Indonesian Journal of Chemometrics and Pharmaceutical Analysis

Original Article

Oral Pre-cancer and Oral Cancer Detection by ATR-FTIR Spectrometry using Blood Serum and Multivariate Data Analysis

Deep Kumari Yadav, Shayma Shaikh, Rakesh Rawal*

Department of Lifesciences, School of Sciences, Gujarat University, Ahmedabad, India

*Corresponding author: Rakesh Rawal | Email: rakeshrawal@gujaratuniversity.ac.in

Received: 22 August 2021; Revised: 3 October 2021; Accepted: 4 December 2021; Published: 26 December 2021

Abstrak: Oral cancer is the most prominent cancer in men and third most common cancer in women in India. Tobacco is one of the leading factors for cancer. There are various conventional methods in practice to determine the cancer. Unfortunately all of them are invasive method, hence it would become essential to develop a non-invasive method which can be used easily for screening of these diseases. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) is proposed to be an efficient minimally invasive method which uses serum for detection of oral cancer and pre-cancer. Serum samples of 12 oral cancer patients, 11 oral submucous fibrosis patients and 10 healthy controls were taken and analysed. Unsupervised hierarchical cluster analysis (HCA) were performed to process the serum IR spectra. HCA obtained higher efficiency though two oral cancer variables were misclassified as healthy control. ROC curve has also shown a higher accuracy among oral cancer, pre-cancer and healthy controls. It was observed that, the healthy controls had prominent peaks at 1339, 1316, 1074, 1739 and 1770 cm^{-1} wavenumber regions which were not present in serum samples collected from oral cancer and oral submucous fibrosis patients. These ranges correspond to the vibrations of several functional groups of DNA, RNA, collagen, amino acid and ester group which play a pivotal role in segregation of oral cancer and oral pre-cancer. It indicated that this method is possibly useful in diagnosis of malignancy which provide new insights for easier detection methods that are presently expensive and difficult.

Keywords: Oral cancer, ATR-FTIR spectroscopy, Hierarchical cluster analysis, Receiver operating curve

1. INTRODUCTION

Cancer is one of the leading causes of death worldwide. In the year 2018, around 9.6 million people were died due to cancer [1]. Every year around 400,000 new cases of cases of oral and oropharyngeal cancer (OC/OPC) are being diagnosed [2]. Though it is a major cause of death but studies have shown that if early treatment is given to the patients then their chances of survival is very high. Oral submucous fibrosis (OSF) is a premalignant lesion which can be characterized by inflammation and progressive fibrosis of the submucosal tissues, result into marked rigidity and trismus [3]. Tobacco and betel quid are considered as major risk factors for causing oral cancer [4]. Screening tests currently used for diagnosis of oral cancer are conventional oral examination, staining with toluidine blue, oral brush biopsy, and scalpel biopsy coupled with histology [5]. There are also various imaging techniques used for the diagnosis of cancers of oral cavity. The most common methods which play significant role in diagnosis and the planning of treatment are magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET) [6]. Though imaging studies are routinely performed in OSCC but it is not able to interpret the accurate results in the early stage of the disease. Histological biopsy is a gold standard method for diagnosis

of cancer and precancerous lesions. Histopathologic grading gives accurate information about anatomic stages which help in prognosis of disease and also guides in treatment [7]. In this way, study of biopsy is very crucial for cancer diagnosis and tumor classification. This histopathological diagnosis method is an invasive method and requires skill to perform. Improper staining often leads to misdiagnosis [8]. Hence there is a need of a non – invasive technique arose, where early detection of diseases can be carried out without involving complex procedure.

Attenuated total reflectance - fourier transform infrared (ATR-FTIR) could be an alternative method to replace the conventional invasive method. It has an optical element composed of interferometer which allows acquisition of wavenumbers in short intervals. It is already applied for many biological molecules where it has given rapid and accurate result. Non- destructive nature towards biological samples of IR rays makes it more suitable for analysis of tissues, fluids and cells as compare to UV, gamma and X rays [9]. Fourier transform infrared (FTIR) enables the biochemical fingerprinting of metabolites such as carbohydrates, polysaccharides, amino acids, proteins, fatty acids and lipids [10]. The peaks are associated with the vibration of a specific chemical bond or a single functional group within the molecules which provide accurate insights of biochemical composition of samples [11]. There are many other studies carried out earlier using this method to showcases its promising impact in detection various cancers like kidney, breast, cervical, lung, liver and prostate [12-20]. ATR-FTIR technique is very simple to use, cost effective and highly reproducible technique. One of the major advantage is it does not require any sample preparation for analysis. Various bands obtained at different frequencies and shapes of bands are directly used for interpretation results [21]. FTIR can efficiently detect changes in molecular compositions according to diseased state, providing fingerprints of biological samples and biological fluids [22]. The only drawback is that the ATR spectra requires prior processing to obtained physical significance at molecular level. Hence, it can be overcome with the calculation of optical constants using K-K transformation [21]. This study aimed to systematically evaluate the accuracy and performance of HCA and ROC were measured to discriminate between the normal, precancer and oral cancer samples. Unsupervised hierarchical cluster analysis (HCA) dendrogram was generated using Euclidean distance and ward's method. It allows us to visualized the overall groupings structure and thereby it subgroups the spectra according to their similarities. ROC curve was created to assess the predictive ability of 'biochemical fingerprint' region. As considering that sensitivity and specificity are basic characteristics to determine the accuracy of a diagnostic test.

2. MATERIALS AND METHODS

2.1. Sample Collection

This study involves age matched 12 oral cancer patients, 11 oral precancer/ oral submucous fibrosis (OPC/OSF) and 10 healthy controls. Serum samples were collected from Sterling Cancer Hospital, Ahmedabad. This study is approved by the local ethical committee [GUJIEC_8_2017] and duly informed written consent was obtained from all the patients and donors prior to sample collection. Both patients and donors were negative for HBsAg, hepatitis C, and HIV antigen. Clinicopathological characteristics are illustrated in Table 1.

Table 1. Clinicopathological characteristics of oral cancer, oral sub-mucous fibrosis and control patients.

Characteristics	Oral cancer (N= 12)	Oral sub-mucous fibrosis (N=11)	Controls (N=10)
Age (Mean ± SD)	34.7 ± 1.62	36.7 ± 4.03	32.9 ± 2.24
Gender			
Male	9	11	7
Female	3	None	3
Habit			
Tobacco chewers	7	9	None
Smokers	5	2	None
Site			
Ca. Buccal mucosa	9	NA	NA
Ca. tongue	3	NA	NA
Grade			
Grade 1	9	6 (OSF grading)	NA
Grade 2	3	0	NA
Grade 3	0	5 (OSF grading)	NA
Stage			
Stage 1	7	NA	NA
Stage2	5	NA	NA
Stage3	0	NA	NA
Stage4	0	NA	NA

2.2. Sample Preparation

Blood was collected in a serum separator tube. Samples were allowed to stand for 20-30 minutes before centrifugation at 3000 rpm. After centrifugation, supernatant serum was carefully pipetted out of the vial and stored at −80 ° C for further use.

2.3. Instrument Set Up

The spectra were acquired using a Bruker alpha ATR-FTIR spectrophotometer equipped with diamond crystal; wavenumber range of 1800-900 cm−1, at a resolution of 4 cm-1 and averaged over 24 accumulations. Twenty microliter of serum sample was spread into a diamond crystal and dried at room temperature for 15 min. ATR-FTIR measurements were carried out in transmittance mode. Opus software (v.7.5) was used for recording spectrum in the mid infrared range. Before processing for sample analysis baseline correction, smoothing, and normalization were carried out. The baseline correction was done through rubber band method, smoothing was performed using the Savitzky-Golay algorithm with nine points and normalization was carried out by vector normalization method in OPUS software. The sample spectra were collected in triplicates for each sample and mean were consider for interpretation. After each sample analysis, ATR crystal was cleaned with isopropanol.

2.4. Data Processing and Chemometric Techniques

The study is focused on assessing the changes in the fingerprint spectral wavenumber region between 1800 to 900 cm^{-1}. Study does not involve any intra-patients variability. After pre-processing the spectra multivariate analysis was carried out. Unsupervised hierarchical cluster analysis (HCA) dendrograms were produced using the origin 2019 software. ROC curve analysis of pre-processed mean spectra of fingerprints region (1800 to 900 cm^{-1}) was performed using GraphPad Prism versions 8.00 (GraphPad Software, USA). *P* value less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSIONS

3.1. Sample Selection

A total of 33 subjects were studied and categorized into three groups; group I oral cancer (n-12, age - 34.7 ± 1.62, male - 9, female- 3), group II OSF (n-11, age- 36.7 ± 4.03, male- 11, female- 0) and group III healthy controls (n-10, age- 32.9 ± 2.24, male - 7, female- 3) (Table 1).

3.2. ATR-FTIR analysis of serum spectra between oral cancer, pre-cancer and healthy controls

ATR-FTIR was used to generate transmittance spectra in the frequency region 1800 to 900 cm^{-1} to establish potential metabolic differences in oral cancer, oral sub-mucous fibrosis and controls. This region is consistent with changes in carbohydrates, proteins (amides) and lipids present in the serum. Representative ATR- FTIR mean spectra of Group I, II and III in the regions of 1800-900 is shown in fig 1. Among all these three groups, group III (healthy controls) shows prominent peaks at 1339, 1316, 1074, 1739 and 1770 cm^{-1} which are absent in group I (oral cancer) and group II (oral sub-mucous fibrosis) (Table 2). Group I (OC) contain peaks at 1642, 1643, 1639, 1638, 1636 cm^{-1} indicating the presence of amide I and it also shows peaks at 1543, 1542, 1541, 1549 cm^{-1} which indicate presence of amide II. Group II (OSF) contains peaks at 1637 and 1550 cm^{-1} indicating the presence of C=C uracyl, C=O and amide II groups respectively. Group III (HC) contains peaks at 1644 and 1541 cm^{-1} indicates the presence of amide I and amide II peaks respectively. It was also observed that mean relative intensity of amide I and II peaks are more intense and higher in healthy controls as compared to oral cancer and oral pre-cancer groups. These changes confirm the cellular structures changes associated with surface proteins and receptor proteins of a progressing malignant cell (Table 3).

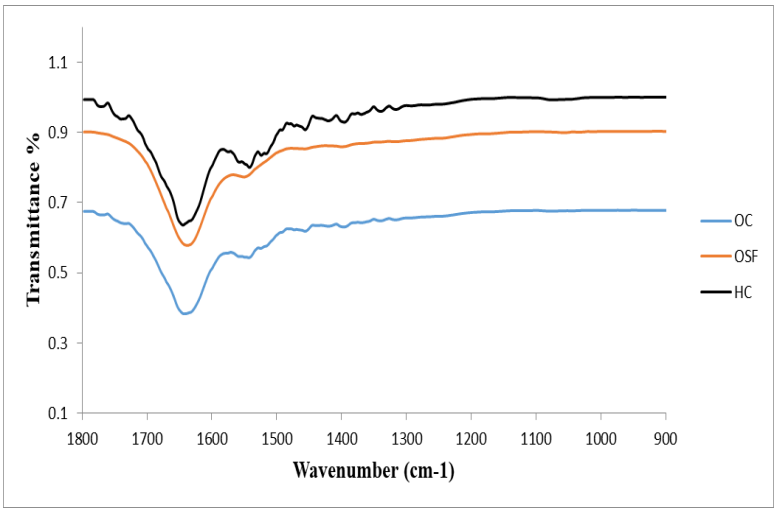

Figure 1. Representative ATR-FTIR mean spectra of oral cancer, oral submucous fibrosis and healthy control in the region of 1800-900 cm^{-1}

Table 2. Frequency (cm^{-1}), assignment and relative intensity of peak of prominent wavenumbers in healthy controls

Peaks cm^{-1}	**Assignments of bands**	**Relative intensity**	**References**
1339	Collagen	0.014	Fung et al., [23]
1316	Amide III, symmetric stretch	0.01	Li et al., [24]
1074	DNA/RNA	0.007	Pachaiappan et al., [25]
1739	C=O, polysaccharides, hemicellulose	0.018	Ruiz-Chica et al., [26]
1770	C=O stretching/vinyl/phenyl ester group	0.014	Nandiyanto et al., [27]

Table 3. Mean relative intensity of amide I and amide II peaks in all the groups

Groups	**Amide I (Mean relative intensity)**	**Amide II (Mean relative intensity)**
Oral cancer (group I)	0.28 ± 0.02	0.01 ± 0.003
Oral sub-mucous fibrosis (group II)	0.030 ± 0.019	0.01 ± 0.009
Healthy controls (group III)	0.35 ± 0.001	0.06 ± 0.002

3.3. Multivariate Analysis

3.3.1. Cluster Analysis

Hierarchical cluster analysis was carried out to determine the similarity between the observations or clusters. The HCA dendrogram for all 33-serum samples is shown in figure 6. Three large distinct clusters can be seen in the dendrogram indicating that these wavenumbers separate OC, OSF and control groups efficiently with high accuracy. The first major cluster in the left-hand side contains all the OC, OSF followed by controls cases in the right. Though two oral cancer samples were not classified in cancer group as HCA could not discriminate these samples based on features of serum profiles.

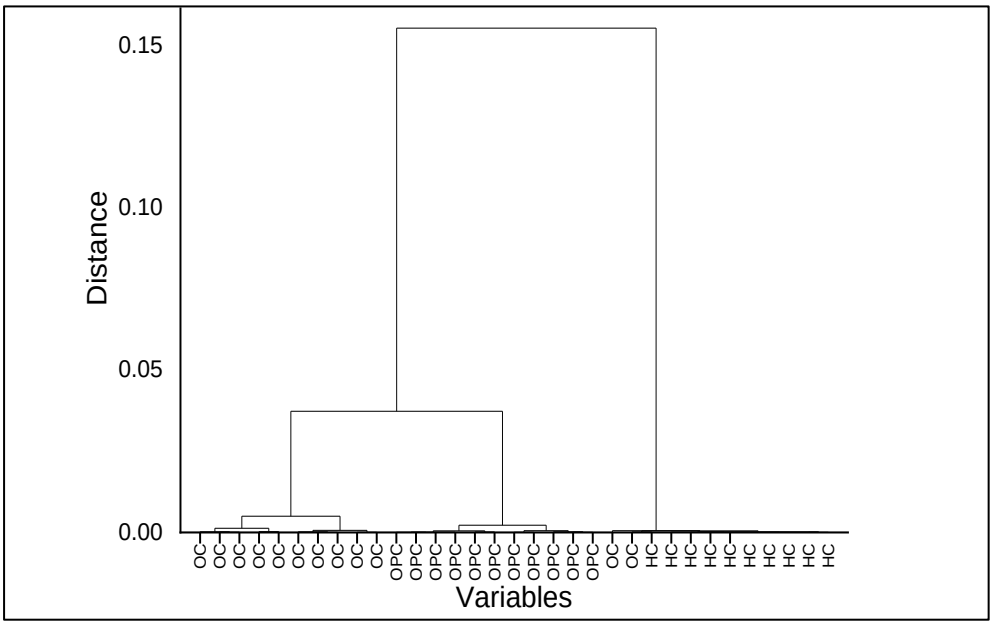

Figure 2. Ward dendrogram produced by agglomerative hierarchical clustering (HCA) of representative FTIR spectra of oral cancer (OC), oral premalignant (OPC) and healthy controls (HC)

3.3.2. Receiver Operating Curve (ROC)

Pre-processed mean IR spectra in wavenumber region 1800-900 cm^{-1} were employed to ROC curve analysis to evaluate the diagnostic potential of this fingerprint region. This IR region corresponds to the conformational changes in lipids, proteins and carbohydrates. The ROC curve shows a significant accuracy of ATR – FTIR tool to segregates oral cancer from pre-cancer and control, with an AUC of 0.8107 for oral cancer vs. oral sub-mucous fibrosis, an AUC of 0.9546 for oral sub-mucous fibrosis vs control and an AUC of 0.9805 for oral cancer vs control (Figure 7).

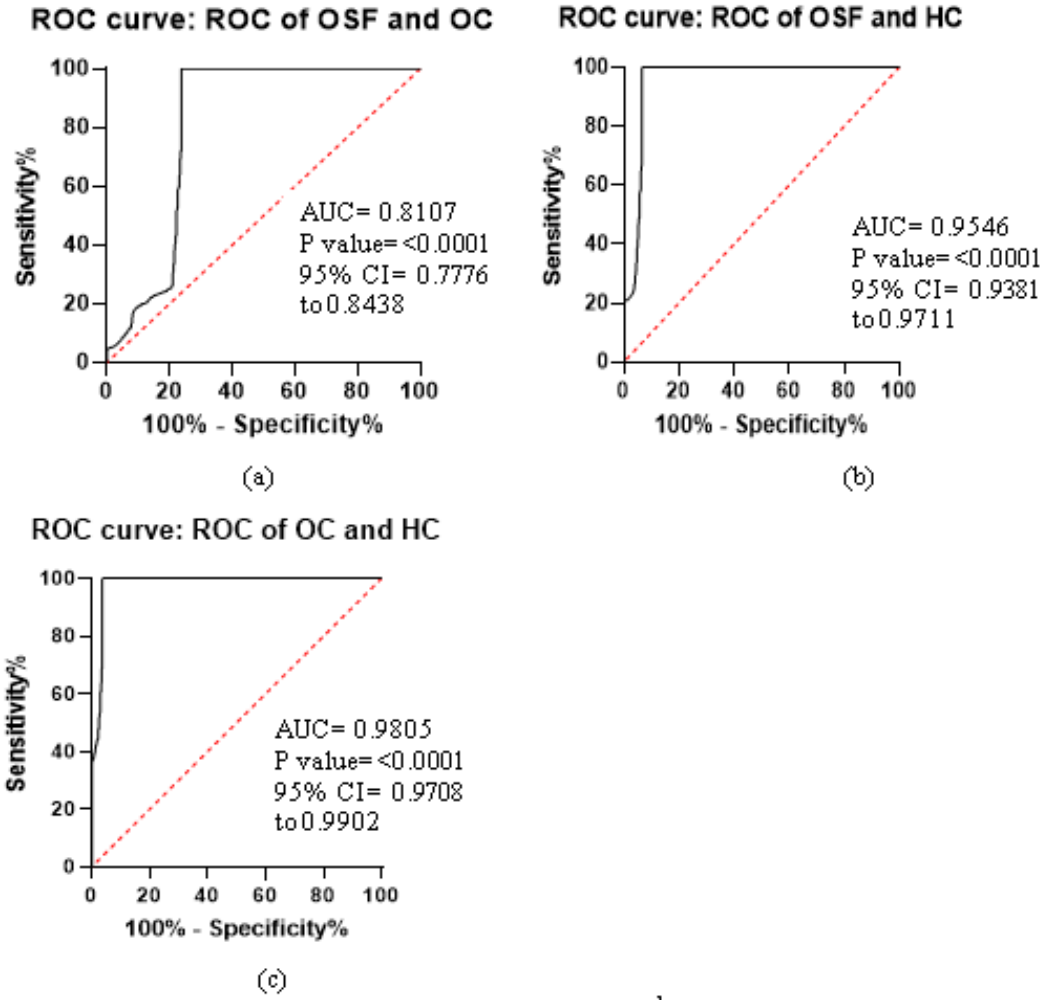

Figure 3. ROC curves made from the wavenumber 1800-900 cm^{-1} for (a) oral submucous fibrosis vs. oral cancer (b) oral submucous fibrosis vs. healthy controls and (c) oral cancer vs. healthy controls. Results about area under the curve (AUC), *P* value and 95% confidence interval are being shown near the ROC curve.

ATR-FTIR played an important role in the identification of different cancers by means of blood plasma / serum samples [28-30]. Dorling et al. emphasized the benefits and significance of attenuated total reflection Fourier transform infrared spectroscopy in blood serum study for disease detection [31]. This study has highlighted a frequency range of 1800-900 cm^{-1} wavenumber as it represents the conformation changes in lipids, proteins, nucleic acids and carbohydrates with respect to oral cancer

and pre-cancer. Peaks at 1339 cm^{-1},1316 cm^{-1}, 1074 cm^{-1}, 1739 cm^{-1} and 1770 cm^{-1} was observed in normal whereas it was found to be absent in oral cancer and oral sub-mucous fibrosis.

Further, to discriminate cancer from pre-cancer and healthy controls various multivariate analysis was performed such as HCA and ROC. The variables corresponding to CH2 deformation of the lipid acyl chain (~1316 cm^{-1}) and amide II (~1545 cm^{-1}) exhibited significant involvement in separating the normal and cancer groups. Fukuyama et al. has investigated the differences of tissue samples from oral leucoplakia and oral squamous-cell carcinoma using FTIR technique. They have shown a significant contribution to discriminate normal and oral malignant groups by the second derivative of amide II bands and lipids at 1550 and 1465 cm^{-1}. They mentioned briefly about the biomolecules present in tissues and their absorption depending on the conditions of the disease (normal, oral leucoplakia and oral squamous cell carcinoma) [32].

Our study, showed a prominent peak of ATR-FTIR band at 1316 cm^{-1} (collagen and lipid) , 1074 cm^{-1}(DNA/RNA), 1770 cm^{-1} (C=O stretching/vinyl/phenyl ester group), 1739cm^{-1} (C=O, polysaccharides) in healthy control. Absence of these bands in cancer and pre-cancer may be may be suggestive of DNA structural disorders [33]. However, Rai et al., noted absorbance bands in the protein region of FTIR spectra in amide-I/Collagen (1650 and 1035 cm^{-1}), amide-II (1544 cm^{-1}) suggesting increase in collagen is typical of various malignant disorder such as OSF [34]. The decline in the FTIR range of lipids in OC can be justified in terms of the cancerous cells' energy requirements, where a high lipid intake serves the nutritional needs of the rapidly growing and intrusive cell population [34].

We have evaluated the diagnosis performance through the ROC curve and it achieved significantly accurate as it segregated cancer from pre-cancer and healthy controls hence, can be used for the biomarker performance. Hierarchical clusters analysis observed the three clusters differentiating control with OSF and OC in our study. Furthermore, Chiu, Li-Fang, et al also demonstrated the infrared spectral biomarkers of oral buccal mucosa can be employed for screening among healthy, pre-cancerous, primary and metastatic cancers via linear discriminant analysis (LDA), with accuracy of 77% [35]. Similarly, Rai et al. applied FTIR spectroscopy combined with hierarchical cluster analysis (HCA), principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) to examine serum samples for the diagnosis of oral sub-mucous fibrosis and obtained a prominent significant wavenumbers which separate spectra between malignancy and normal serum samples [34].

4. CONCLUSION

From the present study it may be concluded that ATR-FTIR can be used as a potential diagnostic tool for identification of molecular changes in the serum. It also emphasizes on the validity of multivariate analysis for separating the malignancy groups from control. Furthermore, on the basis of projected higher accuracy, our results encourage the use of the ATR-FTIR technique in screening of oral cancer and pre-cancer. However, our study needs to be established in larger samples to achieve its reproducibility and robustness.

Deep Kumari Yadav: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation. **Rakesh Rawal**: Visualization, Investigation, Supervision. **Shayma Shaikh:** Writing- Reviewing and Editing, Software, Validation

Declaration of competing interest

The study does not have any conflict of interest.

Acknowledgements

We are grateful to all patients who gave their consent to this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Cancer. Available online: https://www.who.int/news-room/fact-sheets/detail/cancer
2. Glick, Michael, and Newell W. Johnson, Oral and oropharyngeal cancer: what are the next steps? The Journal of the American Dental Association. 142.8 (2011),pp. 892-894.
3. Passi, Deepak et al., Oral submucous fibrosis: Newer proposed classification with critical updates in pathogenesis and management strategies. National journal of maxillofacial surgery vol. 8, 2 (2017), pp. 89-94.
4. Cox, S., Oral cancer in Australia--risk factors and disease distribution. Annals of the Royal Australasian College of Dental Surgeons. 15 (2000), pp.261-263.
5. Singh, S. P., et al. Recent advances in optical diagnosis of oral cancers: Review and future perspectives. Head & neck 38.S1 (2016), pp. 2403-2411.
6. Pałasz P, Adamski Ł, Górska-Chrząstek M, Starzyńska A, Studniarek M. Contemporary Diagnostic Imaging of Oral Squamous Cell Carcinoma - A Review of Literature. Pol J Radiol. 82 (2017), pp.193-202.
7. Greene, Frederick L., et al., eds. AJCC cancer staging handbook: TNM classification of malignant tumors. Springer Science & Business Media, 2002.
8. Epstein, Joel B., Lewei Zhang, and Miriam Rosin, Advances in the diagnosis of oral premalignant and malignant lesions. Journal-Canadian Dental Association. 68.10 (2002), pp. 617-621.
9. Sahu, R. K., and S. Mordechai., Fourier transform infrared spectroscopy in cancer detection. (2005), pp. 635-647.
10. Mukherjee, Rashmi, et al., Altered metabolic profile in early and late onset preeclampsia: an FTIR spectroscopic study. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health. 4.1 (2014), pp. 70-80
11. Cohenford, Menashi A., and Basil Rigas, Cytologically normal cells from neoplastic cervical samples display extensive structural abnormalities on IR spectroscopy: implications for tumor biology. Proceedings of the National Academy of Sciences. 95.26 (1998), pp. 15327-15332.
12. Argov S, Ramesh J, Salman A, et al., Diagnostic potential of Fourier-transform infrared microspectroscopy and advanced computational methods in colon cancer patients. J Biomed Opt. 7.2 (2002), pp. 248-254.

13. Baker, Matthew J., et al., FTIR-based spectroscopic analysis in the identification of clinically aggressive prostate cancer. British journal of cancer. 99.11 (2008), pp. 1859-1866.
14. Barlev, Eyal, et al., A novel method for screening colorectal cancer by infrared spectroscopy of peripheral blood mononuclear cells and plasma. Journal of gastroenterology. 51.3 (2016), pp. 214-221.
15. Eckel, Rainer, et al., Characteristic infrared spectroscopic patterns in the protein bands of human breast cancer tissue. Vibrational Spectroscopy. 27.2 (2001), pp. 165-173.
16. Lewis, Paul D., et al., Evaluation of FTIR spectroscopy as a diagnostic tool for lung cancer using sputum. BMC cancer. 10.1 (2010), pp. 1-10.
17. Mordechai, S., et al., Possible common biomarkers from FTIR microspectroscopy of cervical cancer and melanoma. Journal of microscopy. 215.1 (2004), pp. 86-91.
18. Thumanu, Kanjana, et al., Diagnosis of liver cancer from blood sera using FTIR microspectroscopy: a preliminary study. Journal of biophotonics. 7.3-4 (2014), pp. 222-231.
19. Urboniene, Vidita, et al., Identification of kidney tumor tissue by infrared spectroscopy of extracellular matrix. Journal of biomedical optics. 19.8 (2014), pp. 087005.
20. Pezzei, Christine, et al., Characterization of normal and malignant prostate tissue by Fourier transform infrared microspectroscopy. Molecular BioSystems. 6.11 (2010), pp. 2287-2295.
21. Grdadolnik, Jože. ATR-FTIR spectroscopy: Its advantage and limitations. Acta Chimica Slovenica. 49.3 (2002), pp. 631-642.
22. Bunaciu, Andrei A., Vu Dang Hoang, and Hassan Y. Aboul-Enein. Applications of FT-IR spectrophotometry in cancer diagnostics. Critical reviews in analytical chemistry. 45.2 (2015), pp. 156-165.
23. Fung, Michael Fung Kee, et al, Pressure-tuning fourier transform infrared spectroscopic study of carcinogenesis in human endometrium. Biospectroscopy. 2.3 (1996), pp. 155-165.
24. Li, Qing-Bo, et al, Use of Fourier-transform infrared spectroscopy to rapidly diagnose gastric endoscopic biopsies. World Journal of Gastroenterology: WJG. 11.25 (2005), pp. 3842.
25. Pachaiappan, Rekha, Aruna Prakasarao, and Ganesan Singaravelu, Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) in the discrimination of normal and oral cancer blood plasma. Optical Biopsy XV: Toward Real-Time Spectroscopic Imaging and Diagnosis. Vol. 10060. International Society for Optics and Photonics, 2017
26. Ruiz-Chica, A. J., et al, Characterization by Raman spectroscopy of conformational changes on guanine–cytosine and adenine–thymine oligonucleotides induced by aminooxy analogues of spermidine. Journal of Raman spectroscopy. 35.2 (2004), pp. 93-100.

27. Nandiyanto, Asep Bayu Dani, Rosi Oktiani, and Risti Ragadhita, How to read and interpret FTIR spectroscope of organic material. Indonesian Journal of Science and Technology. 4.1 (2019), pp. 97-118.
28. Hands, James R., et al, Investigating the rapid diagnosis of gliomas from serum samples using infrared spectroscopy and cytokine and angiogenesis factors. Analytical and bioanalytical chemistry. 405.23 (2013) pp. 7347-7355.
29. Khanmohammadi, M., et al, Cancer diagnosis by discrimination between normal and malignant human blood samples using attenuated total reflectance-fourier transform infrared spectroscopy. Cancer investigation. 25.6 (2007), pp. 397-404.
30. Gajjar, Ketan, et al., Fourier-transform infrared spectroscopy coupled with a classification machine for the analysis of blood plasma or serum: a novel diagnostic approach for ovarian cancer. Analyst. 138.14 (2013), pp. 3917-3926.
31. Dorling, Konrad M., and Matthew J. Baker, Highlighting attenuated total reflection Fourier transform infrared spectroscopy for rapid serum analysis. Trends in biotechnology. 31.6 (2013),pp. 327-328.
32. Fukuyama, Yoshikuni, et al., A study on the differences between oral squamous cell carcinomas and normal oral mucosas measured by Fourier transform infrared spectroscopy. Biospectroscopy. 5.2 (1999), pp. 117-126.
33. Simonova, Desislava, and Iliana Karamancheva., Application of Fourier transform infrared spectroscopy for tumor diagnosis. Biotechnology & Biotechnological Equipment. 27.6 (2013), pp. 4200-4207.
34. Rai, Vertika, et al., Serum-based diagnostic prediction of oral submucous fibrosis using FTIR spectrometry. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 189 (2018), pp. 322-329.
35. Chiu, Li-Fang, et al., Oral cancer diagnostics based on infrared spectral markers and wax physisorption kinetics. Analytical and bioanalytical chemistry. 405.6 (2013), pp. 1995-2007.