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Conclusions and future perspectives
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Oral cancer is a major health problem in India and other South-Asian countries. India tops the prevalence of oral cancer list in the world. Although the oral cavity is easily accessible to inspection, patients with oral cancer most often present at an advanced stage when treatment is less successful thereby leading to high morbidity and mortality. Early detection remains the best way to ensure patient survival and quality of life. The current gold standard for clinical diagnosis of oral lesions is biopsy and subsequent histopathological confirmation. The process is invasive, time-consuming and prone to inter-observer variability. An alternate method of diagnosis is therefore warranted, that will enable non-invasive diagnosis of oral cavity in individuals with suspicious oral lesions. It is now well recognized that techniques based on optical spectroscopy can play a very important role towards this end. Raman spectroscopic methods are ideal for in vivo diagnosis because, is nondestructive, does not require external dyes, and photons can be delivered / collected via fiber-based instrumentation. These methods can provide rapid, in situ, objective and near real-time evaluation of a disease with high degree of accuracy. The work reported in the thesis aims at developing and evaluating potentials of in vivo Raman spectroscopic methods for early and non-invasive oral cancer diagnosis. The major highlights of the work are as follows:

1. **Raman spectroscopy of ex vivo tissues**

Fiberoptic probe coupled Raman spectroscope for in vivo applications was procured and assembled. This set up was adapted for ex vivo measurements by attaching probe holder and XYZ precision stage.

In order to standardize data acquisition and analysis protocols as well as to assess the reproducibility of spectral features, spectra of ex vivo normal and tumor tissues were acquired. A total of 683 spectra from 36 pairs of biopsies were obtained. Lipid rich features were observed in
normal spectra while tumor showed predominantly protein bands. Classification with PC-LDA was explored and findings were validated by LOOCV and independent test data. Reproducibility of spectral features was established and objective classification between both groups was obtained. Misclassifications between both groups were analyzed by correlating spectral predictions of normal and tumor biopsies against their respective histopathology. Findings suggest that misclassification between both groups can be primarily attributed to the tissue heterogeneity i.e. presence of normal regions in a tumor biopsy and vice-versa.

Origin of Raman signals in normal tissues was explored by acquiring spectra of intact and incised oral biopsies. Findings demonstrated that morphological and architectural arrangements of different layers in a tissue contribute to the spectral signatures. Influence of surface orientation of normal tissues on classification with tumors was also assessed and it was found that orientation does not have any bearing on classification with tumor.

Spectral features of normal and tumor tissues were correlated with underlying biochemical composition. Area associated with protein (1450 cm\(^{-1}\) and 1660 cm\(^{-1}\)) and lipid 1440 cm\(^{-1}\) bands were computed by curve fitting / deconvolution methods. These were correlated with biochemical composition of the tissue by estimating amount of total lipids, proteins and phospholipids. Spectral features as well as biochemical estimation suggest that the lipid to protein ratio is high in normal tissues in comparison to tumors. Spectral parameters derived from curve resolved protein and lipid Raman bands were found to be highly correlating with biochemical measurements.

2. **In vivo Raman spectroscopy of oral cancers**

To the best of our knowledge, for the first time, we have demonstrated the feasibility of acquiring good quality in vivo Raman spectra under clinically implementable time in Indian population. The fiberoptic probe was adapted for in vivo measurements by attaching a detachable, metallic spacer of
length 5 mm was attached at the tip of the probe to maintain constant focus during all measurements and to avoid inter-subject contaminations. In order to ensure similar acquisition sites in all subjects, spectra were acquired from buccal mucosa as per the teeth positions. A total of 444 contralateral normal, 337 tumor and 206 premalignant spectra from 163 subjects were obtained. In addition to this 300 spectra were also acquired from 30 healthy controls (15 with and 15 without tobacco habits). Mean and difference spectra suggest predominant lipid features in normal conditions while proteins are rich in tumors. Standard models for contralateral, premalignant and tumor conditions were developed and evaluated with independent test data. Discrimination of premalignant conditions against closely associated habitual tobacco users was also demonstrated. Findings suggest that premalignant conditions in the oral cavity can be objectively classified against normal, tumor as well as closely associated habitual tobacco users. Classification of OSMF and leukoplakia, two of the most commonly occurring precancerous conditions in Indian population was explored and feasibility of classification between both conditions was demonstrated.

Various clinically or histologically unrecognizable micro-architectural changes generally precede the development of a clinically visible precancerous lesions and are attributed to ‘malignancy-associated-changes’ (MACs) or cancer-field-effects (CFEs)’, terms often used interchangeably. Identification of CFEs or MACs may serve as a novel screening tool to reduce the morbidity and mortality associated with multiple potentially malignant transforming fields. In order to evaluate the feasibility of \textit{in vivo} Raman spectroscopic identification of early changes which may be an indicative of neoplastic transformation, 722 spectra of 84 subjects under five categories namely healthy control (no tobacco habit, no cancer), contralateral normal (cancer and tobacco habit), non-habitués contralateral (cancer and no tobacco habit), habitués healthy controls (no cancer, tobacco habit) and tumor (cancer and tobacco habit) were analyzed. Mean and difference
spectra are suggestive of changes in protein, lipid content as well as tobacco induced hypercellularity. PC-LDA results suggest that Raman characteristics of mucosa of healthy controls are exclusive, while that of habitués healthy controls are similar to the contralateral normal mucosa, suggesting carcinogen induced field changes can be identified. It was also found that cluster of non-habitués contralateral normal mucosa is different from habitués healthy controls, indicating malignancy associated changes may be different from carcinogen induced changes and can be identified with Raman spectroscopy. The non-invasiveness and use of harmless excitation wavelength impart several advantages to this method, and thus prospectively has potential to become an ideal mass screening tool in public health programs.

3. **Raman microspectroscopy of oral cancer cells**

Keratins are one of the most widely used markers for oral cancers. Keratin 8 and 18 are expressed in simple epithelia and perform mechanical and regulatory functions in cell. Their expression is not seen in normal oral tissues but is often expressed in oral squamous cell carcinoma. Aberrant expression of keratins 8 and 18 is the most common change in human oral cancer. Study on tongue cancer derived AW13516 cell-line was taken up to evaluate potentials of Raman spectroscopy in identifying minor changes associated with differential level of keratin expression. Cells with reduced expression of keratin 8 protein were termed as ‘K8 knockdown’ and with normal expression termed as vector controls. In the first step spectra of K8 knockdown and vector control cell pellets were acquired using fiberoptic probe set up. Spectral features of both groups are suggestive of differences in the protein content and secondary structures. These differences were utilized for classification using PC-LDA followed by LOOCV.

Contrasting morphological differences between both groups could be the main reason behind classification. Individual cell morphology was analyzed using live cell imaging and confocal
microscopy. Findings suggest that vector control cells have more actin based filamentous protrusions and they are elongated in shape. In contrast to vector controls, knockdown cells show very few actin-based protrusions and have symmetric contracted epithelial appearance.

Morphological differences between K8 knockdown and vector control cells were further established by generating Raman maps of single cells. Spectra were acquired at 532 nm excitation with a Raman microspectrometer and maps were generated by K-means cluster analysis method. Different clusters corresponding to membranous, cytoplasmic, perinuclear and nuclear regions of the cell were obtained. Spectra of the perinuclear and nuclear region were dominated by nucleic acid bands while that of cytoplasmic regions were found to be rich in proteins. Corroborating earlier observations, K8 knockdown cells show very few actin based filaments and have symmetric contracted epithelial appearance in contrast to elongated appearance along with multiple membrane protrusions of vector control cells.

Overall findings of our study demonstrate the efficacy of Raman spectroscopic methods in conjunction with multivariate analysis tools for unambiguous and non-invasive identification of normal and pathological conditions as well as the early invisible changes which may be an indicative of neoplastic transformation in oral cancers.

**Future Directions**

The ultimate goal of optical spectroscopic methods is to provide an objective, real-time adjunct/alternative to cancers diagnosis. Studies carried out in the present thesis have successfully demonstrated the feasibility of classifying normal and pathological conditions in oral cancers using Raman spectroscopy in a laboratory/hospital set up. In the coming years, large scale clinical trials
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must be conducted to gain the amount of data necessary for developing adequate size training and test set for robust algorithm development and analysis. These models should be tested very vigorously, preferable double-blinded, as multicentric studies, before they are contemplated for routine use. Incorporating a marking system which could be intrinsic to the probe itself should also be investigated. This will help in realizing the surgical boundary demarcation and site-wise histopathology applications. Further improvements in data analysis algorithms is also required for developing less cumbersome, rapid, unambiguous, objective and user friendly interfaces from the point of view of routine clinical use where a clinician or a technician can analyze a given spectrum against all available models to diagnose a case.

The prospective adaptation of Raman spectroscopy for routine clinical diagnosis would decrease the number of follow-up clinic visits and patient anxiety as long wait for histopathological diagnosis would be minimized to a great extent. The technology poses no known risks to the patients, and therefore could be a safe alternative/adjunct to the current diagnostic methods.