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Research Article

RAMAN SPECTROSCOPY STUDY OF HEALTHY AND CANCEROUS HUMAN BREAST TISSUE FOR CANCER DETECTION

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ABSTRACT: The present study focuses on identifying the features and parameters of Raman spectroscopy for the diagnosis of cancer in human breast surgical samples. Thirty-five clinically unprocessed, fresh human breast tissue specimens (20 cancerous and 15 normal tissues) were obtained under a bioethical protocol approved by the institution's ethical committee. Confocal spontaneous Raman spectroscopy in reflection mode was performed using an incident excitation laser monochromatic beam of 532 nm. The differences observed among Raman profiles of cancerous tissues are more prominent compared with normal breast tissues. Notable spectral differences were present in both the absolute and relative intensities of the Raman peaks in the spectral profile. In the present study, three ratios of Raman intensities 1587 cm-1/ 1637 cm-1, 1587 cm-1/ 1000 cm-1 and 1587 cm-1/ 1375 cm-1 with statistically reliable differences are reported in the present study. It is found to be sensitive enough to differentiate between normal and cancerous breast tissues.

Key words: Breast cancer, Raman spectroscopy, Tissue, Spectrum.

INTRODUCTION

Breast cancer is leading cancer among women today with a reported mortality of 627,000 women from breast cancer globally in 2018 (Bray *et al*. 2018, WHO 2021). Cancer patients have a poorer prognosis in low resources countries due to relatively low awareness about cancer among the public, delay in cancer detection, and inequitable access to affordable treatment as compared to those in high-resourced countries (Chalkidou *et al*. 2014, Dhillon *et al*. 2018, Feng *et al*. 2019). Early detection of cancer may save many lives. However, it is a big challenge and there is an urgent need for a non-invasive, simple, fast, easy-to-use, inexpensive method suitable for mass population disease detection.

Mammography is the most common and widely practiced breast cancer screening method (Oeffinger *et al*. 2015). However, mammography involves the risk of repeated exposure to harmful ionizing radiation.

Currently, histopathology is considered the gold standard for the detection and staging of all cancers. During the procedure, tissue specimens are obtained from patients and critically tested by pathologists using several staining methods. Histopathology analysis of tissue has some inherent limitations. This includes delays in reporting diagnostic results for any therapeutic intervention and it heavily relies on human microscopic inspection of the stained tissue for detection leading to the potential for the interobserver discrepancy (Haka *et al*. 2009). A well-trained clinically qualified professional is required for histopathology analysis of tissue for the detection of cancer. Immunohistochemical tests of tissue apply a molecular targeting strategy and utilize protein-specific antibodies that can allow increased sensitivity for cellular abnormality for the detection of cancer. However, Immunohistochemical procedures are more time-consuming and costly and again suffer from an interobserver discrepancy in reporting diagnostic results (Bird *et al*. 2009). To overcome these limitations of conventional diagnostic techniques for the detection of cancer, novel approaches are required to allow cost-effective, non-invasive, rapid, and high patient throughput diagnosis. Raman spectroscopy is an effective method for monitoring the vibrations of molecular groups present in the tissue.

Raman spectroscopy presents strong evidence to overcome these constraints and provide the diagnostic

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potential to detect the presence of cancer by analyzing the biochemical profile of the biological specimens (Sattlecker *et al*. 2014, Jermyn *et al*. 2016, Sinica *et al*. 2019).

Raman spectroscopy is considered a rapid non-destructive and non-ionizing chemical analysis tool providing detailed spectroscopic data regarding the phase, chemical structure, crystal, and molecular interactions present in a tissue. The present study aimed to provide Raman fingerprint data for the detection of breast cancer using Raman spectroscopy among the Asian population. Vibrational Raman signatures of human breast tissue are employed to discriminate between normal and cancerous tissues. The present study focuses on identifying the features and parameters of Raman spectroscopy for the detection of cancer in human breast surgical samples.

MATERIALS AND METHODS

Study model

This was a single-center, multi-disciplinary, randomized, single-site, single-blind, active control, parallel, prospective, experimental, study. Female patients aged between 18-75 years with written informed consent having a confirmed diagnosis of breast cancer were recruited in the study. Male patients with breast cancer, Covid-19 positive patients were excluded from the study.

Patient selection and specimen characteristics

The present study uses human surgical samples of cancer patients aged with Mean ± SD (47±10 years) who gave consent to perform the study. The biological samples were obtained from the modified radical mastectomy (MRM) surgical procedure. The breast was removed during this surgery. The malignant tissue sections were obtained from gross tumor volume. The normal tissue sections (control samples) were obtained from 2 to 3 cm away from gross tumor volume with a safe clean margin of the breast tissue resection during the MRM surgical procedure. The study protocol for the collection of specimens of the human breast including tumor and surrounding normal tissue was approved by the institutional ethical committee at the SMS Medical College, Jaipur (3095/MC/EC/12/04/2017). Written informed consent was obtained from the participants. The specimens were taken during the MRM resection surgery in 10 breast cancer patients from the gross tumor volume and normal tissue with a safe negative margin. All of the fresh tissue samples were transported to the Materials Research Center (MRC) Lab by placing them in a sterile cryovial at temperatures below 4° Celsius. The sample was placed on the glass substrate and Raman spectroscopy has been performed. All the procedures were recorded to complete within 4 hours. The final set of experiments included 105 spectra acquired in 35 clinically unprocessed, fresh human surgical samples (20 cancerous and 15 normal tissues). Specimens were stratified into normal and cancerous tissues. In the present study, all excised breast tumors were invasive ductal carcinomas of the breast. The tissue sample was immediately placed on acetate paper and transferred in a 4 mL air-tight sterile cryovial to withstand low temperatures. The microscope slide was used as a platform for microscopic specimen observation. The slides were nearly transparent to visible light and chemically inert. Following spectral data acquisition, formaldehyde was used to preserve tissue samples for storage of the biological sample.

Raman spectroscopy

The Raman spectra were acquired with a confocal micro Raman spectrometer system with photoluminescence (PL) STR 500 system (AIRIX Corp., Tokyo, Japan) equipped with lasers with wavelengths of 325 nm (UV), 532 nm (Visible), 785 nm (Near IR) and a maximum power of 50 mW. In the present experimental work, confocal spontaneous Raman spectroscopy (SRS) in reflection mode was performed using an incident excitation laser monochromatic beam of 532 nm. It has a diode-pumped solid-state (DPSS) Nd-YAG Green laser (DL 532, AIRIX Corp, Tokyo, Japan). The specimen was focused through a 20x dry objective and a 4.0–2.9 mm operating distance. The laser beam of 532 nm with laser power 5mW was utilized to record Raman spectra with an integration time of 10x3 s. The notch filter (Kaiser Optics, Ann Arbor, MI, USA) was employed to remove Rayleigh's scattered light. The spectra of human surgical tissue samples were obtained and monitored at 22° Celsius temperature. The spectra were acquired at a spectral resolution of 0.5 cm-1 and within the identical spectral range of 700 – 3350 cm-1 (Jain *et al*. 2022). After conformal focus, Raman spectroscopy was performed three consecutive times for each sample and average spectra were obtained to reduce uncertainty in the measurements.

Data processing and analysis

Before the spectral analysis, each Raman spectrum was preprocessed to remove cosmic rays for spectra measurement using frequency and spatial filtering in STR software of the Raman spectroscope system. The sharp spikes in raw spectra attributed to cosmic rays were removed with the use of frequency and spatial filtering. STR Data Collection Software output data was processed

with OriginPro software to quickly analyze and present meaningful information for visualizing, processing, and managing spectroscopy data. Further, all data processing and chemometrics analysis have been performed using the data analysis software OriginPro version 8.5 (OriginLab Corporation, Northampton, MA, United States). Each corrected spectrum was processed and smoothened to gain the signal-to-noise ratio (SNR) by using spectral smoothing (Savitzky - Golay algorithm) procedure over the entire wavenumber region acquired (3350 – 700 cm-1). This increases SNR by eliminating the effect of change in Raman intensity, mainly induced due to variation in cellular density and thickness of the sample. The baseline subtraction was done manually using OriginPro software and the Raman spectra were normalized. Baseline subtraction eliminates the Rayleigh scattering. After the removal of the baseline, the spectra represented the Raman intensity of the identical Raman characteristics, emerging from the varying quantity and vibrations of biochemical substance present in the biological specimen (Lasch 2012). We have recorded the wavenumber region (3350 – 700 cm-1) in the present study. Raman data was pre-processed via normalization and Savitzky–Golay (SG) method. The Raman spectrum was normalized using the Raman intensity values divided by the mean. Further, the Raman spectrum was smoothened by 10 points using second-order differentiation in Origin Pro software. The mean Raman spectrum was estimated as an arithmetic means of the acquired Raman spectrum. Diagnostically significant principal components (PCs) of Raman intensity ratios were identified using Student's t-test with a 95% confidence level with a p-value < 0.05 (Lang *et al*. 2015).

RESULTS AND DISCUSSION

Processing of the Raman spectra

The present study analyzed the average Raman spectrum of human breast tissue, the tissue that contains adipose cells, epithelial cells of ducts, and connective tissues. A Raman spectrum features several crests and troughs, depicting the intensity and wavenumber site of the light with Raman scattering. This is due to organic molecular substances found in tissue having lots of hydrocarbon bonds in proteins and lipids, which are required for cell nutrition. The wavenumber less than 700 cm-1 fluorescence was predominant. Hence, after that Raman spectrum was extracted and processed for the most probable Raman bands of significance for the detection of breast cancer. Several peaks were observed due to molecular structures present in fatty acids, and

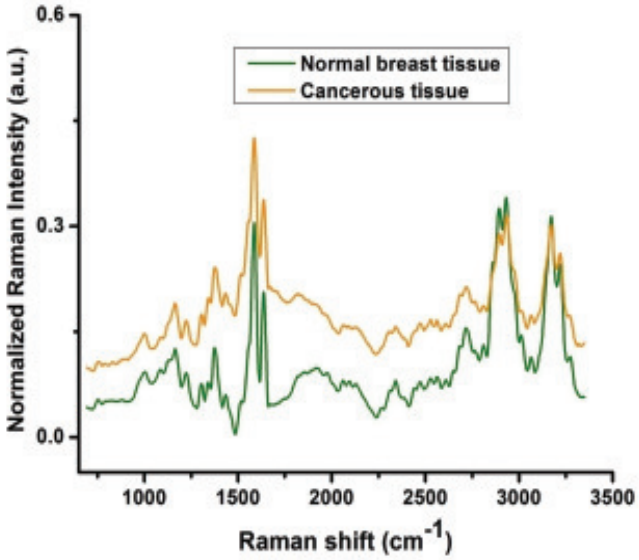

Fig. 1. The comparison of average Raman spectra of the human breast normal tissue (green line) and cancerous breast tissue (orange line).

amino acids which have hydrocarbon and nitrogen bonds. The wavenumber range 830 –1900 cm–1 spectral region provided several identifiable peaks. In our analysis, there were 11 identifiable peaks. Most of the positive bands observed around 1000, 1163, 1375, 1587, 1637, 2346, 2723, 2892, 2937, 3172, and 3223 cm–1 can be assigned to various vibrational modes of proteins and lipids. There was evident variability observed in Raman intensity without notable variation in the spectral profile. The differences observed between Raman profiles of cancerous tissue were more prominent compared with normal breast tissues. Notable spectral differences were observed in both the absolute and relative intensities of the peaks in the Raman spectra. Further, the breast tissue is a heterogeneous mixture of cells and biomolecules differentiating from the biochemical and histological points of view that were reflected in the average spectra of the normal and cancerous breast tissue specimens. Averaging of Raman spectra was performed to reduce the impact of fluctuations in Raman intensities of individual substances at multiple locations of a sample. Each average Raman spectrum was normalized and used for the calculation of the average Raman spectra for each set. The average Raman spectra presented in Fig. 1 demonstrated prominent distinctions between normal and cancerous breast tissues. Moreover, the Raman profiles were predominated due to protein-rich biomolecules in the Raman fingerprint region in the spectral range below 1800 cm–1. The protein-rich region was dominated due to the abundance of adipose tissue in the human breast.

The protein-rich regions were observed to dominate in the molecular composition of the cancerous tissue in comparison to the normal breast tissue.

Raman assignment of the spectral region

To characterize the positions of the Raman peaks and peak assignment was done based on previous Raman spectroscopy studies on cellular organic macromolecules and natural tissues based on available literature data. Average Raman spectra of normal and cancerous breast tissues reflected several characteristic bands for biochemical components of human breast tissue. Further, the spectral interpretations of the most common Raman bands are shown in Table 1. The differences in Raman spectrum between normal and cancerous breast tissues were presented based on the (i) changes in composition and conformation in proteins, and (ii) the presence of other biochemical molecules (nucleic acids, lipids, etc.). The Raman bands of collagen content were found as the potential Raman marker for the detection of breast tissue malignancy. During the cancer progression, cancerous lesions tend to become denser than normal tissue due to the increase in the number of fibrous components. These components are mostly collagen-like proteins, which causes the increase of the Raman band intensity of amide I at 1637 cm-1 in spectra of cancerous breast tissues. In addition to this, the Raman spectra of breast tumors presented with higher nucleic acids (NA) and aromatic amino acids (Phe, Trp) bands at 1587 cm-1 (NA) and 1375 cm-1 (Trp) and 1000 cm-1 (Phe) respectively. Furthermore, the Raman peak at 1163 cm-1 of glycogen may also be explored as the Raman marker for the detection of breast cancer. Cancerous cells utilize glycogen in anaerobic metabolism and glycogen levels can increase in cancerous cells in response to acute hypoxia. A similarly elevated glycogen level was observed in the cancerous breast tissue. These Raman biomarkers can be utilized for cancer detection in the human breast.

The results of the present study suggest that the tumor vascularity kinetics associated with tumor proliferation shall be measured with the hemoglobin peak at 1587 cm-1. Moreover, the peak has a major contribution from vibrations in hemoglobin originating from blood vessels. It acts as a key and effective Raman marker for the

Table 1. Position and assignment of major peaks in the Raman spectra of specimens
(Zúñiga *et al*. 2019, Eshraghi-Arani and Dehghani-Bidgoli 2021, Liu *et al*. 2013, Rehman *et al*. 2007, Rehman *et al*. 2013, Chaturvedi *et al*. 2016, Talari *et al*. 2015, Brozek-P³uska *et al*. 2016, Kopec *et al*. 2019).

Sl. No.	Raman shift of peak position (cm^{-1})	Molecular assignment	Compounds
1	1000	υ(C-C) Symmetric ring breathing, C-O-C skeletal mode	P (Phe)
2	1163	C-C (&C-N) stretching of proteins, C-O stretching of carbohydrates	L, P (Tyr), Gly, Cho
3	1375	δCH_2 symmetric, CH_3 bending mode	P (aliph AA, Trp), L, GlcNAc
4	1587	δ(C=C) and υ(C=C) bending mode and C=N stretching	P (Trp, Amide II), deoxy-Hb, NA (A,G)
5	1637	C=C stretching mode of lipids, C=O stretching mode of proteins	P (Amide I), L (unsat. FA, trigly)
6	2346	Asymmetric stretching band of CO_2-hydrates	L, P
7	2723	Stretching vibrations of CH, NH, and OH groups	L, P
8	2892	CH_2 asymmetric stretch of lipids and proteins	L (FA, TG), P, lys, Gly
9	2937	CH_3 asymmetric stretch of proteins; aliphatic and aromatic CH stretching vibrations in nucleic acids	L, Met lys
10	3172	CH stretching	L, P
11	3223	O-H & N-H stretching vibrations	L, P

[Abbreviations: υ (stretching mode), δ (bending mode), P (proteins), L (lipids), Phe (phenylalanine), Tyr (tyrosine), Gly (glycogen), Cho (carbohydrates), Trp (tryptophan), aliph. AA (alliphatic amino acids), GlcNAc (N-acetyloglucosamine), deoxy-Hb (deoxyhemoglobin), NA (nucleic acids), A (adenine), G (guanine), FA (fatty acids), TG (triglycerides), Met lys (methylated lysine)].

Table 2. Raman intensity ratios, I_{1587}/I_{1000}; I_{1587}/I_{1375}; and I_{1587}/I_{1637}, for normal and cancerous breast tissue samples.
[Sample: 20 cancerous Tissue (C), 15 normal tissues (N)].

Ratio	Assignment	Tissue type	Intensity ratio (Mean ± SD)	p value
1587/1000	Phosphorylated proteins/phenylalanine	N	1.79 ± 0.46	0.04
		C	1.63 ± 0.14	
1587/1375	Phosphorylated proteins/tryptophan	N	1.29 ± 0.18	0.01
		C	1.19 ± 0.05	
1587/1637	Phosphorylated proteins/proteins	N	1.06 ± 0.09	0.005
		C	0.98 ± 0.06	

identification of the phosphorylation status in the rapidly dividing nature of cancer cells. Cancerous breast tissue has greater tumor vascularization and greater oxygen consumption than normal breast tissue. The peaks 1587 cm-1 and 1637 cm-1 were especially pronounced in the spectra of cancerous breast tissue samples. The prominent hemoglobin peak at 1587 cm-1 in cancerous breast tissue may indicate that RS can be used to detect cancer and tumor progression. The Raman spectra changed dramatically in cancerous specimens, with various molecular bands becoming predominant and evident in Raman fingerprint spectral region.

Ratios of the characteristic bands

The discrimination between normal and cancerous breast tissue samples was evaluated using the intensity ratios of characteristic Raman bands. Furthermore, the average Raman spectra of normal and cancerous tissues demonstrated significant spectral differences, and the intensity ratio of Raman biomarkers in the Raman fingerprint region range 1000–1637 cm-1 provided pivotal information for the detection of breast cancers. Further analysis was carried out and various Raman intensity ratios were tested with the level of significance (p-value) for Raman spectra biomarker for cancer detection. Comparing the Raman intensities of several characteristic Raman bands for normal and cancerous breast tissue samples in the present study, we report three ratios of Raman intensities 1587 cm-1/ 1637 cm-1, 1587 cm-1/ 1000 cm-1 and 1587 cm-1/ 1375 cm-1 that showed statistically reliable differences and found to be sensitive enough to differentiate between normal and cancerous breast tissue. The results of the present study are summarized in Table 2.

Several researchers investigated the application of Raman spectroscopy in diagnosing cancer in human tissue. The present study is to collect Raman spectroscopy data to serve the purpose. There were three key observations made in our study. First, there were 11 identifiable Raman peaks were found in the analysis of Raman spectroscopy experiments of normal and cancerous breast specimens. Second, Raman peak assignments were performed to detect major components of breast specimens in the Raman spectral profile useful to differentiate cancerous breast tissue from normal breast tissue. Third, Raman intensity ratios were identified using the intensity ratios of characteristic Raman bands.

In our study, we observed the pronounced hemoglobin peak at 1587 cm-1 to determine tumor proliferation depending upon the degree of vascularization within tumor volume. Zúñiga *et al*. (2019) evaluated breast tumor Raman spectra and measured the peak at 1560 cm-1 associated with hemoglobin present in the biological specimen. The probable cause of the difference observed in hemoglobin peak can be attributed to the excitation wavelength used. The present study used 532 nm visible laser excitation wavelength to report the findings. However, Zúñiga *et al*. (2019) conducted the Raman experiments using a 1024nm and 785nm near-infrared laser excitation source.

In this paper, three ratios of Raman intensities 1587 cm-1/ 1637 cm-1, 1587 cm-1/ 1000 cm-1, and 1587 cm-1/ 1375 cm-1 were found to be sensitive enough to differentiate between normal and cancerous breast tissue. Our results were found similar to that Abramczyk *et al*. (2019) that the 1586/829 ratio is the best Raman biomarker for monitoring phosphorylation status. Indeed, the Raman bands at 1587 cm-1 and 1637 cm-1 were assigned primarily to nucleic acid and Amide I vibrations, which were markedly more pronounced in the case of cancerous breast tissue samples. Similarly, the other two Raman bands at 1375 cm-1 and at 1000 cm-1 assigned to Tryptophan and Phenylalanine vibrations, having contributions of mixed protein-lipid vibrations, were observed more intense for cancerous breast tissue samples.

CONCLUSION

The Raman spectra were acquired and analyzed for the human normal breast and cancer tissues. This paper provides strong evidence for the diagnostic potential of Raman spectroscopy for monitoring of Raman method in the detection of cancerous tissue in human breast surgical samples.

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