DIAGNOSIS OF CERVICAL CANCER USING CONFOCAL RAMAN SPECTROSCOPY

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ABSTRACT

According to W.H.O., cervical cancer is the leading cause of fatality in women in India, though it can be effectively treated if diagnosed at an early stage. Pap smear test carried out regularly can aid in early diagnosis but the accuracy of this test is around 70%. Hence to this date, histopathology study is regarded as the golden standard but it is a cumbersome process and is left at the expertise of the pathologist. Accurate, rapid and non-invasive detection and diagnosis of malignant disease in tissues is an important goal of biomedical research and is the need of the hour. Optical methods such as diffuse reflectance, fluorescence spectroscopy and Raman spectroscopy are actively explored to this end. Raman spectroscopy, a method based on Raman scattering, is a powerful technique that can be applied to many tissue sites. Raman spectroscopy is a molecular-specific technique that probes the vibrational or rotational transitions in chemical bonds and provides detailed information about the biochemical composition of a sample. The use of Raman spectroscopy in the detection and classification of malignancy within the human uterine cervix would be evaluated in this work. The excitation wavelength being 785nm from a diode laser source is selected for this study. The wave number region to be analysed would be from 600-1800cm^{-1} which is the fingerprint region for bio molecules. The spectra would be corrected for noise and then analysed by statistical significances.

Keywords: *Raman Spectroscopy, metaplasia ,precancer, cervical cancer, Cervical Intraepithelial Neoplasia.*

1. INTRODUCTION-1:

Spectroscopy is the study of the interaction between matter and radiated energy. Spectrometry and spectrography are terms used to refer to the measurement of radiation intensity as a function of wavelength and are often used to describe experimental spectroscopic methods.

1.1 Various Optical Spectroscopy Techniques:

Various techniques of optical spectroscopy can be used to study biological tissues, and in particular the surfaces of the various parts and organs of the human body. Many of these techniques such as fluorescence, Raman, diffuse reflectance have already been employed to study biological systems and tissues both in vitro and in vivo. Each one has its own special features, advantages, and potential applications.

Reflectance Spectroscopy:

Reflectance spectroscopy is the study of light that has been reflected or scattered from a solid, liquid, or gas. As photons enter a material, some are reflected from grain surfaces, some pass through the grain, and some are absorbed. Those photons that are reflected from grain surfaces or refracted through a particle are said to be scattered. Scattered photons may encounter another grain or be scattered away from the surfaces so they may be detected and measured.

Reflectance measurements can be divided into two basic categories- internal or external reflectance. The technique of Attenuated Total Reflection (ATR) is employed for internal reflectance measurements as the beam of infrared radiation passes through an ATR element (crystal) in contact with the sample.

For external reflectance measurements, the infrared beam of radiation is reflected directly from the sample surface. This type of external reflectance measurement can be divided into two types- Diffuse or Specular.

Absorption spectroscopy:

Absorbance spectroscopy, commonly referred to as spectrophotometry, is the analytical technique based on measuring the amount of light absorbed by a sample at a given wavelength. Spectrophotometry, particularly in the VIS and UV portions of the electromagnetic spectrum, is one of the most versatile and widely used techniques in chemistry and the life sciences.

Molecular absorption spectroscopy in the ultraviolet (UV) and visible (VIS) is concerned with the measured absorption of radiation in its passage through a gas, a liquid or a solid. The wavelength region generally used is from 190nm to about 1000nm, and the absorbing medium is at room temperature. However, in some cases (e.g. in enzyme assays) measurements at temperatures above or below room temperature may be advantageous or necessary.

Transmission spectroscopy:

Transmission spectroscopy is highly interrelated to Absorption spectroscopy. This technique can be used for solid, liquid, and gas sampling. Here, light is passed through the sample and compared to light that has not. The output depends on the path length or sample thickness, the absorption coefficient of the sample, the reflectivity of the sample, the angle of incidence, the polarisation of the incident radiation, and, for particulate matter, on particle size and orientation.

Fluorescence spectroscopy:

Fluorescence spectroscopy uses higher energy photons to excite a sample, which will then emit lower energy photons. This technique has become popular for its biochemical and medical applications, and can be used for confocal microscopy, fluorescence resonance energy transfer, and fluorescence lifetime imaging.

The basis for the fluorescence technique for tissue studies is to excite molecules and to examine the re-emitted fluorescence light. The fluorescence characteristics are determined by the transition probabilities between different electronics, vibrational and rotational configurations of the molecule, illustrated by its Jabolanski diagram. Fluorescence spectroscopy can thus be a useful tool to identify the type and concentration of molecule in a sample. Measurable fluorescence properties are useful in such an analysis, which include the excitation and emission spectra, and the fluorescence lifetime.

Diagnostic techniques based on optical spectroscopy have the potential to link the biochemical and morphological properties of tissues to individual patient care. In particular, these techniques are fast, non-invasive and quantitative.

2. RAMAN SPECTROSCOPY-2:

When light is scattered from a molecule most photons are elastically scattered -the scattered photons have the same energy (frequency) and therefore wavelength is same as the incident photons. However, a small fraction of light (approximately 1 in 106-108 photons) is scattered at optical frequencies different from and usually lower than, the frequency of the incident photons. The process leading to this inelastic scatter is termed the Raman effect.

Raman scattering can occur with a change in vibrational or rotational energy of a molecule. If the substance being studied is illuminated by monochromatic light, for example from a laser, the spectrum of the scattered light consist of strong lines (the exciting line) of the same frequency as the incident illumination together with weaker lines on either side shifted from the strong line by frequencies ranging from a few to about 4000cm-1. The lines of frequency less than the exciting lines are called stokes lines, the others anti-stokes lines. Accurate, rapid and non-invasive detection and diagnosis of malignant disease in tissues is an important goal of biomedical research. Optical methods, such as diffuse reflectance, fluorescence spectroscopy, and Raman spectroscopy, have all been investigated as ways to attain this goal. Diffuse reflectance utilizes the absorption and scattering properties of tissues, particularly from cell nuclei and stroma. Changes in the scattering properties of tissues arise as the tissue becomes more dysplastic due to variations in haemoglobin content and neovascularisation. Fluorescence spectroscopy is also influenced by the changes in the optical properties of tissues and has been used to diagnose dysplasia. However, there are a number of disadvantages to these techniques, including the need for extensive sample preparation or excision, as well as low sensitivity and specificity rates.

Many research groups have used Raman spectroscopy to detect and diagnose disease in vivo without the need for tissue removal or the addition of exogenous agents. Raman spectroscopy, a method based on Raman scattering, is a powerful technique that can be applied to many tissue sites. Raman spectroscopy is a molecular-specific technique that probes the vibrational or rotational transitions in chemical bonds and provides detailed information about the biochemical composition of a sample. The sensitivity of this technique is so high that a Raman spectrum is effectively a precise fingerprint of the biochemical makeup of the tissue.

2.1 Principle of Raman Spectroscopy-1:

Raman spectroscopy is a spectroscopic technique based on inelastic scattering of monochromatic light, usually from a laser source. Inelastic scattering means that the frequency of photons in monochromatic light changes upon interaction with a sample. Photons of the laser light are absorbed by the sample and then reemitted. Frequency of the reemitted photons is shifted up or down in comparison with original monochromatic frequency, which is called the Raman effect.

This shift provides information about vibrational, rotational and other low frequency transitions in molecules. Raman spectroscopy can be used to study solid, liquid and gaseous samples.

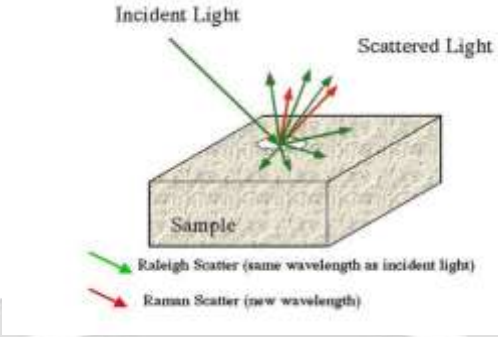


Fig -1 Principle of Raman spectroscopy

2.2 Origins of Raman-2

The Raman effect is based on molecular deformations in electric field E determined by molecular polarizability α. The laser beam can be considered as an oscillating electromagnetic wave with electrical vector E. Upon interaction with the sample it induces electric dipole moment P=αE which deforms molecules. Because of periodical deformation, molecules start vibrating with characteristic frequency νm.

When the scattered photon has same energy as the incident photon, the process is referred to as elastic scattering which is the Rayleigh Scattering.

When the scattered photon has less energy than the incident photons, the process is referred to as Raman Stokes Scattering.

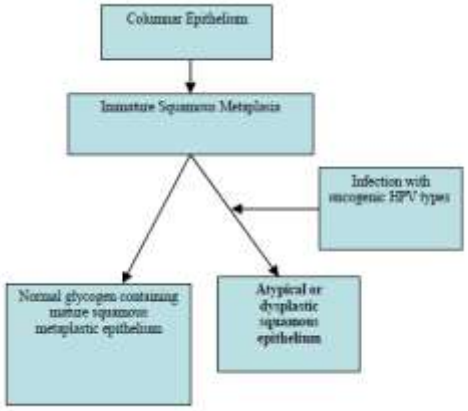
When the scattered photon has more energy than the incident photons, the process is referred to as Raman anti-Stokes Scattering.

About 99.999% of all incident photons in spontaneous Raman undergo elastic Rayleigh scattering. This type of signal is useless for practical purposes of molecular characterization. Only about 0.001% of the incident light produces inelastic Raman signal with frequencies higher or lower frequencies. Spontaneous Raman Scattering is very weak and special measures should be taken to distinguish it from the predominant Rayleigh scattering. Filters such as edge filter or notch filters are used to reduce Rayleigh scattering.

2. Cervical Cancer-2

The cervix is located at the lower part of the uterus that opens into the vagina. The cervix measures 3-4 cm in length and is approximately 2.5cm in diameter. There are two different types of cells that can grow on the cervix, either columnar or squamous.The size and shape of the cervix varies depending on age, parity and hormonal status,a typical normal cervix. The cervix is covered by two types of epithelia: the multi-layered squamous epithelium covers most of the ectocervix and is separated from the stroma by the basal layer. The columnar epithelium consists of a single layer of columnar cells, and covers the surface of the endocervical canal. The ectocervix consists of 15-20 layers of cells, has a large amount of glycogen, very few nerve endings and is typically pale pink in color. The endocervix on the other hand, is a single cell layer thick, has extensive sensory nerve endings, very little glycogen content and usually appears reddish in color.

The interface of the two epithelia is called the squamous-columnar junction. Overtime, the columnar epithelium is replaced by squamous epithelium, which causes the squamous-columnar junction to move towards the opening of the cervix called the os. This transitional epithelium is called squamous metaplasia. [2] Squamous metaplasia isan irreversible process shown in figure 2.1. The transformation zone (TZ) is the region where the columnar epithelium has been replaced or is currently being replaced by the new squamous epithelium. In younger women, the transformation zone is visible but as a woman ages the cervix shrinks due to decreased estrogen levels, the transformation zone may only be partially or not at all visible. This region (transformation zone) is where most new cervical dysplasia occurs. Cervical Disease and Progression Cervical



intraepithelial neoplasia (CIN) refers to the development of Neoplasia arising from the epithelium of the cervix. CIN refers to the precancerous stages of cervical carcinoma and is often also referred to as cervical dysplasia or

Fig-2 Progression of Columnar Epithelium to Squamous Epithelium

squamous intraepithelial lesion (SIL). Precancers may be categorized as mild, moderate and severe precancers (or dysplasia).The next step in the progression of this disease is carcinoma-in-situ (CIS) which is one step before the transformation of the dysplasia (precancer) to cancer. Clinically, cervical lesions can be divided into low grade lesions (mild precancers) and high grade lesions (moderate and severe precancers and CIS).

It has been observed that some cases of dysplasia regress and return to normal while other cases persist and develop into CIS and potentially cancer. The Fig.2 Progression of Columnar Epithelium to Squamous Epithelium distinction between high and low grade is important because patients with low grade lesions are typically followed but not treated whereas patients with high grade lesions are usually treated immediately with extended

Human papilloma viruses (HPV) are viruses that predominantly infect skin and mucosal membranes and produce characteristic epithelial proliferation, which may undergo malignant transformations. Most HPV infections have no symptoms and go away over the course of a few years. Similarities observed in the morphological changes of the epithelial cells between those induced by HPV and precancers has led to the suggestion that certain strains of HPV may be involved in the early stages of cervical precancer and other strains may aid in the progression of the disease.

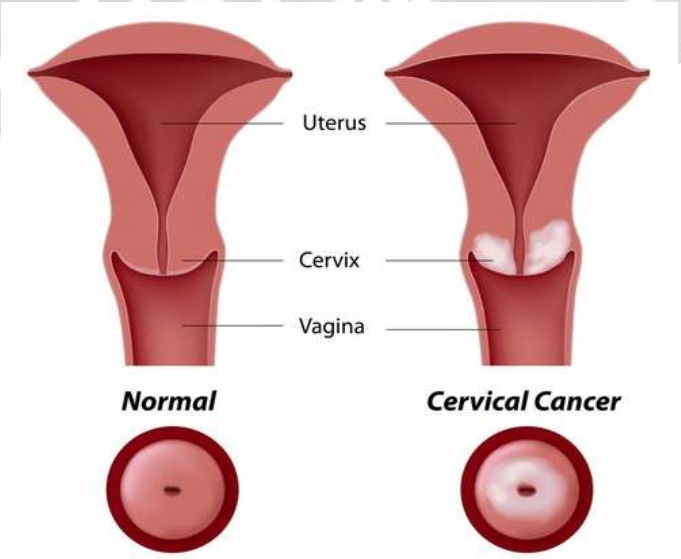


Fig 3 Normal Vs Cervical cancer

2.1Types of Cervical Cancer

The two types of cervical cancer are squamous cell carcinoma and adenocarcinoma, which are distinguished based on their appearance under a microscope. Both squamous cell and adenocarcinoma begin in the cells that line hollow organs, but squamous cells have a thin, flat appearance while adenocarcinoma involve cells with secretory functions. Squamous cell carcinoma is far more common and makes up approximately 90% of cervical carcinoma cases. Both types have similar risk factors and treatments.

2.2 Precancerous cervical lesions

A regular Pap test can detect a precancerous cervical lesion so that it can be removed before it becomes cancer. Pap test takes a sample of cervical cells for examination in a lab. If the results of this cervical cancer screening test are abnormal, then it may have changes in the cells of cervix that could indicate a precancerous cervical lesion.A precancerous cervical lesion, which is also called an intraepithelial lesion, is an abnormality in the cells of cervix that could eventually develop into cervical cancer.There are two main types of cervical cells, squamous and glandular and abnormalities can occur in either type. The most common types of precancerous cervical lesions include:Atypical squamous cells: It means that the abnormalities have been detected in the squamous cells, of cervix. This indicates that have been caused by a Human Papillomavirus (HPV) infection.

2.2 Squamous intraepithelial lesion (SIL)

This lesion means there is changes in the cervix that may be precancerous. SIL lesions are classified as either low-grade (LSIL) or high-grade(HSIL),with high-grade lesions being more likely to progress to cervical cancer.

2.3 Atypical glandular cells:

This indicates that a possible precancerous lesion in the upper area of the cervix or inside the uterus. Cervical cancer is the most common cancer affecting women in India and accounted for 17% of female cancer deaths. Cervical cancer is the leading cancer among women in terms of incidence rates in 2 out of the 12 in

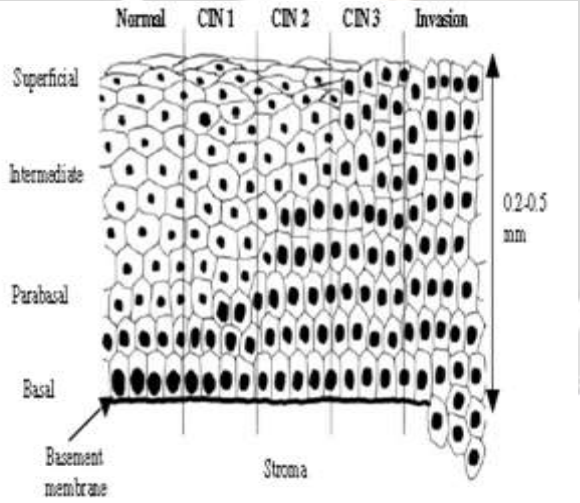


Fig 3 Structure of epithelium

India. One woman dies of cervical cancer every 7 minutes in India as of 2010. While pap smears is the most common screening method for early diagnosis but has an accuracy of 40-60% only.

The patient is followed by colposcopic examination, biopsy and histopathological diagnosis for all the abnormal Pap smear. All these procedures give valuable clinical information. But it is a time consuming process, subjective and not real time.. The Papanicolaou test is a screening test used to detect cancer and pre-cancer in the general female population but it is widely acknowledged that sensitivity values for this test are low.The aim of this study was to investigate the potential of Raman spectroscopy to detect biochemical changes in cervical

Epithelium consists of Basal, Parabasal, Intermediate, and Superficial cells. The above figure shows the changes in tissue structure when comparing to the normal and various stages of Cervical Intraepithelial Neoplasia. The Cervical Intraepithelial Neoplasia1 (CIN1) shows the changes in Basal and Parabasal. The stage2 (i.e.) Cervical Intraepithelial Neoplasia2 (CIN2)

shows the changes in Intermediate cells also. Whereas, in stage3 the changes can be seen in superficial cells. CIN1 is mild dysplasia it can be treated.

Changes in tissue structure associated with the progression of CIN in cervical squamous epithelium low risk to become invasive and CIN2 is moderate dysplasia and it can also be treated. But CIN3 is very risky dysplasia. This stage cannot be treated because its very invasive .Even it extends to Stroma, which has collagen devoid of endometrial glands. Stromal layer contains fibroblasts, inflammatory cells and capillary beds.

3.MATERIALS AND METHODS

The tissue samples were obtained from Aringar Anna Cancer hospital, Karapettai, Kancheepuram. These samples were fixed in 10% formalin solution. Prior to acquiring the raman spectra, these samples were washed meticulously in normal saline solution and was immersed in saline for 30 minutes to reduce the influence of formalin.The Instruments LabramHR 800, Raman spectroscopic confocal microscope was used, with a Diode laser operating at an excitation wavelength of 785nm. A 50X long working distance objective lens was used. The laser power at the sample was measured and found to be 13.3±0.05mW. A 60 second accumulation time was used for the various tissues. The Raman system is equipped with peltier cooled CCD camera with a spectral region of 200-1050nm. The grating used was 600gr/mm blazed at 500nm. A total of 4 spectra were recorded from different spots on each sample. Signals were recorded in the range from 300cm-1 to 1800cm-1. Selected spectra were baseline corrected, dark current subtracted and were smoothed using a 3 point moving average.

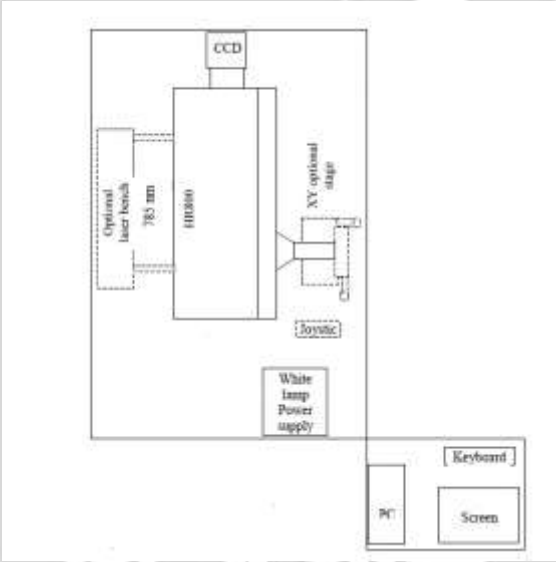


Fig 4 Schematic diagram of Raman spectroscopic technique

The diode laser of wavelength 785nm was used and this is outside the raman system and the beam is directed into the Raman system through a hole. This laser now passes through interference filter and enters a set of optics where neutral density filters can also be placed in the path of the beam to reduce the intensity of the beam. The beam is then directed to the microscope and thus is incident on the sample placed on the XYZ stage of the microscope. The same microscope collects the back scattered photons. The grating disperses the photons and the edge filter filters out the Rayleigh scattered photons. This beam is then directed to the CCD camera.

Data pre-processing was carried out using Labspec 5 software. Then were imported to text format and further normalisation and averaging was done using Matlab 2009a.

4.RESULTS AND DISCUSSION

Figure 5 compares the Raman spectra collected from normal (2) and cancer (2) subjects. The spectra of invasive carcinoma also show characteristic nucleic acid bands. These include prominent bands at 1578 cm-1 and 1240 cm-1. Distinct bands were also seen at 1366 cm-1, 1482 cm-1 and a band at 1578 cm-1. The increased nucleic acid and protein bands are a result of the increased proliferation of these tumour cells. The amide 3 band is well pronounced in normal cervical samples when compared to cervical cancer samples. The amide 1 vibration of collagen at 1662cm-1 is of higher intensity in both the normal samples when compared to the cancer samples.

5.STATISTICAL ANALYSIS

Inorder to use spectral information for diagnosis, a mathematical algorithm or series of algorithms must be developed to extract clinically relevant information from the spectra and calculate a score or set of scores to classify the given spectrum into a category based on a predetermined set of criteria. Classification of the spectra in the test set determines the unbiased accuracy of the algorithm.

The first step in using Raman spectra is to develop a basic algorithm to discriminate between abnormal and normal tissues. First, the mean and standard deviation at each wave number of the spectra within each pathology group was calculated to characterize the overall spectral trends for each group. A students t-test was performed at selected wave numbers between individual pairs of pathology groups to identify regions of spectral distinction between two different pathologies. Any major peak that showed statistical differences at the level of $p < 0.001$ between normal ectocervix spectra and high-grade dyplasia spectra was chosen as an input for the algorithm.

Thus, the inputs to the algorithm are the normalized intensity values at 1240cm^{-1}, 1318cm^{-1},1364cm^{-1}, 1450cm^{-1},1482cm^{-1},1553cm^{-1},1662cm^{-1}.The classification model was constructed to automatically classify spectra into one of two categories (high-grade dysplasia or benign cervix) using a student 1- test model. This algorithm was developed to distinguish normal pathology from cancer, however at 1482cm-1 did not yield a valid p value. The rest of the wave numbers yielded a p value of <0.001.

The results show clearly that Raman spectroscopy can be used for identification and discrimination of normal and abnormal cervical cells. Raman spectroscopy shows enormous clinical potential for cervical cancer screening.

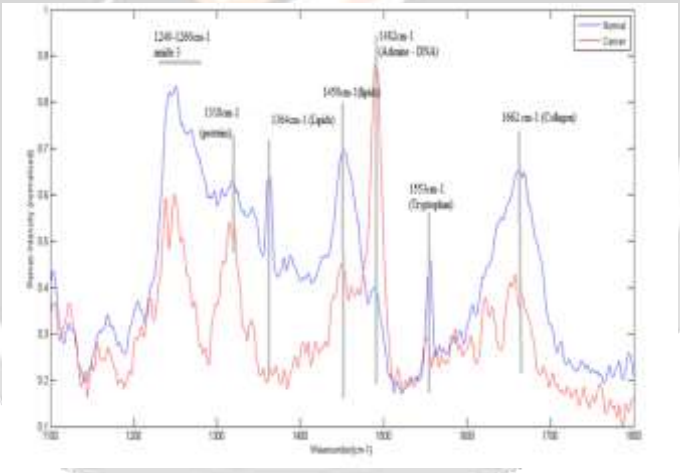


Fig 5 Raman spectra of normal and cervical cancer tissue

Wave number (cm-1)	Raman Vibrational modes
1106	DNA O-P-O backbone stretch (1101)
1176	Cytosine/guanine/adenine
1240-1290	Amide3 deformation
1318	CH_2 deformation in lipids/adenine/cytosine/amide3
1339	Polynucleotide chain(DNA/RNA)
1364	C-C skeletal stretch of lipids
1450	CH_2 deformation in lipids
1482	Adenine
1553	Tryptophan
1630-1665	Amide 1 stretching
1662	Amide 1 stretching of collagen

Table-1 Wave number of Raman vibrational modes

6.CONCLUSION:

In this study, we have analyzed two normal and two cervical cancer tissues and it shows significant changes between normal and cancer samples. Spectral features like broad amide 1 and peaks at 1662cm^{-1} characteristic of collagen are present in normal samples. The cancer samples have well defined DNA peaks at 1318 cm^{-1}, 1339cm^{-1} and 1482cm^{-1}. Hence the present study validates Raman spectroscopic method for cervical cancer diagnosis.

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