for radioresponse. Optical spectroscopic techniques, sensitive to molecular alteration, are being pursued as potential diagnostic tools. Present pilot study aims to explore the fiber-optic-based Raman spectroscopy approach in prediction of tumor response to CCRT, before taking up extensive *in vivo* studies. *Ex vivo* Raman spectra were acquired from biopsies collected from 11 normal (148 spectra), 16 tumor (201 spectra) and 13 complete response (151 CR spectra), one partial response (8 PR spectra) and one nonresponder (8 NR spectra) subjects. Data was analyzed using principal component linear discriminant analysis (PC-LDA) followed by leave-one-out cross-validation (LOO-CV). Findings suggest that normal tissues can be efficiently classified from both pre- and post-treated tumor biopsies, while there is an overlap between pre- and post-CCRT tumor tissues. Spectra of CR, PR and NR tissues were subjected to principal component analysis (PCA) and a tendency of classification was observed, corroborating previous studies. Thus, this study further supports the feasibility of Raman spectroscopy in prediction of tumor radioresponse and prospective noninvasive *in vivo* applications.

Keywords: Concurrent chemoradiotherapy; tumor response; principal component linear discriminant analysis; principal component analysis; response evaluation criteria in solid tumors.

‡Corresponding author.

1. Introduction

Cervical cancer is the third most frequent malignancy among females worldwide.[1] In India it is the leading cancer among women with 74,825 deaths annually.[2] Most often, approximately 80% cervical cancer patients, presents at advanced stages (stage IIA and above) and five-year survival rate is below 60%.[2,3] For stage IIA and other locally advanced tumors, concurrent chemoradiotherapy (CCRT) is the choice of treatment which includes radiotherapy and concurrent 40 mg/m^2 of weekly cisplatin-based chemotherapy.[4] A standard criterion for evaluation of treatment outcome is response evaluation criteria in solid tumors (RECIST), which is based on unidimensional size measurement of a lesion. Treatment response is evaluated at patient's first visit after treatment which is about four months.[5]

Patients with identical clinical and histological stages exhibit diverse response to CCRT. These differential tumor responses are ascribed to factors like tumor oxygen status, proliferation potential, vascularity and intrinsic radiosensitivity.[6] There are evidences of correlation of tumor markers like SCC-Ag (squamous cell cancer antigen), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and glutathione levels in serum to poor prognostic outcome or tumor response.[7–9] Tumor size and tumor volume regression rate has also been suggested as an important predictor of local control and long term survival.[10] Imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT), 18-flurodeoxyglucose-positron emission tomography ([^{18}F] FDG-PET) and single-photon emission computed tomography (SPECT), which provides an indication of the metabolic and proliferative activity within tumors, have also been pursued for prediction of tumor response. Though these techniques have shown to be potential, wide variation in data collection and analysis are some of the known limitations.[11]

Fluorescence, infrared and Raman are optical diagnostic methods that have been pursued as potential tools in detection of cancers.[12,13] Major advantages of these methods are molecular level information with least sample preparation, objectivity and significantly less time consuming. Among these, Raman spectroscopy which is least influenced by water and less harmful NIR excitation sources are utilized, is the potential tool for *in vivo* applications. Previous *in vivo* studies on cervix have successfully shown efficacy of this methodology in classifying normal, cancer and premalignant conditions in clinically implementable time.[14–20] Few *ex vivo* Raman spectroscopic studies are reported on prediction of tumor response to CCRT.[21–24] Vibrational microspectroscopic studies on formalin-fixed malignant cervix tissue sections has demonstrated the feasibility of classifying formalin-fixed tumor tissues which were collected before and 24 h after patient was exposed to 2nd fraction (2RT) of CCRT.[21] Though classification between normal, pre- and post-RT biopsies could be achieved, since all subjects accured in the study were complete responders, no conclusion on prediction of treatment response could be drawn. Raman spectroscopic study on cervix biopsies before and after 2nd fraction 2RT of CCRT showed that principal component analysis (PCA) of untreated tumor tissue spectra failed to provide classification towards tumor response to CCRT. But PCA post-CCRT spectra gave clear classification between responding (complete and partial response) and nonresponding conditions.[22]

Radiation resistance is a serious hurdle in CCRT and there are no methods to predict tumor response at early stages of treatment regimen. Therefore, considerable efforts are made to develop new methods which are objective, rapid and preferably noninvasive. In this context, the present study aims to explore the feasibility of fiber-optic-based Raman spectroscopic approach for the evaluation of treatment outcome in cervical cancers. This pilot study was carried out with an outlook for an *in vivo* approach. In this study, 11 normal, 16 tumor and 15 RT tissues were analyzed by PC-LDA followed by LOOCV. PCA of all 5RT (tissues after 5th fraction of radiation) spectra were used for PCA classification to explore the classification pattern in the data. Findings of the study are discussed in the paper.

2. Methods and Materials

2.1. *Sample details and Raman spectroscopy*

Study was approved by ethics committee of Shirdi Sai Baba Cancer Hospital, Manipal. Histopathologically certified 42 cervix tissues were collected in phosphate buffer saline (PBS) and stored in liquid nitrogen. Out of these 16 tumor tissues were collected from locally advanced cancer subjects before

undergoing treatment (tumor) and 15 tumor tissues were collected from the subjects 24 h after the 5th fraction of radiation treatment (5RT). Of these 16 tumor subjects, one of the subjects did not turn up for treatment. Normal tissues were collected from 11 subjects undergoing hysterectomy. The clinical assessment was done by three clinicians independently as per World Health Organization guidelines. Extent of tumor volume shrinkage was measured as index of radioresponsiveness. Subjects with 100% shrinkage of tumor at the primary site were considered as complete response (CR), where as higher than 50% shrinkage as partial responders (PR) and nonresponders (NR) had a lower than 50% shrinkage. Out of 15 5RT tissues, 13 were tissues of subjects with CR after treatment; one tissue was from the subject with PR and one tissue from the subject with no response (NR).

Raman spectra were acquired by placing tissue on CaF_2 window. On average, 10 spectra per tissue were acquired from 42 tissues using HE-785 commercial spectrometer (LabRam, Jobin-Yvon-Horiba, France). This system consists of a diode laser (Process Instruments) of 785-nm wavelength as excitation source, a HE-785 spectrograph coupled with a CCD (Synapse) as dispersion, and detection elements. The spectrograph is equipped with a fixed 950 gr/mm and has no movable parts. Spectral resolution, as specified by the manufacturer, is $\sim 4\,cm^{-1}$. Commercially available InPhotonics (Downy St., USA) probe consisting of 105 μm excitation fiber and 200 μm collection fiber (NA 0.40) was used to couple the excitation source and detection system. As per InPhotonics probe manufacturer specifications, the theoretical spot size and depth of field are 105 μm and 1 mm, respectively. Spectral acquisition parameters were: λ ex 785 nm, laser power 50 ± 0.5 mW, spectra were integrated for 10 s and averaged over six accumulations. Spectra were recorded with a spacing of $\sim 1-2$ mm using a manual XYZ precision stage.[25]

2.2. *Data analysis*

Preprocessing of spectra was carried out by correcting for CCD response with a NIST certified SRM 2241 material followed by subtraction of background signal from optical elements and CaF_2. Corrected spectra were subsequently first derivatized followed by vector normalization.[26–28] Spectra in $1000-1800\,cm^{-1}$ region were used for PCA-based linear discriminant analysis. PC-LDA was performed operating in-house MATLAB-based software.[29] In PC-LDA, set of significant principal components (PCs) with maximum variance are used as input data for LDA-based classification. This aids to filter out noise and utilize optimum variables for classification. As a thumb rule, to avoid over-fitting of the data, number of factors selected for analysis should be less than half the

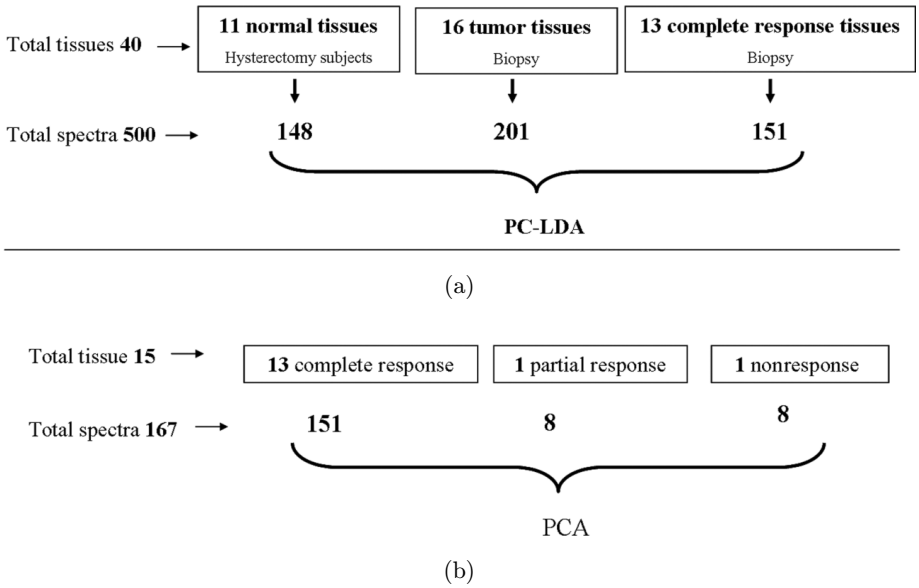

Fig. 1. Schematic representation of methodology: (a) total number of tissues and spectra used in PC-LDA, (b) total number of tissues and spectra used in PCA.

number of the spectra in the smallest group.[30,31] Therefore, PC-LDA was carried out using seven factors accounting for ∼77% variance. Data analysis was performed in three steps: in the first step, standard classifier models belonging to each class i.e. normal, tumor and 5RT were developed using all spectra from each class. In second step, the classifier model was verified by LOO-CV method. In third step, PCA of 167 spectra of 13 CR, 1 PR and 1 NR tissues was accomplished (see Fig. 1).

3. Results and Discussion

The mean spectra of normal, tumor, complete responder, partial responder and nonresponder cervix tissue are shown in Fig. 2. Spectral features of normal tissue with broader amide I and the features of amide III suggest dominance of collagenous protein whereas the amide I and amide III in tumor spectra indicating presence of noncollagenous protein features and DNA which corroborate previous studies.[32–34] These spectral features can be ascribed to presence of proliferating cells in tumors where as normal tissue comprises of a layer of epithelial cells and connective tissue, connective tissues are rich in collagen.[34] Mean spectra of CR, PR and NR tissue exhibited very small but significant variations, such as in amide I, with respect to tumor (see Fig. 2). It can be explained as tissues of same tumor type and 5RT specimens were collected 24 h after radiation treatment, which provides system with sufficient time to repair most of the radiation-induced effects.[22]

To elucidate the potential of Raman spectroscopy to discriminate this residual radiation changes in the tissue before and after radiation; normal, tumor and complete responder tissues were subjected to PC-LDA. Since PR and NR spectra were few in number, hence they were not included. PC-LDA was carried out with seven factors accounting for ∼77% variance as shown in scree plot [see Fig. 3(a)]. Three discrete clusters belonging to normal, tumor and complete responder spectra were observed in 3D scatter plot [see Fig. 3(b)].

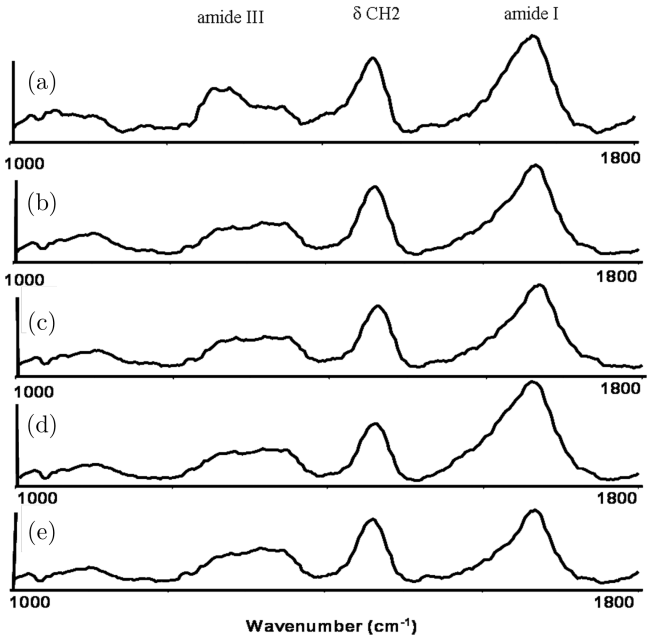

Fig. 2. Mean Raman spectra of: (a) normal, (b) tumor, (c) complete responder specimens, (d) partial responder, and (e) nonresponder.

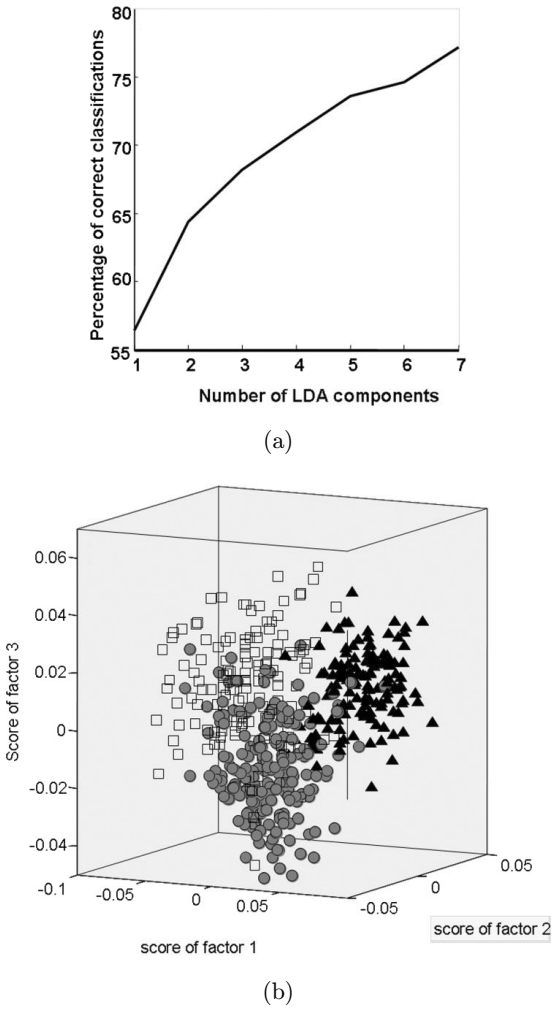

Fig. 3. PC-LDA of normal, tumor and CR tissues: (a) Scree plot, (b) 3D scatter plot of normal (▲), tumor (●) and CR spectra (□).

Table 1. PC-LDA for normal, tumor and complete responders: (a) standard model, (b) leave-one-out cross-validation (diagonal elements are true positive predictions and ex-diagonal elements are false positive prediction).

		Normal	Tumor	CR
(a)	Normal	137	10	1
	Tumor	5	148	48
	CR	9	41	101
(b)	Normal	137	10	1
	Tumor	5	148	48
	CR	9	44	98

PC-LDA results are also summarized in confusion matrix shown in Table 1(a). Out of 148, 137 normal spectra were correctly predicted, whereas 10 spectra were misclassified as tumor and 1 CR. In case of tumor, 148 out of 201 spectra were correctly classified as tumor while 5 and 48 were misclassified as normal and CR, respectively. 101/151 CR spectra were correctly classified as CR whereas, 9 as normal and 41 as tumor. To verify the robustness of PC-LDA standard model, LOO-CV was carried out [Table 1(b)]. Cross-validation is also referred as rotation estimations, in which one spectrum is excluded from the dataset and the remaining data is used as a training set. When training is fulfilled, the excluded spectrum is used as independent test dataset to evaluate the performance of the training model. The performance of model is estimated as number of correct predictions over all samples used in the dataset. In LOO-CV, 137/148 normal tissue spectra were correctly classified while 10 and 1 were misclassified as tumor and CR, respectively. 148/201 tumor tissue spectra were correctly classified where as 5 were misclassified as normal and 48 as CR. 98/151 CR tissue spectra were correctly classified while 9 were misclassified as normal and 44 were misclassified as tumor spectra. Results indicate that classification between normal and abnormal spectra (tumor and CR) was more efficient as compared to that of tumor and CR. As mentioned earlier, tissues were collected 24 h after radiation; majority of radiation-induced damages may perhaps be repaired and hence higher rate of misclassification between tumor spectra and CR spectra was observed.

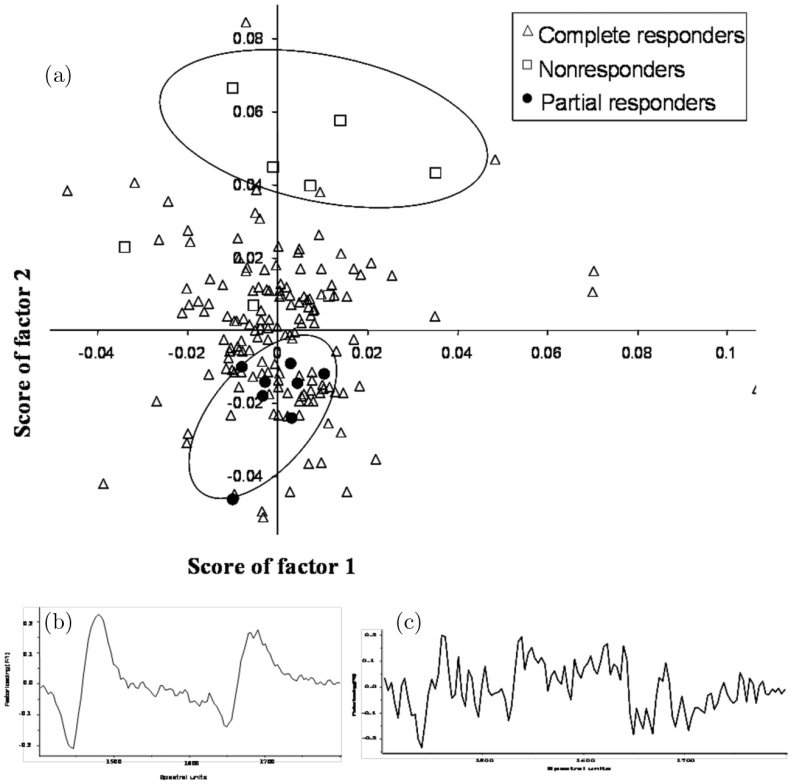

Fig. 4. PCA for complete, partial and nonresponder spectra. (a) Scatter plot of complete responders (▲), nonresponders (□) and partial responders (●). (b) Loadings of factor 1. (c) Loadings of factor 2.

Radiation induced damage to DNA, proteins and membranes oxidation is a well-known phenomenon. System reacts to radiation-induced damage by producing antioxidants to counter these effects. The radiation-induced antioxidant variations are believed to be one of the reasons in differences in treatment response regardless of same pathological conditions. To explore the feasibility of Raman spectroscopy to discriminate CR, PR and NR tissue spectra, PCA of 5RT tissue (151 CR, 8 PR and 8 NR) spectra were carried out. The PCA scatter plot of complete, partial and nonresponder spectra is shown in Fig. 4, which indicate a tendency of classification. Despite lower number of tissues of partial and nonresponders, the observed pattern is quite encouraging and corroborate findings of our earlier studies.[22−24] In our earlier studies using conventional Raman spectroscopy in combination with PCA, we have shown that normal and tumor tissues can be classified, however tumor and RT tissues show overlap. As is well known, PCA is data overview tool rather than discrimination tool and commonly employed to visualize outliers and trends in the data. In the present study, we could classify normal, tumor and RT tissue spectra using discriminating tool, PC-LDA. Further, this study was carried out using a fiber-optic probe coupled commercial Raman system which can be adapted for prospective noninvasive *in vivo* monitoring of the tumor response. Prospectively, studies on larger sample size and use of discriminant tools like PC-LDA can bring out classification among all three responding conditions.

4. Conclusions

CCRT is the choice of treatment for locally advanced cervical cancer whereas resistance to treatment is a serious hurdle. Therefore, efforts are being made to develop objective, rapid and preferably noninvasive novel method. Early prediction of tumor response leads to individualizing treatment regimen. Present study aims to explore the feasibility of fiber-optic-based Raman spectroscopic prediction of tumor response to CCRT in cervical cancers. Spectral features like broader amide I and amide III suggest dominance of collagenous proteins in normal tissue spectra whereas the amide I and amide III in tumor spectra indicating presence of noncollagenous protein and DNA features which corroborate previous studies. PC-LDA of normal, tumor and CR biopsies suggest that normal tissues can be efficiently classified from both pre- and post-treated tumor tissues, which were validated using LOO-CV. Observed PCA classification pattern for CR, PR and NR tissue are encouraging. Thus, this study further supports the feasibility of Raman spectroscopy in prediction of tumor response to CCRT and prospective noninvasive *in vivo* applications.

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