RAMAN SPECTROSCOPY: NON-INVASIVE TECHNIQUE FOR CANCER DETECTION

By

SAMINA JAVAID

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

(2016)

Raman spectroscopy
Semiconductors.
Raman effect

RAMAN SPECTROSCOPY: NON-INVASIVE TECHNIQUE FOR CANCER DETECTION

SAMINA JAVAID

Reg. No. 126-FBAS/MSBT/F14

Supervisor	Co-Supervisor
Dr. Shaheen Shahzad	Dr. Mushtaq Ahmad
Assistant Professor	Director (NILOP)

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University Islamabad

(2016)

Department of Bioinformatics and Biotechnology
International Islamic University Islamabad

Dated: 10-8-2016

FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Samina Javaid and it is our judgement that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

COMMITTEE

External Examiner

Dr. Rabia Habib

Assistant Professor

COMSATS, Islamabad

Internal Examiner

Dr. Asma Gul

Assistant Professor

International Islamic University, Islamabad

Supervisor

Dr. Shaheen Shehzad

Assistant Professor

International Islamic University, Islamabad

Co-Supervisor

Dr. Mushtaq Ahmad

Director

NILOP

Head of Department

Dr. Naveeda Riaz

Associate Professor

International Islamic University, Islamabad

Dean FBAS

Dr. Muhammad Sher

A thesis submitted to Department of Bioinformatics and Biotechnology,

International Islamic University, Islamabad

as a partial fulfilment of requirement for the award of the degree of

MS in Biotechnology

DEDICATION

Special dedication to

The Lord of the Lords; Allah the Almighty and His beloved Prophet

(PBUH)

My parents for their unconditional support at every step

My supervisor for being so humble and supportive in tough times

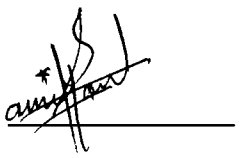
And specially to

My Co-supervisor a true mentor for me.

DECLARATION

I hereby declare that the present work in the following thesis is my own effort except, where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 10-8-2016



Samina Javaid

126-FBAS/MSBT-F14

CONTENTS

ACKNOWLEDGMENTS..... i

LIST OF ABBREVIATIONS..... ii

LIST OF FIGURES..... v

LIST OF TABLES..... vi

ABSTRACT..... vii

1.INTRODUCTION.....**1**

2.LITERATURE REVIEW.....**6**

2.1 Mechanism of cancer formation 6

2.2 Types of cancer.....7

2.2.1 Head & Neck Cancer.....8

2.2.1.1 Global incidence of HNC.....8

2.2.1.2 Risk factors.....9

2.2.1.3 Diagnostic methods for HNC.....9

2.3 Different optical tools used for differential diagnosis of cancer.....11

2.3.1 Spectroscopy.....11

2.3.2 Scattering process.....12

2.3.3 Raman Spectroscopy.....13

2.3.3.1 Basic Principle.....15

2.4 Raman Instrumentation 16

2.4.1 Excitation source.....18

2.4.2 Sample holder and light collection system.....18

2.4.3 Detection System.....18

2.5 Analytical techniques for Raman data collection.....19

2.5.1 Feature extraction.....20

2.5.2 Principle Component Analysis (PCA).....20

2.5.2.1 Aims of PCA.....21

2.6 Clinical applications of Raman Spectroscopy.....21

3. MATERIALS AND METHODS.....24

3.1 Study Approval.....24

3.2 Blood Samples 24

3.3 Subjects and protocol.....24

3.4 Serum preparation.....24

3.5 Materials.....25

3.6 Methods.....25

3.6.1 Optimization for seeking spectra.....25

3.6.2 Optimization of integration time.....25

3.6.3 Optimization of laser power.....25

3.6.4 Optimizing the focusing lens.....26

3.6.5 Choosing the surface and shape.....26

3.6.6 Seeking the spectra.....26

3.6.7 Spectral processing.....26

3.6.7.1 Background removal.....27

3.6.7.2 Substrate removal.....27

3.6.7.3 Baseline correction.....27

3.6.8 Analysis and plotting.....27

3.6.9 Acquiring spectra.....28

3.6.10 Statistical Analysis.....28

4. RESULTS.....29

4.1 Data pre-processing.....29

4.2 Raman Spectral Analysis.....31

4.3 Correlation Calculations.....33

4.4 Principle Component Analysis.....35

4.5 Data Classification and Analysis.....35

4.6 Statistical analysis of clinical and demographic parameters.....35

5. DISCUSSION.....39

6. REFERENCES.....44

ACKNOWLEDGMENTS

In the name of "Allah", the most gracious, the most merciful, the one and the only, the creator, "who is perfect in all characteristics, worthy of worship. All praises for Allah who guides us when we bewilder in darkness of ignorance and helps us in enlightening our ways". Coutnless salutaion in the city of knowledge Holy Prophet Muhammad (Peace Be Upon Him), who declared it an obligatory duty of every Muslim to seek and acquire knowledge.

I wish to express my warmest, sincerest thanks and profound gratitude to my supervisor Dr. Shaheen Shahzad, Assistant Professor (IIUI) it was because of her guidance, encouragement and dynamic supervision throughout research.

I am also thankful to my co-supervisor Dr.Mushtaq Ahmad, Director NILOP for facilitating and providing continuous guidance.

I wish to express my deepest gratitude to Dr. Saranjam Khan, Principle Scientist and Dr. Rahat, Principle Scientist, NILOP for their invaluable assistance, support and guidance to complete this research work.

I am also thankful to Dr. Farwa Narjis, Senior Scientist, NILOP for facilitating during the start of research.

I would like to acknowledge staff of Oncology Department of NORI hospital specially Dr. Humaira for facilitating regarding blood samples of cancer patients. Special thanks to all the affected families for their cooperation in the research work, May Allah relieve their pain.

I am extremely thankful to Dr.Naveeda Riaz, Chairperson of the Department of Bioinformatics and Biotechnology for her facilitation and support throughout the research.

It would have been impossible for me to accomplish this study without the incredible support of my family, my very special thanks to my parents, brothers and sisters for making me what I am today. And my special gratitude goes to my Mother who always supported me throughout my studies. Infact, it is the result of her love, affection and encouragement that kept me going. I cannot thank them enough for what they have done throughout my entire life. Last but not the least I am sincerely thankful to my friends for their encouragement and nice company.

SAMINA JAVAID

Chapter 1

INTRODUCTION

Introduction

Cancer is the second leading cause of death in economically developed countries (WHO, 2008). It is a group of diseases that cause body cells to change and grow uncontrollably. There are different types of cancer which all start with uncontrollable growth and instead of dying start proliferating to form new abnormal cells. Cancer cell possess uncontrolled growth and invades other tissues. (Abeloff et al., 2008).

Tumors are either benign or malignant. Benign tumors can't invade or metastasize to other tissues of the body and never life threatening (Wolff et al., 2008). Malignant word comes from Latin which means "born to be bad" this implies that cancer is unavoidable production of cells and fated to death (Pories et al., 2009). It contain cells that have several aberrant properties like uncontrolled proliferation, resistance to cell death signals, inappropriate migratory and invasive capability, capacity to alter the tissue microenvironment to promote angiogenesis and evasion of immune surveillance (Rodier et al., 2007).

Cancerous cells are unable to perform regular function and spread to nearby organs or to whole body by a process known as metastasis which leads them towards death. Transformation of normal cell into malignant occurs in different stages. These are biological (parasites,bacteria,viruses), physical (ultraviolet radiation) and chemical carcinogens (arsenic, aflatoxin) (Stewart and Wild, 2014). DNA is the genetic material in all living organisms and in every cell directs all its actions. Whenever DNA gets damaged in a normal cell, the cell repairs the damage. In cancer cells, damaged DNA is not repaired and start proliferating abnormally. Like first cell all new cells will have same damaged DNA (Lichter et al., 2008). The production of DNA lesions and the resulting activation of DNA damage response (DDR) pathways are both pretentious by the chromatin status at the site of damaged DNA. As a result, DDR activation affects the chromatin both at the damaged site and across the whole genome.

Cellular senescence and cancer are linked with the arrangement of the DDR pathways and chromatin changes (Sulli et al., 2012). Other mechanisms involved in the carcinogenesis process is loss of tumor suppressor gene function, which normally acts as a negative regulator of cell proliferation and conferring certain advantages to growth that lead to tumor progression (Caldeira et al., 2006).

Generally cancers are the result from the accumulation of multiple clonal changes in genes that regulate cell growth and differentiation (Osborne et al., 2004). Since tumors with similar histopathological diagnosis can follow different clinical courses and show different responses to therapy. One of the leading causes of morbidity and mortality worldwide also includes cancer (Shackney et al., 2003; Beckmann et al., 1997).

There are over 10 million new cases of cancer annually (4.7 million in developed countries; 5.5 million in less developed countries) and more than 6 million cancer deaths. It is estimated that there will be 15 million new cancer cases every year by 2020, and 10 million cancer deaths (Journal of cancer prevention, 2004). In the US about 575000 people die because of cancer and more than 1.5 million are diagonosed with cancer. The incidence rates for all cancers combined were 80.5 per 100,000 for males and 91.8 per 100,000 for females (Bhurgri, 2006). World Health Organization (WHO) has stated that cancer status will increase by 70 % in the next 20 years.

Ageing is an important risk factor for the development of cancer. Other risk factors may include unhealthy diet, alcohol, drugs, low physical activity, tobacco, genetic and non-genetic factors. Cancer is result of interaction between person's genetics and cancer causing agents (WHO, 2012). The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well. In addition to inherited mutations, single nucleotide polymorphisms (SNPs) of various genes are defined to be related with cancer risk and disease progression as well as the treatment response.

Cancer is classified on the basis of cell affected first and now there are more than 100 different types of cancer. Lung, prostate, colon, rectum, stomach, liver, breast, colon, rectum, lung, cervix and stomach are most affected areas with cancer (Bhurgri, 2004).

Head and neck cancer is the second leading cause of cancer in Pakistan. It is also due to the abnormal growth of cells in the regions like Oral cavity, nasal cavity, paranasal sinuses, larynx, trachea, hypopharynx, nasopharynx and oropharynx (Masood et al., 2012). Approximately 42 % of head & neck cancer are due to factors like smoking, tobacco, alcohol consumption and human papilloma virus (Cmelak, 2012).

Colorectal cancer is a major cause of morbidity and mortality and the third leading cause of cancer related deaths in the United States (American Cancer Society, 2008). Worldwide, 1.2

million new colorectal cancer cases and 609,000 deaths were expected to occur in 2008 (Parkin et al., 2000). Major risk factors for CRC are obesity, diet with high rate of fats and low vegetables, non physical activity and smoking (Fernandez et al., 2002; Hjartaker et al., 2013; Robsahm et al., 2013). Evidence showed that CRC is a genetic disease and 10- 15 % of CRC cases have genetic cause so that one out of 200 individuals has genetic high risk allele for CRC (Fernandez et al., 2002; Turati et al., 2013; Han et al., 2014).

Breast cancer is the most common cancer among American women, except for skin cancers. About 1 in 8 (12%) women in the US will develop invasive breast cancer during their lifetime. The American Cancer Society's estimates for breast cancer in the United States are for 2013; About 232,340 new cases of invasive breast cancer will be diagnosed in women. About 64,640 new cases of carcinoma in situ (CIS) will be diagnosed and about 39,620 deaths of women are expected from breast cancer (Breast Cancer Facts and Figures 2011-2012).

Liver cancer in men is the fifth most frequently diagnosed cancer worldwide. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death. An estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008 (Armstrong et al., 2006). Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males, accounting for 14% of the total new cancer cases and 6% of the total cancer deaths in males (Ferlay et al., 2010).

Doctors and scientists are always looking for better ways to treat people with cancer. To do this, they are constantly developing and studying new techniques. Actually, as with all kinds of cancers, the early detection of cancer is fundamental for the fast and successful treatment of the disease. Despite these interventions, more than 50% of patients with cancer will experience local relapse and distant metastasis. Recurrences and distant metastases are associated with poorer prognosis (Khuri et al., 2000). Furthermore, surgical intervention causes facial contour defects and can lead to functional impairment and psychological trauma in cancer patients. This is disappointing and points to the need for novel therapeutic approaches. International public health agencies and private and government donors can play significant roles in strengthening existing cancer control programs and/or implementing new programs to arrest the growing burden of cancer in economically developing countries (Anderson et al., 2008).

There has been some slight progress recently with recent studies showing a decrease in cancer incidence of 1.7% during the recent period of 2001–2005; and some cancers such as breast cancer have shown a reduction in cancer related mortality over the last 10 years.

Significant development has been observed in computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) in recent decades. To decrease cancer related difficulties and deaths much more development is needed in the areas of diagnosis and treatment. Advancement in imaging technologies, radiation therapy, chemotherapy and surgical procedures is needed (Keereweer et al., 2011). Early neoplasia or subtle lesions cannot be identified with conventional screening methods. Multiple suspicious lesions cannot be diagnosed with excisional biopsies because this method is invasive and impractical. Greater cost and inconvenience has been observed in case of colonoscopy screening (McLoughlin and Morain, 2006).

Identification at different stages of cancer in tissue and for differentiation of cancerous and normal tissue, a nondestructive diagnostic tool named as Raman spectroscopy has emerged. This is being used for identification of cancer at various body sites like cervix, bladder, stomach, parathyroid, lung, prostate and breast (Utzinger et al., 2001).

Raman spectroscopy maybe preferred over other techniques since, it is noninvasive and requires no complex instrumentation or sample preparation. Since the power and wavelength of the laser required in Raman spectroscopy as an excitation source do not cause injury to patients, therefore, it is preferred for in vivo analysis. Raman spectroscopy have been used in biomedical analysis of tissue, serum and plasma samples to confirm the presence of macromolecules including proteins, nucleic acid, and lipids to assess several diseases including Atherosclerosis, Diabetes, and Alzheimer's in addition to cancer (Ellis et al., 2013).

The purpose of the present study is to use Raman spectroscopy combined with multivariate analysis as a screening method to detect Head and Neck cancer in early stages. The difference between the control and cancerous blood samples were assessed by recording the spectra from Raman spectrometer with laser excitation wavelength 532 nm. The spectra from cancerous blood samples were then compared with the spectra from control samples with and without using multivariate statistical tool i.e. Principle Component Analysis (PCA) and Linear Discrimination Analysis (LDA).

The main objectives of the current research are:

- Plasma screening of Head and Neck cancer using Raman shifts produced by Raman spectroscopy in selected Pakistani patients.
- Analysis of changes in molecular and chemical composition of molecules in the sample.
- Correlation of biological molecules in diseased and control samples.

Chapter 2

LITERATURE REVIEW

Literature review

Cancer is a potentially lethal disease that poses a major challenge to longevity in organisms with renewable tissues. Cancers, or malignant tumors, contain cells that have acquired several aberrant properties, often by somatic mutation or epigenetic changes in gene expression. These properties include uncontrolled proliferation, resistance to cell death signals, inappropriate migratory and invasive capability, and the capacity to alter the tissue microenvironment to promote angiogenesis and evasion of immune surveillance. A number of mammalian genes functions to prevent the development of cancer, some of which are directly implicated in longevity assurance (Rodier et al., 2007).

Most types of cancer cells eventually form a lump or mass called a tumor, and are named after the part of the body where the tumor originates (American Cancer Society, 2011). Not all tumors are cancerous. Tumors that aren't cancer are called benign. Benign tumors can cause problems – they can grow very large and press on healthy organs and tissues. But they cannot grow into (invade) other tissues. Because they can't invade, they also can't spread to other parts of the body (metastasize). These tumors are almost never life threatening (Wolff et al., 2008).

2.1 Mechanism of Cancer Formation

Cancer is a complex disease that is very variable in its presentation, development and outcome from one patient to the other. The same heterogeneity and variability exist at the cellular and molecular level. Cancer is a multi-step process during which cells undergo profound metabolic and behavioral changes, leading them to proliferate in an excessive and untimely way, to escape surveillance by the immune system, and ultimately to invade distant tissues to form metastases (Reid et al., 2006). It is now clear that there are thousands of point mutations, translocations, amplifications and deletions that may contribute to cancer development, and that the mutational range can differ even among histopathologically identical tumors (Cairns et al., 2011).

One of the mechanisms involved in the carcinogenesis process is loss of tumor suppressor gene function, which normally acts as a negative regulator of cell proliferation. Tumor suppressor gene inactivation contributes to carcinogenesis by conferring certain advantages to growth that lead to tumor progression (Caldeira et al., 2006).

Cells become cancer cells because of damage to DNA. DNA is in every cell and directs all its actions. In a normal cell, when DNA gets damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn't die like it should. Instead, this cell goes on making new cells that the body does not need. These new cells will all have the same damaged DNA as the first cell does (Lichter et al., 2008).

The generation of DNA lesions and the resulting activation of DNA damage response (DDR) pathways are both affected by the chromatin status at the site of damaged DNA. In turn, DDR activation affects the chromatin at both the damaged site and across the whole genome. Cellular senescence and cancer are associated with the engagement of the DDR pathways and with profound chromatin changes (Sulli et al., 2012).

Cancer cell attains an independent survival advantage by escaping the laws and rules governing cell life and becomes a rogue cell by adopting an aggressive and invasive behavior. These cells are so good at adapting themselves to new conditions that they resist many attempts at killing including cytotoxic drugs or radiation treatments. This is the reason that most cancers are best treated at an early stage, at a time when cancer cells still have limited adaptive capacity and are thus unable to bypass the effects of treatment (Weinberg et al., 2000).

2.2 Types of Cancer

There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. The incidence rates for all cancers combined were 80.5 per 100,000 for males and 91.8 per 100,000 for females (Bhurgri, 2006). Cancer continues to persist as one of the most common causes of death throughout the world and remains the second leading cause of death in the United States (Richardson et al., 2010). This rise in cost coincides with estimates

that 1,529,560 new cases of cancer were diagnosed, and 569,490 cancer-related deaths had occurred in 2010 in the US alone (Siegel et al., 2010).

According to the World Health Organization (WHO), the numbers of new cancer cases is expected to rise by about 70% over the next 20 years. The most common sites of cancer among men are lung, prostate, colon, rectum, stomach and liver and the most common sites of cancer among women are breast, colon, rectum, lung, cervix and stomach.

This section provides basic information on risk factors, symptoms, early detection, and treatment, as well as statistics on incidence, mortality, and survival, for the most commonly diagnosed cancers.

2.2.1 Head and Neck Cancer

Head and neck cancer (HNC) also result from abnormal growth of cells in the regions like oral cavity, nasal cavity, paranasal sinuses, larynx, trachea, hypopharynx, nasopharynx and oropharynx. HNC is sixth common cancer worldwide and in Pakistan it is the second leading cancer (Masood et al., 2012).

2.2.1.1 Global incidence of Head and Neck Cancer

Head and neck cancer is the 6th leading cancer in humans (Marcu et al., 2009). Under developed countries like India, Brazil and Thailand are most affected regions (Jemal et al., 2004). 75,000 head and neck cancer cases are reported and 3% of all cancers are head and neck cancer in USA. Frequency of larynx, hypopharynx, oropharyngeal mucosa, cervical esophagus and lip has been decreased. On the other hand frequency of cancers of nose, tongue, soft tissues, bone, tonsil, salivary and bone has been increasing (Bruce and Everett, 2011). Occurrence of cancers in the regions of oropharynx among females has been increased in Japan while cancers of tongue, hypopharynx and oropharynx have been increased in males (Akiko et al., 2005). Most prevalent form of head and neck cancers in India is oral cancer. Increased frequency of head and neck cancer in young adults was not found in India (Elango et al., 2006). In Pakistan there is a striking increase in HNC in males and females. In males increase in tongue cancer is observed while decrease in lip cancer. Disease onset at early age is evident in Pakistani

patients. 65 years and older patients' accounts for 23 % while 40 years and younger HNC patients account for 30 %. (Chaudhry et al., 2008).

2.2.1.2 Risk Factors

Smoking and tobacco products, alcohol use, and infection with human papilloma virus (HPV) are among the major risk factors for head and neck cancer; globally, smoking and alcohol have historically been aetiologies for approximately 42% of head and neck cancer. (Cmelak, 2012).

Malignant and benign oral lesions are also caused by human papillomavirus (HPV) (Gillison et al., 2000). Red chilli and meat are considered risk factors for head and neck cancer while dietry iron vegetables and fruits rich in vitamin C and A play protective role (Negri et al., 2000).

Many genes play role in altered pathways in head and neck cancer leading towards malignancy. Collectively these changes in tumor suppressor genes, oncogenes and proto oncogenes can lead towards cancer. Protein expression can decrease and increase due to genetic and epigenetic changes. Genes being altered in head and neck cancer pathways can serve as therapeutic targets e.g. signal transducer and activator of transcription 3 (STAT3), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and p53 (Jonah and Jennifer, 2010).

2.2.1.3 Diagnostic Methods for Head and Neck Cancer

- **Fine-Needle Aspiration Biopsy**

For this test mostly fine or thin needles are used (22 to 27 guage). The FNA biopsy technique uses aspiration to obtain cells or fluid from a mass as compared to large needle biopsy,that obtain tissue specimen and requires histologic fixation (Gharib et al., 2007). The needle is passed into the lesion and multiple fast jabbing movements in and out of the lesion as well as in different directions are performed. Once the material is seen in the hub of the needle, there is usually sufficient material (Kulkarni et al., 2002).

- **Computed Tomography**

CT was developed by Hounsfield et al in 1967, driven in part by the improvement in computer processing power along with volumetric x-ray imaging (Saif et al., 2008). Using available computing technology, CT generated high resolution three dimensional reconstructions of patient anatomy using multi-plane x-ray imaging and mathematical computer image reconstruction algorithms. Non-specific iodinated contrast agents are routinely used to visualize blood flow and washout, often delineating areas of abnormal enhancement and uptake which can be used as a surrogate for neoplasm or infection/inflammation (Sharma et al., 2005).Despite the exquisite anatomic details CT provides, it imparts little to no information regarding functional or metabolic activity of tissues.

Malignant potential is construed from lesion size, and distortions and displacements of normal tissue. As a result, benign and malignant lesions can overlap in appearance (Sharma et al., 2005).Early metastatic lymph nodes are often too small to be accurately characterized by CT, resulting in frequent false negative results. Post-surgical changes can distort normal anatomy and may change the structure and morphology of the anatomy sufficiently to mask remaining tumor; these findings on CT scan incorrectly suggest areas of abnormal growth or disguise areas of true malignant disease (Delbeke et al., 2009).

- **MRI**

MRI is an accurate test for the diagnosis of Nasopharyngyeal cancer. MRI depicts subclinical cancers missed at endoscopy and endoscopic biopsy and identifies patients who do not have NPC and who therefore do not need to undergo invasive sampling biopsies (Bhatia et al., 2011). NPCs usually present with intermediate signal intensity, higher than the muscle signal, on T2-weighted images, low signal intensity on T1- weighted images, and enhance to a lesser degree than does normal mucosa. Eighty-two percent of NPCs arise in the posterolateral recess of the pharyngeal wall (Rosenmüller fossa), and 12% arise in the midline. In 6–10% of patients, the nasopharyngeal mucosa appears normal at endoscopy (Glastonbury, 2007).

2.3 Different optical tools used for differential diagnosis of cancer

There has been some slight progress recently with recent studies showing a decrease in cancer incidence of 1.7% during the recent period of 2001–2005; and some cancers such as breast cancer have shown a reduction in cancer related mortality over the last 10 years. However, much more progress is needed to improve diagnosis and treatment of cancer in order to ultimately reduce cancer-related suffering and death. Research for cancer diagnosis and treatment has continued to yield advances in chemotherapy regimens, radiation therapy, surgical procedures, and imaging technologies. Biomedical imaging modalities such as Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), and Computed Tomography (CT) for cancer diagnosis have progressed significantly in recent decades (Keereweer et al., 2011).

Screening methods are available which can reduce the incidence by removal of adenomas and can reduce deaths in diagnosed cancer cases by earlier stage detection. However, there are many disadvantages in conventional cancer screening methods. For instance, colonoscopy screening method involves greater cost and inconvenience to the patient (whole bowel cleansing). Excisional biopsies currently remain the standard approach for cancer diagnosis, though this method is invasive and impractical for mass screening of high-risk patients with multiple suspicious lesions. In brief, using the conventional cancer screening tools it is difficult to identify early neoplasia or subtle lesions (McLoughlin and Morain, 2006).

2.3.1 Spectroscopy

Spectroscopy is basically the study of radiation interaction with the matter to perform an analysis. The matter can be solid or liquid which can either absorb, reflect or re-emit incident radiation. Initially it originates from the study of visible light when dispersed according to its wavelength by a prism. Later on, the concept was further generalized to interaction of all type radiation with matter as a function of wavelength or frequency (Harvey, 2010). The data that is obtained from spectroscopy is called a spectrum. A spectrum is a plot of the intensity of energy detected versus the wavelength of the energy. A spectrum can be used to obtain

information about atomic and molecular energy levels, molecular geometries, chemical bonds, interactions of molecules, and related processes (Pecsok et al., 1994).

There are many different types of spectroscopy depending on the nature of interaction and types of energy used. When radiations impinge upon sample, it will either be absorbed, pass through. Different types of interaction leads to various types spectroscopic techniques. Some of these techniques are absorption spectroscopy, fluorescence spectroscopy, Raman spectroscopy, and surface-enhanced Raman spectroscopy. All these techniques use laser light as an energy source. Since in current research Raman spectroscopy is used for early diagnosis of cancer. It is therefore necessary to understand the nature of interaction and more specifically in the scattering before describing the basic mechanism of Raman spectroscopy.

2.3.2 Scattering Process

As mentioned before that when the light interact with the material, it will absorb, reflect or scattered. Scattering refers to the change in the direction of light as a result of its interaction with matter. Scattering may or may not be associated with energy transfer. Various physical properties of a substance can be studied by the amount of light scattered at different angles, wavelengths and polarization angles by that substance.

There are three different types of scattering, i.e., Rayleigh scattering, anti-strokes and strokes scattering. Most of the radiations scattered from a sample comprised Rayleigh scattered radiations.

An interaction where the energy of the radiation remain same after an interaction with the matter is termed as Rayleigh or elastic scattering. In contrast a situation where energy is transferred either from the molecule to the photon or vice versa, the scattered photon has less or more energy than that of incident photon. Such scattering is called inelastic scattering. There two of types of inelastic scattering i.e. Stokes and Anti-Stokes. (Zeng et al., 2003).

An inelastically scattered photons can be either of higher or lower energy, depending upon the vibrational state of the target molecule. If the scattered photon has higher wavelength, i.e., lower energy than the incident light, this is known as Stokes-Radiation. Unlike Stokes an anti-

Stokes radiation have high energy and lower wavelength than the incident radiation. This decrease or increase in the energy is directly related to the initial vibrational energy levels in the ground state of the molecule. The difference in the initial and final energy of the photon is known as Raman shift. Raman shift (wave numbers) is expressed in unit of cm−1. This Raman shift provides a finger prints from the molecular composition of contributing molecules can be determined. The schematic diagram of different types of scattering is illustrated in Figure 2.1 (Ferraro et al., 2003).

2.3.3 Raman Spectroscopy

Raman spectroscopy was first observed in 1928 by Sir Chandrasekhara Venkata Raman, an Indian physicist, who received the Nobel Prize 2 years later for work in the field of light scattering (Gardiner, 1989). Raman spectroscopy, a molecular and chemical detective, can be used to optically probe the molecular changes associated with diseased tissues (Singh et al., 1998).

Raman spectroscopy is a promising technique in biomedical studies due to its non-invasive character and high specificity. It is based on inelastic scattering of monochromatic light, usually from a laser source by the sample. In addition to provide unique finger print information about a sample, Raman spectroscopy offers several advantages than other techniques including:

- Sample preparation is very easy and can be prepared directly through glass containers
- Non-destructive technique and requires minimum interference of water
- No interference from atmospheric parameters such as carbon dioxide and water (Itzkan et al., 1999).

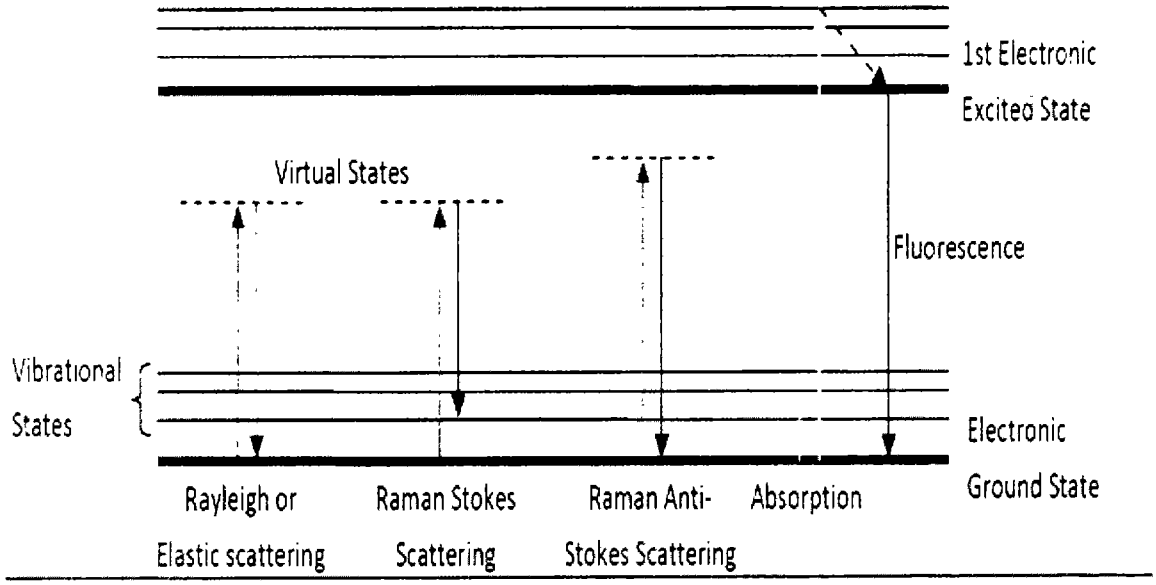


Fig. 2.1 Energy level diagram for Rayleigh and Raman scattering (Ferraro et al., 2003).

2.3.3.1 Basic Principal

When light is scattered from a molecule, most photons are elastically scattered. The scattered photons, therefore, have the same energy (frequency) and wavelength as the incident photons. However, a small fraction of light (approximately 1 in 10^7 photons) is scattered at optical frequencies different from, and usually lower than, the frequency of the incident photons. The process which leads to this inelastic scatter is termed the Raman Effect. Raman scattering can occur due to a change in vibrational, rotational or electronic energy of a molecule. If $\lambda_{incident}$ is the wavelength of the incident photon and $\lambda_{scattered}$ is the wavelength of the scattered photon. The mathematically the energy difference between the initial and final vibrational levels, which termed as Raman shift ($\bar{\upsilon}$), can be calculated as

$$\bar{\upsilon} = 1/\lambda_{incident} - 1/\lambda_{scattered} \quad \ldots\ldots\ldots \quad (1)$$

The relative intensities of Raman signal depends on the number of the molecule in different states. Inside sample there are different types of molecules. For example in biological sample (body fluids) there are lot of Raman active molecules such as proteins, lipids etc. Each of these molecules give rise a specific peak or peaks in the Raman spectrum. Sometime different molecules contribute to the same peak. In such a situation other peaks is alos considered for the exact molecular assignment. The peak height in the spectrum represents the relative concentration of that particular type of molecule in the sample. The intensity of Raman signal is very weak as compared to (Durdevic et al., 2013).

Classically Raman Effect can be explained on the basis of polarizability of a molecule. When a molecule is placed in a static electric field, the electric field polarizes the molecule by attracting the positive charged nucleus towards the negative pole and negatively charged electrons cloud towards the positive pole. This separation of charged centers an induced dipole moment is directly related to the applied electric field as long as the field is not too strong;

$$P = \alpha . E \ldots\ldots\ldots\ldots (2)$$

Where α is the polarizability of the molecule that decreases with increasing electron density, increasing bond strength and decreasing bond length.

Light is considered as electromagnetic radiation, which contains an oscillating electric field component. When light is impinged on a sample, the electric field component of the incident polarizes the molecules in the sample and they behave as an oscillating dipole, the oscillating dipole is the source of different types of scattering signals including Raman signals;

$$E = E_o \cos(\omega t) \ldots\ldots\ldots\ldots (3)$$

$$P = \alpha E_o \cos(\omega t) \ldots\ldots\ldots\ldots\ldots (4)$$

The Raman Effect arises when a photon is incident on a molecule and interacts with the electric dipole of the molecule. In quantum mechanics, the scattering is described as an excitation to a virtual state lower in energy than a real electronic transition with nearly coincident de-excitation and a change in vibrational energy. The scattering event occurs in 10-14 seconds or less. When the sample is de-excited to the ground state, a Raman signal is emitted with a shift in wavelength (Derek, 2002).

2.4 Raman Instrumentation

All Raman spectrometer consists of three main components (Motz, 2012):

- Excitation source, usually a laser
- Sample holder and light collection system
- Detection and computer control system

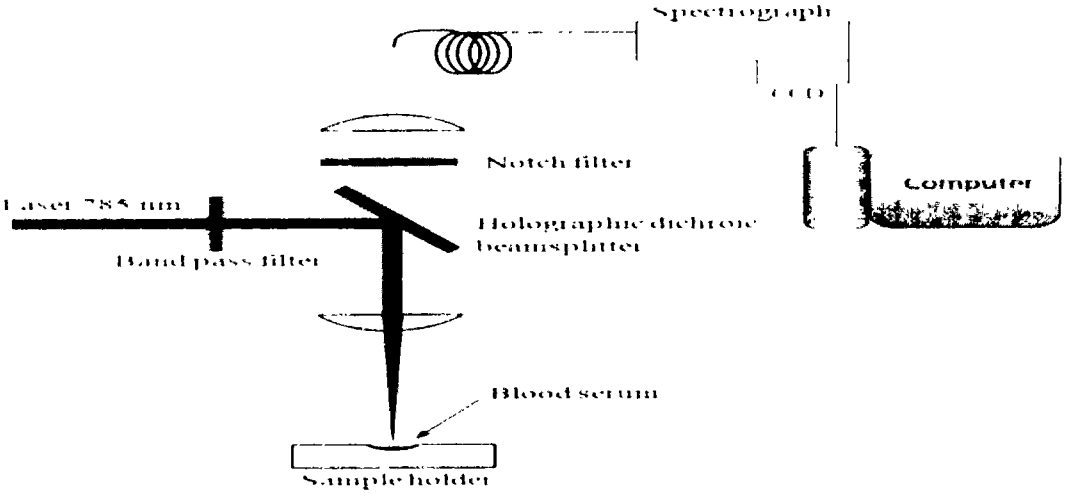


Fig. 2.2 Raman Spectrometer

2.4.1 Excitation Source

Raman spectrometer consists of excitation source for sample illumination. All modern system are using laser as an excitation source. Similarly it is also necessary for the laser to have sharp bandwidth and stable frequency. Three different wavelength are normally used, i.e. 532, 785 and 1064 nm. Each wavelength has its own advantages and disadvantages. For example shorter wavelength produces a powerful Raman signal but have the problem of fluorescence in case of organic molecules. Most of the time the fluorescence single overwhelm the weak Raman signal. In contrast to 532nm, for 785 lasers the fluorescence signal decreases prominently, making detection of the weak Raman signal easier. In present system, excitation source is diode laser emitting at 785 nm (Brown et al., 2003).

2.4.2 Sample holder and Light collection system

Like an excitation source, sample holder and light focusing and collecting system are also an important part of Raman spectrometer. Normally there is a proper stage for keeping the sample. Normally a droplet of samples is put on aluminum substrate or glass slide. The light from laser source is usually focused on the surface of the sample through an objective lens. In most cases the same objective is used for the collection Raman scattered light. In our laboratory we have (Peak Seeker PRO-785) Raman system. The system coupled with microscope (RSM-785, Agiltron, USA). Light from diode laser is focused on the sample's surface through the microscope objective lens and the lens is used for collecting the Raman scattered light. There is dichroic mirror in the optical path for the separation of scattered light from the excitation light. The main function of dichroic mirror is that, it reflect one wave length while transmit other wavelength (Dinh, 2003).

2.4.3 Detection System

The detection system mainly comprises spectrometer coupled with computer. The fundamental c function of a spectrometer is break an incoming light into its spectral components, convert it to digital signal as a function of wavelength and readout the digitize signal. The final result is then and display through a computer. The light enter the spectrometer through a narrow slit called entrance slit. The role of the slit is critical to spectrometer's performance as it determines

number photons (photon flux) and is important for the determination of spectral of the spectral resolution. The divergent light is then collimated through concave mirror and imaged onto grating. The diffraction grating disperses the incident light into its components at different angle. The selection of an accurate grating is a key factor in optimizing your spectrometer for the best spectral results in your application. The diffracted light is then imaged onto the detector via second concave mirror. Once the light is imaged onto the detector the photons are then converted into electrons which are digitized and read out through a serial port to a computer. The spectrum, which the plot of intensity as a function of wavelength is computed through inbuilt software, and finally display through computer (Motz, 2003).

2.5 Analytical Techniques for Raman data classification

Raman spectroscopy recently shows advancement in capability of distinguishing difference between RNA/DNA, lipids and proteins which makes it unique technique for measuring changes at cellular level as well as segregating between different tissue and cells types which provides extensive variety of biomedical uses. Raman spectroscopy as a spectral and nondestructive method, gives enormous data on chemical configuration of probed material that usually aided well-known technique for inspecting most challenging biological systems (Zhu et al., 2013). However, the Raman spectrum usually comprised many overlapping bands, so its interpretation cannot be simply made by visual observation for changes in tissue pathology (Hanlon et al., 2000). Hence, different statistical techniques would need to be applied on the Raman data in order to use the biomolecular Raman signal for tissue analysis and classification. The Raman spectrum data usually comprised the results of observations of many different variables (i.e. Raman shift) for different cases (i.e., normal and malignant). Each of these variables represents a different dimension. It is natural that we should seek to design and build machine which can recognize pattern and for the comfort of human (Duda et al., 2000). Classification is a binary process which involves the building, the testing and the use of classification model. It maps the data into some groups or samples which is called classifying the data (Duda et al., 2000). Brief description of different algorithms commonly applied for tissue diagnosis in Raman spectroscopy describes below:

2.5.1 Feature Extraction

Feature extraction is used to find the peaks and valleys of the intensity. It just gathers the necessary information which is used to describe the complete target of interest and to lose the irrelevant information of the target. In short, it is fundamentally an information reduction process (Duda et al., 2000). There are two techniques for feature classification and dimension reduction i.e. Principal Component Analysis (PCA) and Linear Discrimination Analysis (LDA) (Balakrishnama et al., 1998). The major applications of PCA and LDA are face recognition, image retrieval and microarray data classification. The major difference is that LDA maximizes the ratio of between-class variance to the within-class variance in any particular data set thus achieving maximum Discrimination (Ye et al., 2005) while PCA analyzes a data set representing observations which are described by several dependent variables, which are inter-correlated (William et al., 2010).

2.5.2 Principal Component Analysis (PCA)

Principal component analysis (PCA) is the multivariate statistical technique and is used by almost all scientific disciplines. It is an unsupervised learning technique. The aim of PCA is to extract the important information from the data table and to express this information as a set of new orthogonal variables called principal components. Basically PCA is used for feature classification means to find the peaks and valleys of the intensity (William et al., 2010: Duda et al., 2000). It analyzes the data matrix which can be explained by several dependent variables and these variables are inter-correlated (William et al., 2010).

The quality of the PCA model can be evaluated using cross-validation techniques such as the jackknife. PCA generalized the correspondence analysis (CA) and multiple factor analysis (MFA). CA used to handle the qualitative variables while MFA used to handle heterogenous sets of variables. Mathematically, PCA depends upon the Eigen-decomposition of positive semi-definite matrices and upon the singular value decomposition (SVD) of rectangular matrices (William et al., 2010).

2.5.2.1 Aims of PCA

The aims of PCA are:

- To extract the important information from the data table.
- To compress the size of the data table by keeping only this important information.
- To simplify the detail of the data set.
- To analyze the structure of the observation and the variables.

For achieving these goals, PCA calculates new variables called principal components and these components are obtained as linear combinations of the original variables. The first principal component is required to have the largest possible variance. The second component is computed under the constraint of being orthogonal to the first component and to have the largest possible inertia. And the other components are calculated as same. The value of these new variables for the observations called factor scores, these factor scores can be interpreted geometrically as the projection of the observation onto the principal components (William et al., 2010).

2.6 Clinical Applications of Raman Spectroscopy

Raman spectroscopy is a promising technique in biomedical studies due to its non-invasive character and high specificity. The use of multivariate analysis can improve their applicability and extract the useful information that Raman spectroscopy can provide to biomedicine. It has emerged as a novel diagnostic tool for cancer detection and identification of malignancy at different stages of the evolution of neoplasia in tissue. (Utzinger et al., 2001).

It can provide a wealth of spectrally narrow features that are related to the structural and biochemical composition of the sample (Huang et al., 2003).Spectrum of tissue and blood samples is generated and compared with the spectrum of healthy tissues. Difference between these spectras provide an information about the abnormalities.

In biomedical research Raman spectroscopy has been recognized as a valuable analytical tool and depends on energy shift due to interaction with vibrational modes of molecules. The

Raman spectrum is a direct function of the molecular composition of the tissue and provides a fingerprint of the tissue (Crow et al, 2003; Stone et al, 2004).

It can also provide useful biochemical information regarding cells, without the need of fixatives, markers or stains (Kraft et al., 2003). Our approach is based on the hypothesis that the biochemical composition of each body fluid is unique and Raman spectroscopy can easily recognize the difference (Sikirzhytski et al., 2010).

Raman spectroscopy was extensively used to study atherosclerosis (Laarce et al., 2000). Raman fiber-optical probes are under development to classify normal breast tissue, benign disease and malignant breast cancer in vivo during breast lumpectomy surgeries (Motz et al., 2005). The instrument is capable of collecting and processing Raman spectra in less than 2s.

Raman spectroscopy can be used for microstructural characterization of drug delivery systems, as well as to understand drug–excipient interactions in the formulation (Bleye et al., 2014).

Tablets and capsules are still considered the preferred dosage forms. The manufacture of tablets involves several unit operations such as milling, powder blending, granulation, drying, compaction and coating. It is crucial to control the quality of the intermediate products generated during various unit operations to manufacture the final quality product. Raman spectroscopy has been found to be a very useful PAT tool for in-process monitoring to test for the desired intermediate product after each unit operation (Remon et al., 2011).

Raman spectroscopy enabled in-line and real-time monitoring of the blend homogeneity and also led to a better understanding of the process. The effect of particle size, shape, density and light absorption capability on the Raman sampling depth during in situ monitoring of API-excipient blending by wide area illumination (WAI) (Allan et al., 2013).

Raman spectroscopy has been used to measure the concentrations of chemicals in blood serum (glucose, urea, proteins, cholesterol, triglycerides etc.), with results in good correlation with reference values (Berger et al., 2007). A recent study employed Raman spectroscopy for diagnosis of type II diabetes by detecting molecular changes in the membranes of erythrocytes (Lin et al., 2014).

Raman spectroscopy was used to establish reliable biomarkers that can be used to distinguish between non-infectious systemic inflammatory response syndrome (SIRS) and sepsis (Bocklitz et al., 2014).

Materials and Methods

3.1 Study Approval

The present study was approved by Ethical Review Committee of International Islamic University and Ethical Committee of Nuclear Oncology and Radiotherapy Institute (NORI), Islamabad, before conducting the research.

3.2 Blood Samples

Before the start of the study proper informed consent from the patients were obtained. 50 Blood samples of Head and Neck cancer patients along with equal number of age matched healthy controls were collected at different days from Nuclear Oncology & Radiotherapy Institute (NORI) Hospital, Islamabad through proper questionnaire (Annex1) which includes different parameters like name, age, sex, stage of the cancer, past medical history, biopsy report and hormonal report etc. Blood samples (5cc) were collected in EDTA filled vacutainers (HebeiXinle, Sci&Tech Co. Ltd., China) using disposable BD syringes, after collection each sample has been transported to National Institute for Laser and Optronics (NILOP) for preparation and stored at -16°C in the refrigerator till use for spectroscopic technique.

3.3 Subjects and Protocol

The present study comprised two groups: 50 H&N cancer patients with a confirmed clinical and pathological diagnosis from Nuclear Oncology & Radiotherapy Institute (NORI). The second group includes equal number of healthy control volunteers comprised both female and male with normal history.

3.4 Serum Preparation

Serum from both cancer patients and control group were extracted according to the standard protocols. The blood was centrifuged at 3500 rpm for 10-20 minutes to obtain the fine sera

of the samples. The serum samples after extraction were stored at -16^{0}C refrigerator at National Institute of Laser and Optronics (NILOP) before performing Raman Spectroscopy analysis.

3.5 Materials

In this study following materials were used;

- Serum samples from Cancer patients
- Serum samples from healthy persons
- A peak-seeker PRO-785 Agiltron, Raman Spectrometer
- MathWorks - MATLAB 2015a Software for digital signals processing.

3.6 Methods

3.6.1 Optimizations for Seeking Spectra

The Raman Spectrometer made by Agiltron model peak seeker pro, with fixed wave length 785nm, was used to collect the spectra. This model uses a Helium Neon super cold (-20°C) laser to excite samples. This system is attached to microscope to view and focus the point of target for laser excitation.

3.6.2 Optimization of Integration Time

Minimal excitation time of 5s and maximum time 60s with a gradual increase of 5s each was selected. Three spectra were recorded on each integration time. Mean plot of each group was taken and peaks in graph were compared. Results showed that integration time of 15s is best suitable for recording the spectra and its further analysis. All further samples were then set to be excited for time of 15s.

3.6.3 Optimization of Laser Power

Second critical point was to optimize the laser power because if more laser power is provided the sample will heat up and results in denaturation. But in case of reduced laser power low or no signal will be omitted. For optimization range of 150mv was selected. On each step 10 spectra for each sample were recorded and then later analyzed. Mean plot of each group was taken and results showed that best spectra can be recorded on 150 mv laser. Further all steps were set to be analyzed on 150 mv laser power.

3.6.4 Optimizing the Focusing Lens

There were three optical zoom ranges 5X, 10X and 50X available in microscope to focus the laser excitation point. All of three were checked one after another and results showed that optical zoom of 10X is best suitable to focus on sample and determine the point of excitation for laser. All of the samples were then set to be focused on 10X optical zoom.

3.6.5 Choosing the Surface and Shape

A number of type of surfaces and shapes, for example glass slide and aluminum surface and then shapes like oval and round were analyzed. Using previously optimized conditions, a criteria was to take spectra of a 15-20µl drop of same sample. The purpose was to check where we can get the best signal. Analysis showed that sample on an aluminum surface which omits the best signals with least noise. All further samples were then set to analyze on the same shape on aluminum base slide.

3.6.6 Seeking The Spectra

After conformation of positive samples, sample and control were brought to Biophotonics Laboratory NILOP for spectra collection. A drop of 15 micro liter was dropped on Aluminium base slide as specified earlier. Slide was then placed under microscope for focusing where focus was set in the middle of moving layers in a droplet. Minimal

10spectra were recorded for each sample with laser power 150 mv and integration time of 15s as optimized earlier.

3.6.7 Spectral processing

After recording the spectra next step was processing of the data, in which Math Works – MATLAB version 2015a was used. A special code running PCA was developed in collaboration with statistician and software experts. Spectral processing is a complex work and include following steps further.

3.6.7.1 Background Removal

Some of the background light is added in process of recording any spectra of a sample. That creates problems in analysis and comparison to original references and recognition of any new spectral feature. Because true peaks that are resulting from vibrational moments of biomolecules are either hindered or altered. So for that it is necessary to remove all the background. It was removed by taking a dark view and subtracting that dark view forms the recorded spectra leaving original spectra behind.

3.6.7.2 Substrate Removal

In raw spectra some portion of the signal is produced by aluminum base itself. These spectral lines deform the spectral signatures. So for that it must be removed from it. All the substrate background was removed by taking spectra of aluminum surface and subtracting that from the spectra of sample.

3.6.7.3 Baseline Correction

Raman spectroscopy plots a graph that is not always smooth in accordance with baseline. So for that base line correction is applied which makes the entire graph straight along with x-axis.

3.6.8 Analysis and plotting

To make results more applicable and representable mean of all the groups were taken. Graphs were plotted for the results. Scatter plots were also made and Principle Component Analysis (PCA) was performed on the data. It is an unsupervised learning technique and used in all scientific disciplines for multivariate statistical analysis. Important information is extracted from the data table and expresses as a set of new orthogonal variables called Principle Components. Basically this analysis is used for feature classification means to find the peaks and valleys of the intensity. It analyzes the data matrix which can be explained by several dependent variables and these variables are inter-correlated.

3.6.9 Acquiring spectra

All the upper mentioned conditions were applied and 10 spectra for each sample were taken to avoid any variation or change, mean of all spectra were taken and then for one by one comparison of cancer and healthy samples, mean of all cancer and healthy spectra was taken.

3.6.10 Statistical Analysis

The demographic and clinical characteristics of patients like age, sex, stage of cancer, past medical history, biopsy report and, hormonal report were also recorded. The mean and percentages were calculated for Head and Neck cancer and graphs were plotted.

Chapter 4

RESULTS

RESULTS

Head and neck cancer is the second leading cause of death in Pakistan. It is also due to the abnormal growth of cells in the regions like Oral cavity, nasal cavity, paranasal sinuses, larynx, trachea, hypopharynx, nasopharynx and oropharynx (Masood et al., 2012). Approximately 42 % of head & neck cancer are due to factors like smoking, tobacco, alcohol consumption and human papilloma virus (Cmelak, 2012).

In the present study, serum samples of Head and Neck cancer patients were analyzed through Raman spectrometer using wavelength 785nm, integration time 10-15 seconds and power 150 milli-watts, the results showed multiple Raman shifts. Of these multiple shifts the main focus was mainly on some of the particular shifts which differ in healthy and diseased samples in order to make a good comparison. This project looks for strong, weak, conformational or additional peaks that can give us good key of comparison.

The Raman Shifts in the selected samples show some changes in the molecular conformations and chemical composition of molecules at some points which could be analyzed further in diseased and healthy blood samples. Pre-processing of spectrum that was obtained can be done by following methods:

4.1 Data pre-processing

Raman spectra of both normal as well as diseased samples were illustrated in figure 4.1. The spectra from normal sera have been shown in green color whereas; spectra from the cancerous samples have been shown in red color. These entire samples are almost overlapped, so for the purpose of clarity a space has been intentionally created using a self-written MATLAB code. All these sample looks quite noisy. The reason is that there is always exists different types of noise in the Raman spectra of biological samples. So one cannot get useful information from using noisy spectra. It is therefore imperative to first clean (de-noised) all spectrums. First step in the pre-processing was smoothing of the Raman spectra. Raman spectra shown in figure 4.1 were noisy, so these spectra were smoothed by using sgolay function in MATLAB. Figure 4.2 shows the smoothed Raman spectra.

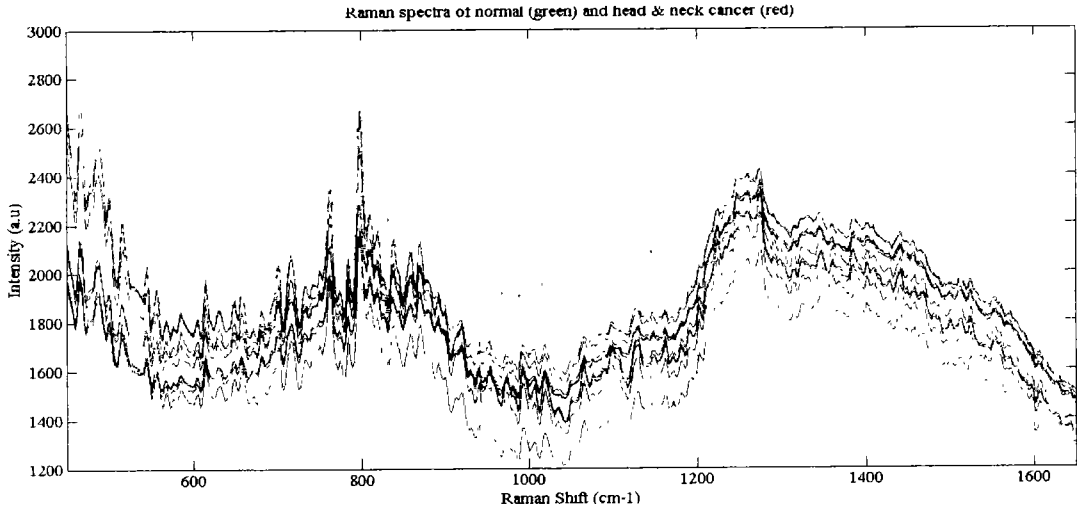


Fig. 4.1 Raman spectra (raw) from healthy human blood sera (green) and Head and Neck cancerous samples (red).

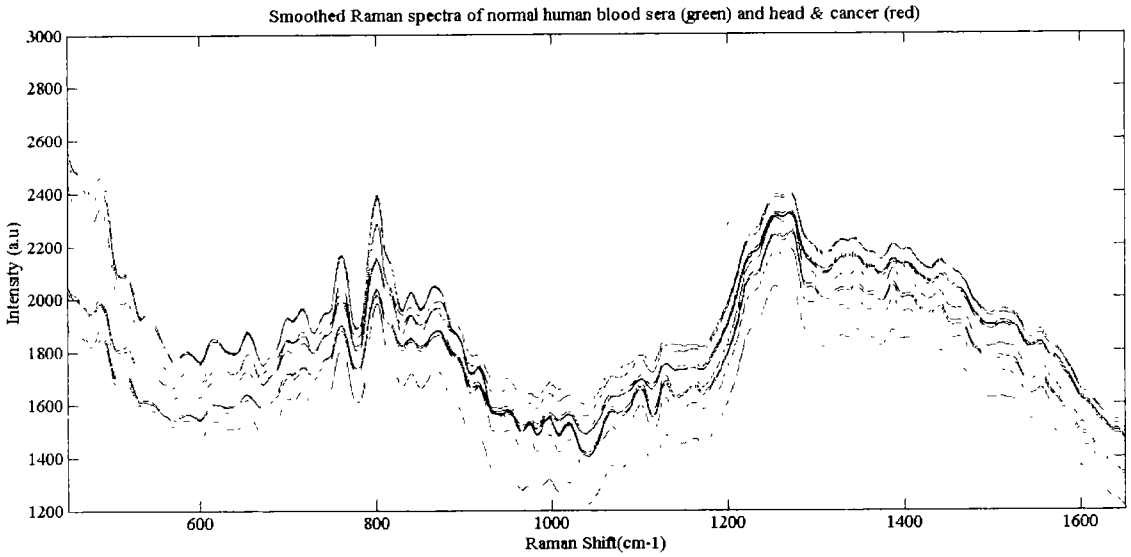


Fig. 4.2 Raman spectra (smoothed) from healthy human blood sera (green) and Head and Neck cancer samples (red).

Apart from noise there exists florescence background. Inside biological samples there exists natural fluorophores. These molecules produce fluorescence when illuminated with the laser. This property is inherent to all biological samples. This fluorescence background is always very strong and most of the time overwhelm the weak Raman signal. Before making an analysis it is necessary to remove this fluorescence background. This is done by using msbackadj command in self written code in MATLAB. From figure 4.2 it was obvious that each spectrum has different background i.e. Different starting point. This adjusts by using the above mentioned command in MATLAB. The fluorescence background adjusted (background subtracted) Raman spectra both for healthy and diseased samples shown in figure 4.3.

As mentioned earlier, the vertical separation between these two types of samples has been intentionally created for the purpose of clarity. Finally this separation has been removed. So the final de-noised and background subtracted Raman spectra shown in figure 4.4.

4.2 Raman spectral analysis

Raman spectra of biological samples (serum) contain large numbers of peaks. Each of these peak represents a specific molecule inside the sample, whereas the peak concentration shows the intensity of molecules in the sample. The first spectra of the Head and Neck cancer samples and healthy Samples ranging from 450cm^{-1} to 1650cm^{-1} Raman shifts as illustrated in Figure 4.4. All these Raman shifts corresponds to some chemical composition but mainly focus on those shifts which show deviation from normal behavior.

- Raman shift at 622 cm^{-1} and 951cm^{-1} shows that Phenylalanine is higher in diseased samples as compared to normal samples.
- Raman shift at 802 cm^{-1} and 840 cm^{-1} shows that Tryptophan and 12-methyl tetradecanoic acid is higher in diseased samples but lower in normal samples.
- Raman Shift at 1020 cm^{-1} depicts that Galactosamine and N-Acetylglucoseamine are higher in cancer blood samples but lower in normal samples.
- Raman shift at 700 cm^{-1} and 712 cm^{-1} shows that lactose and Amylose is higher in cancer samples as compared to normal samples.

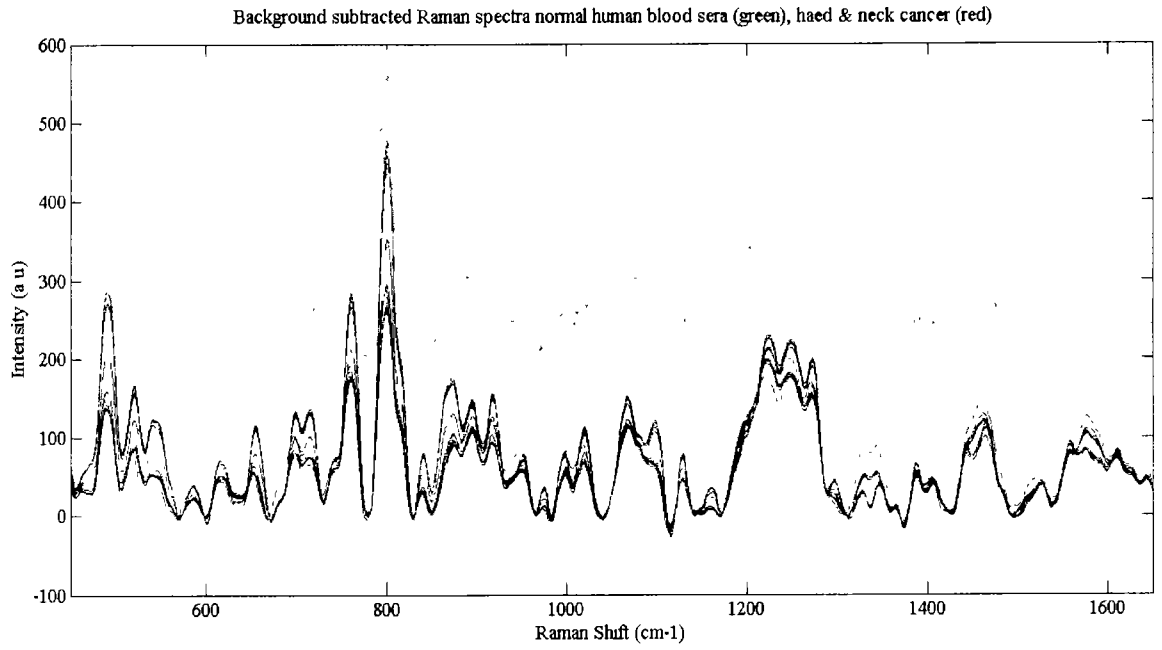


Fig. 4.3 Raman spectra (background adjusted) from healthy human blood sera (green) and Head and Neck cancer samples (red).

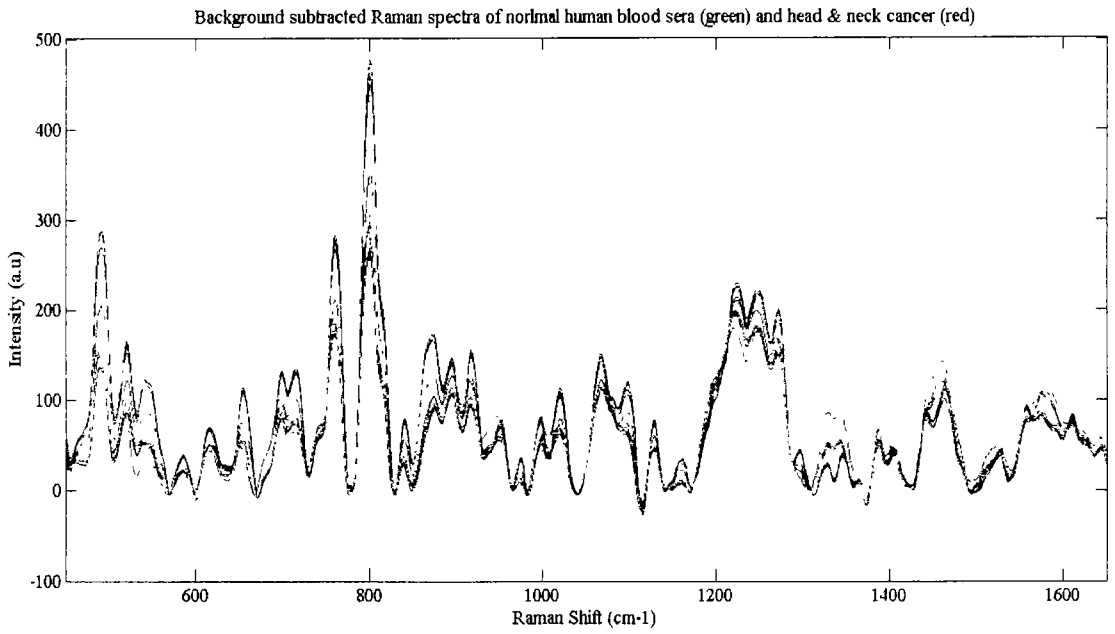


Fig. 4.4 Raman spectra (de-noised and background adjusted) from healthy human blood sera (green) and Head and Neck cancer samples (red).

- Raman Shift at 1222 cm^{-1} and 1270 cm^{-1} depicts that lactose and 14-methyl pentadecanoic acid is higher in diseased samples but lower in normal blood samples.
- Raman shift 1325 cm^{-1} shows that Coenzyme A is lower in cancer samples but higher in normal blood samples.
- Raman shift at 1410 cm^{-1} shows that Proline is lower in diseased samples but higher in normal samples.
- Raman shift 1440 cm^{-1} shows that Oleic acid is lower in cancer samples but higher in normal blood samples.
- Raman shift at 1463 cm^{-1} depicts that Stearic acid is lower in diseased samples but higher in normal samples.

Raman spectroscopy analysis of Head and Neck cancer patients and healthy control are illustrated in table 4.1.

4.3 Correlation calculations

Correlation of biological molecules in cancer samples was calculated by the following method:

1- For Tryptophan molecule deviation of Tryptophan was observed in 4 samples out of 11.

$$\text{Correlation} = \text{No. of Samples} \div \text{Total Samples} \times 100$$

$$\text{Correlation} = 4 \div 11 \times 100 = 36\%$$

2- For Phenylalanine molecule deviation of this molecule was observed in 2 samples out of 11.

$$\text{Correlation} = \text{No. of Samples} \div \text{Total Samples} \times 100$$

$$\text{Correlation} = 2 \div 11 \times 100 = 18\ \%$$

Table 4.1 Comparison of Raman peaks of Normal and Head and Neck cancer patient's

Peak(cm^{-1})	**Healthy Raman shifts**	**Diseased Raman shifts (Increased)**	**Diseased Raman shifts (Decreased)**
622 cm^{-1}	Phenylalanine	Phenylalanine	-------
700 cm^{-1}	Lactose	Lactose	-------
712 cm^{-1}	Amylose	Amylose	-------
802 cm^{-1}	Tryptophan	Tryptophan	-------
840 cm^{-1}	Tryptophan	Tryptophan	-------
951 cm^{-1}	Phenylalanine	Phenylalanine	-------
1020 cm^{-1}	Galactosamine and Glucosamine	Galactosamine and Glucosamine	-------
1222 cm^{-1}	Lactose	Lactose	-------
1270 cm^{-1}	14-Methyl Pentadecanoic acid	14-Methyl Pentadecanoic acid	-------
1325 cm^{-1}	Coenzyme A	-------	Coenzyme A
1410 cm^{-1}	Proline	-------	Proline
1440 cm^{-1}	Oleic acid	-------	Oleic acid
1463 cm^{-1}	Stearic acid	-------	Stearic acid

4.4 Principal Component Analysis

PCA is a statistical technique which transforms a large number of correlated variables into a few uncorrelated variables called PCA. These new uncorrelated variables (PCs) describe the greatest variance of the spectral data. The aim of PCA is to extract the important information from the data table and to express this information as a set of new orthogonal variables called principal components (William et al., 2010). Basically PCA is used for feature classification means to find the peaks and valleys of the intensity (William et al., 2010: Duda et al., 2000). It analyzes the data matrix which can be explained by several dependent variables and these variables are inter-correlated (William et al., 2010).

4.5 Data classification and analysis

Figure 4.5 shows the plot of the data set in the PCA domain. It is clearly visible that by considering only two dimensions in PCA domain, the data set can be easily split into two classes (normally and abnormal). The plot of PC1 vs. PC2 makes a clear distinction between these two types of data points. In the diseased data points the spread is comparatively more as compared to normal data points.

4.6 Statistical analysis of clinical and demographic parameters

The demographic and clinical characteristics of patients like age, sex, stage of cancer, past medical history, biopsy report and, hormonal report were also recorded. The mean and percentages were calculated for Head and Neck cancer and graphs were plotted shown in figures 4.6, 4.7, 4.8 and 4.9.

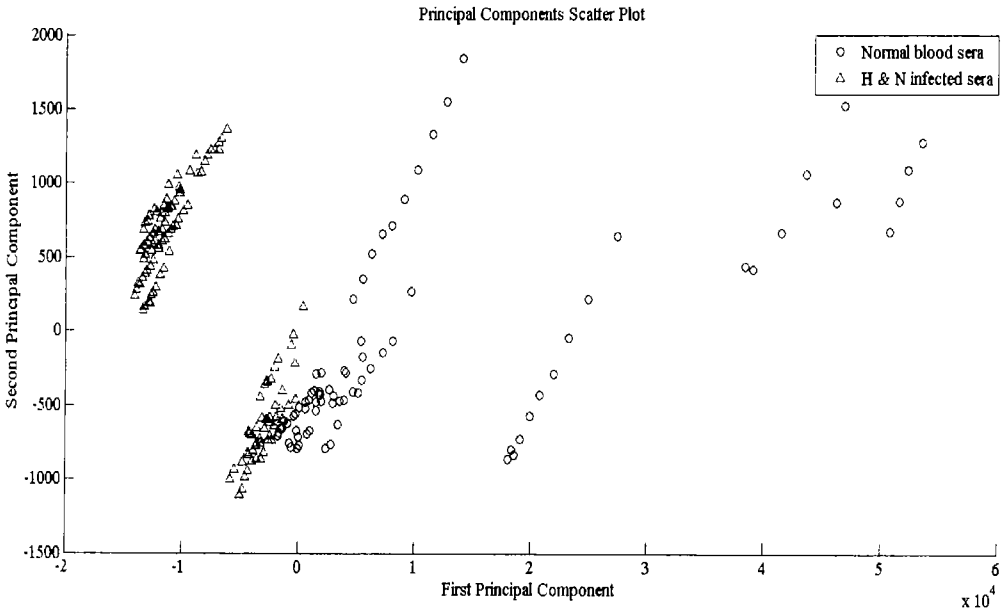


Fig. 4.5 PCA plot between normal and cancer samples

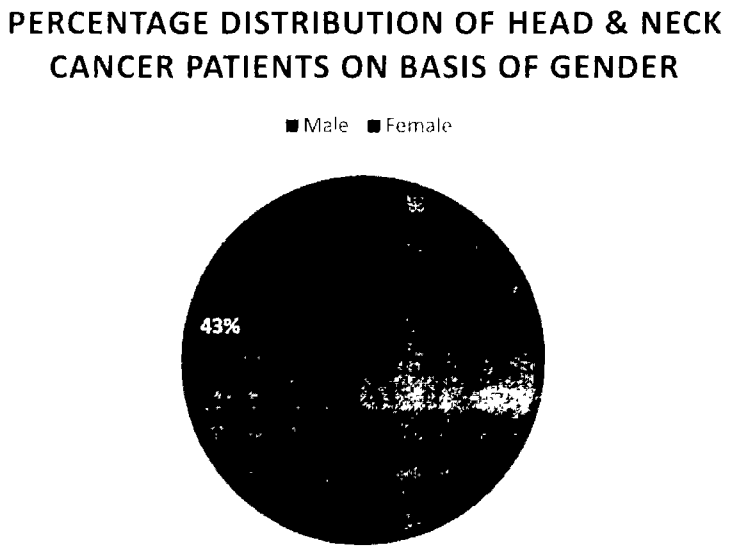


Fig. 4.6 Distribution of Head and Neck cancer patients on basis of gender

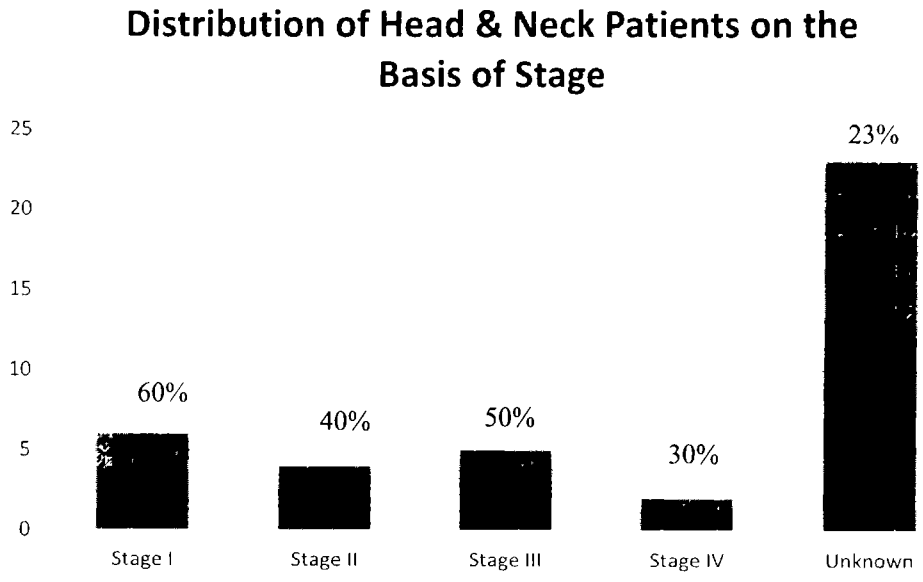


Fig 4.7 Distribution of Head and Neck cancer on the basis of stage

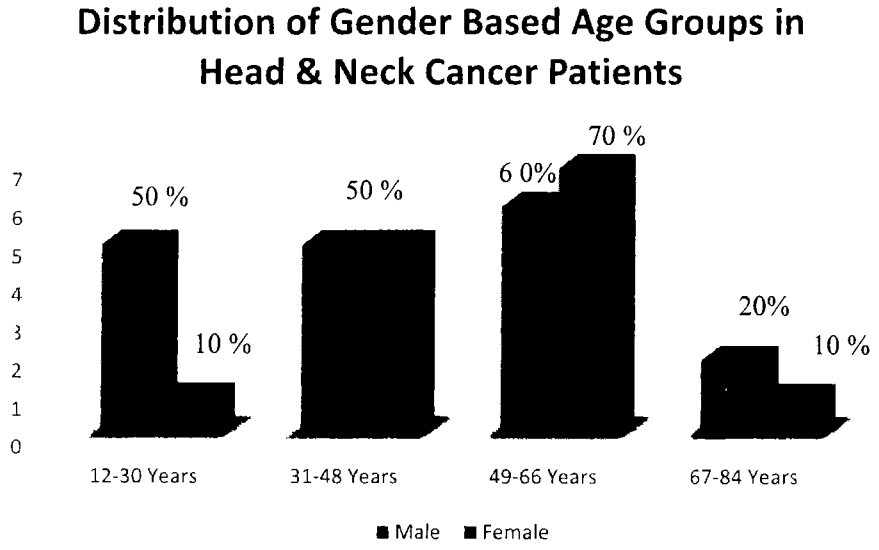


Fig. 4.8 Distribution of gender based age groups in Head and Neck cancer patients

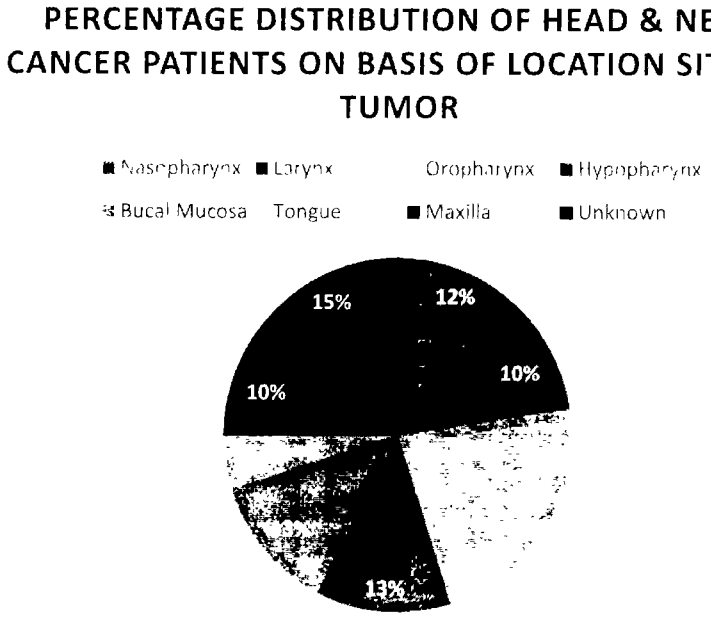


Fig. 4.9 Distribution of Head and Neck cancer on basis of location/site of tumor

Chapter 5

DISCUSSION

DISCUSSION

Head and neck cancer (HNC) results from abnormal growth of cells in the regions like oral cavity, nasal cavity, paranasal sinuses, larynx, trachea, hypopharynx, nasopharynx and oropharynx. HNC is sixth common cancer worldwide and in Pakistan it is second leading cancer (Masood et al., 2012). Head and neck cancer is a complex disease and full understanding has not yet been developed due to multiple factors (environmental and genetic) involved in disease. Clinical presentation of patients with small differences in different patients can mislead in diagnosis of disease.

The results obtained with reference to the chemical changes in the chemistry of blood that were studied and analyzed may provide a way to rapidly diagnose HNC cancer by Raman spectroscopic technique. All groups from diseased and normal samples presented the same characteristic features and set of bands that could differentiate normal samples (Hanlon et al., 2000) from pathological tissues in accord with the relative intensity increase or decrease of some bands, absence or appearance of some weak peaks (Shami et al., 2002).

Characteristic peaks appeared at 622 cm^{-1} shows that Phenylalanine is significantly increased in cancer cells as compared to normal cells of blood. Experimental data of Raman Spectroscopy on blood shows that this peak is associated with the presence of Phenylalanine in blood. A sharp, intense and typical peak at 622 cm^{-1} and 951 cm^{-1} is attributed to symmetric ring breathing mode of phenylalanine (C-C skeletal stretch) which is a protein assignment and plays a vital role in terms of differentiation between normal and pathological conditions and can also be used as a potential molecular marker in cancer biology. Protein metabolism in terms of metastasis, which includes transcription (RNA synthesis) and translation (protein synthesis), can be obtained through biochemical information acquired by Raman spectrum. Several studies have demonstrated that phenylalanine concentration is increased in patients with malignancy, HIV infections, trauma, burn and sepsis (Chang et al., 1983, Roth et al., 1985, Rath et al., 1987, Ollenschläger et al., 1988). In malignant tissues an increased intensity of phenylalanine was significant and reflects an increased amount of protein associated with

tumor. It was found that the as the tissue changes from normal to malignant, intensity of protein dominant peaks also increased.

Similarly Raman shift at 802 cm^{-1} and 840 cm^{-1} shows higher concentrartion of Tryptophan (Trp) which is an indispensable amino acid required for biosynthesis of proteins, serotonin and niacin and is needed for rapidly dividing malignant cells. Malignant tissue samples possess relative increase in peak intensities of tryptophan (Devpura et al., 2011, Tankiewicz et al., 2006). Tumor growth is associated with a number of metabolic abnormalities like protein metabolism is deranged in cancer patients, as revealed by changes of plasma amino acid profile. The increased plasma free tryptophan levels are a frequent finding in cancer patients. Plasma free tryptophan concentrations were found to be significantly elevated with respect to healthy controls in patients with breast, lung, colon, stomach, and cancer from various origin. The sensitivity of this marker in predicting the presence of the tumor was highest for stomach and lung cancer patients. High plasma free tryptophan concentrations seem to be directly related to the presence of the tumor. The increase of plasma free tryptophan concentrations is a common feature of both human solid and hematologic malignancies and some experimental tumors. Increased plasma tryptophan facilitates transport into the brain, hypothalamic serotonergic activity is in fact stimulated in the presence of anorexia of cancer (Laviano et al., 2003).

Raman shift at 1020 cm^{-1} shows that galatosamine and glucosamine is higher in cancer samples but lower in normal blood samples because tumor tissue are by far the most active in accumulating radioactive glucosamine in vivo. It is the accumulation of glucosamine derivatives which results in the toxic effect of glucosamine, as well as tumors should be most sensitive to glucosamine (Molnar et al., 1965). It is possible that the capacity of neoplastic tissues to accumulate large quantities of glucosamine and its phosphorylated derivatives coupled with a relatively slow removal of glycoproteins results in selective cytotoxicity of D-glucosamine to cancer cells (Winzler et al., 1964).

Characteristic peaks at 700 cm^{-1} and 712 cm^{-1} and 1222 cm^{-1} shows that different forms of glucose like lactose, amylase are increased in concentration in cancer samples as compared to normal blood samples of healthy patient. It has long been assumed that the primary function

of glucose is to provide energy to the cell in the form of ATP as an alternative to mitochondrial respiration. The availability of oxygen is a major regulator of glucose consumption, which was first described by Pasteur in 1856 (Krebs et al., 1972). Thus, in hypoxic tissues glucose consumption is higher (Racker et al., 1958). An effect was first described by Warburg in 1930 that Cancers continue to consume glucose at high rates, even in the presence of adequate oxygen (Warburg et al., 1930). Thus, ''aerobic glycolysis'' can be described as a cancer hallmark (Hanahan et al., 2000) and elevated glucose consumption may play an essential role in cancer progression. Genes of glycolysis are ubiquitously overexpressed in cancers (Mikuriya et al., 2007). The dominant perception of energy metabolism in cancer has been that ATP production is fueled by oxidative metabolism of glutamine and glucose and by the anaerobic metabolism of glucose through the Embden–Meyerhof pathway (EMP) of glycolysis, and that this ATP is needed to support a high level of oncogene-induced proliferation.

Experimental data of Raman Spectroscopy on blood shows that Raman shift at 1440 is associated with the presence of Oleic acid in blood. So decrease level of this peak in diseased samples leads to a cancer risk in humans, because OA was found to significantly down-regulate the expression of Her-2/neu (Vellon et al., 2005) a well-characterized oncogene that plays a key role in the etiology, progression and chemosensitivity of various types of human cancer. So the ability of OA to down-regulate Her-2/neu promoter activity and to suppress Her-2/neu protein over expression. The intake of oleic acid rich diet specifically olive oil may have a potential role in lowering the risk of malignant neoplasms especially breast and stomach cancer and also in ovary, colon and endometrium cancer (Simonsen et al., 1998).

Raman shift at 1410 cm^{-1} shows that proline is lower head and neck cancer patient sample as compared to normal samples and leads to increased risk of cancer. Proline plays a special role in cancer metabolism. Proline oxidase (POX), also known as proline dehydrogenase (PRODH), is among a few genes induced rapidly and robustly by P53, the tumor suppressor. In human tumors of the digestive tract and kidney, POX was markedly decreased, suggesting that the suppressive effect of POX was downregulated (Frederick, 2012). Downregulation of proline catabolism is complementary to its biosynthesis and commonly observed in a number of tumor types. The first step of this process is catalyzed in the mitochondria by proline

oxidase (POX), and the expression of this enzyme is markedly reduced in many cancers compared to normal tissue from the same patient (Diwan et al., 2009). POX expression is inhibited by MYC via miR-23b* in lymphoma, renal, and prostate cancers (Wang et al., 2010; Hancock et al., 2012). The widespread repression of POX in cancer indicates that this enzyme may act as a tumor suppressor; however, the specific mechanisms through which POX deficiency promotes tumorigenesis are not yet clear.

Raman shift at 1463 cm^{-1} shows lower Stearic acid in diseased samples as compared to normal samples. Stearic acid is a saturated fatty acid that is two carbon atoms longer than palmitic acid and is similarly cholesterogenic. Low level gives increased fluidity which is associated with active tumor proliferation (Apostolov et al., 1985).

Despite many advantageous features of Raman spectroscopy could offer, there exists some problem usually in cancer diagnosis with this technique. The spectral difference between normal and neoplastic tissues is normally very small. One cannot differentiate them just by visual observation inspite of small variations in intensity. For the interpretation of such a complicated data a powerful data processing algorithms are needed in order to get an effective diagnostic information. Multivariate statistical techniques such as principal component analysis (PCA), linear discriminant analysis (LDA) and partial least squares-discriminant analysis (PLS-DA) have been widely applied to the diagnosis and classification of various tissues Raman spectroscopy. By using multivariate analysis technique can resolve above-mentioned difficulties with a simplified by removing irrelevant or redundant variables from the spectral data set. In this research, principal component analysis has used for the spectral data analysis and data classification of cancer and normal patients.

In the present study a total number of 50 Head and Neck cancer patients with equal number of age matched healthy controls were assessed for gender, age, stage and type of cancer. 70 % of patients in the study were above age of 66 and 60 % of patients were above age of 44. Percentage of Oral cavity cancer or oropharynx was highest, i.e., 22 %. In the present study, the sex ratio was found to be 2:1. This means that males and females are not equally affected by HNC. This finding is matched with the findings from earlier studies in other populations which reported a 2:1 male to female ratio (Toefil et al., 2007). However, Abdulamir et al.,

2008 found no association of age with sex ratio and areas of Head and Neck cancer in Asian patients. Data from this study shows that cancer of oral cavity was most frequently affected area of Head and Neck cancer followed by cancer of pharynx and larynx. Higher incidence for oral cancer compared to other cancers of Head and Neck had also been reported in many countries like England (Llewellyn et al., 2004) and Taiwan (Ping et al., 2007). Major risk factors may include infection of human papilloma virus (HPV), alcohol, tobacco and smoking. 42 % of head and neck cancers are caused by smoking and alcohol (Cmelak, 2012). Many genes also play role in altered pathways in head and neck cancer leading towards malignancy. Collectively these changes in tumor suppressor genes, oncogenes and proto oncogenes can lead towards cancer (Jonah and Jennifer, 2010).

Biochemical information obtained by Raman spectra can be extremely sensitive and may identify specific biomolecules which can be used as biomarkers for cancer detection non-invasively and in a very short time. This study demonstrates the use of Raman spectroscopy combined with Support Vector Machine SVM technique for the classification of the spectral data acquired from the sera of Head and Neck cancer patients. Raman spectroscopy coupled with statistical tools has great potential to contribute significantly in the diagnosis and research of cancer patients in an effective way.

Chapter 6

REFERENCES

References

1. Abeloff M.D, Wolff A.C, Weber B.L., (2008) Cancer of the Breast. In: Abeloff MD, Armitage JO, Lichter AS, eds. Clinical Oncology. 4th ed. Philadelphia, Pa: Elsevier, p. 1875–1943.
2. Abdulamir A. S., Hafidh R. R., Abdulmuhaimen N., Abubkar F. and Abbas K. A., (2008). The distinctive profile of risk factors of nasopharyngeal carcinoma in comparison with other head and neck cancer types. BMC Public Health, (8), p. 400.
3. Akiko I., Hideaki T., Wakiko A., and Akira O., (2005) Trends in Head and Neck Cancer Incidence in Japan during 1965-1999. Japanese Journal of Clinical Oncology, 35(1), p. 45-47.
4. American Cancer Society. Cancer Facts and Figures (2011). Atlanta, Ga: American Cancer Society.
5. Anderson B.O., Yip C.H., Smith R.A., (2007) Guideline implementation for breast healthcare in low-income and middle-income countries: overview of the Breast Health Global Initiative Global Summit. Cancer, **113**, p. 2221–43.
6. Allan P., Bellamy L.J., Nordon A., Littlejohn D., Andrews J., and Dallin P., (2013) In situ monitoring of powder blending by non-invasive Raman spectrometry with wide area illumination. Journal of Pharmaceutical and Medical Analysis, 76, p. 28–35.
7. Apostolov K., Barker W., Catovsky D., (1985) Reduction in stearic acid to oleic acid ratio in leukemia cells- a possible chemical marker of malignancy.blut, 50, p. 349-354.
8. Buschman H.P., Marple E.T., Wach M.L., Bennett B., Schut T.C., Bruining H.A., Bruschke A.V., Laarse A.V., and Puppels G.J., (2000) In vivo determination of the molecular composition of artery wall by intravascular Raman spectroscopy. Analytical Chemistry, 72(16), p. 3771– 3775.
9. Balakrishnama S., and Ganapathiraju A., (1998). Linear Discriminant Analysis- A brief Tutorial, Institute for Signal and information Processing, p. 1-8.
10. Bruce EB., and Everett EV., Head and neck cancer in 2010 Maximizing survival and minimizing toxicity. Nature Reviews Clinical Oncology, 8, p. 72-74.

11. Beckmann M.W., Niederacher D., Schnurch H.G., Gusterson B.A., Bender H.G., (1997) Multistep carcinogenesis of breast cancer and tumour heterogeneity. Journal of Molecular Medicine, 75, p. 429–439.
12. Bhurgri Y., Bhurgri A., and Usman A., (2006) Epidemiological review of head and neck cancers in Karachi. Asian Pacific Journal of Cancer Prevention, 7, p. 195-200.
13. Bhurgri Y., Bhurgri A., Nishter S., Ahmed A., Usman A., Pervez S., Ahmed R., Kayani N., Riaz A., Bhurgri H., Bashir I., and Hassan S.H., (2006a) Pakistan-country profile of cancer and cancer control 1995-2004, Journal of Pakistan Medical Association, 56, p. 124.
14. Bhurgri Y., Bhurgri A., Hassan S.H., Zaidi S.H.M., Rahim A., Sankaranarayanan R., and Parkin D.M., (2000) Cancer incidence in Karachi, Pakistan: first results from Karachi cancer registry, International Journal of Cancer, 85, p. 325-329.
15. Cmelak A.J., (2012) Current issues in combined modality therapy in locally advanced head and neck cancer. Critical reviews in oncology/hematology, 84(2), p. 261-273.
16. Cairns R., Harris I., Mak T., (2011) Regulation of cancer cell metabolism, 11.
17. Chaudry S., Khan A.A., Mirza K.M., Iqbal H.A., Masood Y., N.R., and Izhar F., (2008) Estimating the burden of head and neck cancers in the public health sector of Pakistan. Asian Pacific Journal of Cancer Prevention, 9(3), p. 529-532.
18. Chang X. J., Yang C. C., Hsu W. S., Xu W. Z., & Shih T. S., (1983) Serum and Erythrocyte amino-acid pattern - studies on major burn cases. Burns, 9, p. 240-248.
19. Delbeke D., (2009) Hybrid imaging (SPECT/CT and PET/CT): improving therapeutic decisions. Seminars in Nuclear Medicine, 39(5), 308–40.
20. Derek L., (2002) The Raman Effect, John Wiley & sons.
21. Duda R.O., Hart P.E., and Stork D.G., (2000). Pattern Classification, 2nd Edition, Chapter 1, 11-17.
22. De Beer T., Burggraeve A., Fonteyne M., Saerens L., Remon J.P., and Vervaet C., (2011) Near infrared and Raman spectroscopy for the in-process monitoring of pharmaceutical production processes. International Journal Of Pharmaceutics, 417, p. 32–47.

23. DEVPURA S., THAKUR J. S., SETHI S., NAIK V. M. & NAIK R., (2011) Diagnosis of headand neck squamous cell carcinoma using Raman spectroscopy: tongue tissues. Journal of Raman Spectroscopy.
24. Ellis D.I., Cowche D.P., Ashton L., OHagan S., and Goodacre R., (2013) Illuminating disease and enlightening biomedicine: Raman spectroscopy as a diagnostic tool, Analyst, 138, 3871-3884.
25. Elango J. K., Gangadharan P., Sumithra S., and Kuriakose M. A., (2006) Trends of head and neck cancers in urban and rural India. Asian Pacific Journal of Cancer Prevention, 7(1), p. 108-112.
26. E.B. Hanlon et al., (2000) "Prospects for in vivo Raman spectroscopy," Physics in Medicine and Biology, 45, R1- R59.
27. Fernandez E., La Vecchia C., Talamini R., and Negri E., (2002) Joint effects of family history and adult life dietary risk factors on colorectal cancer risk, Epidemiology, 13, p.360-363.
28. Metabolism and Cancer Susceptibility Section, Laboratory of Comparative Carcinogenesis, Center for Cancer Research, National Cancer Institute at Frederick, Frederick. 2012, 1;17, p. 1835-45.
29. Foran J.M., Sekeres M.A., (2008) Myelodysplastic Syndromes. In: Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE. Kastan MB, McKenna WG, eds. Clinical Oncology. 4th ed. Philadelphia, Pa: Elsevier, 2235-p. 2259.
30. Ferlay J., Shin H.R., Bray F., Forman D., Mathers C., Parkin D.M., Cancer incidence and mortality worldwide, International Agency For research On Cancer, 2010.
31. Gardiner DJ, Introduction to Raman Scattering in practical Raman spectroscopy, Springer, 1989, p. 1-12.
32. Gillison M.L., Koch W.M., Capone R.B., Spafford M., and Westra W.H., (2003) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. Journal of the National Cancer Institute, 92, p. 709–720.
33. Gharib H., Papini E., (2007) Thyroid nodules: clinical importance, assessment, and treatment. Endocrinology Metabolism Clinics Of North America, 36, p. 707-35.

34. Glastonbury CM, Nasopharyngeal carcinoma: the role of magnetic resonance imaging in diagnosis, staging, treatment, and follow-up, Topics in Magnetic Resonance Imaging, 2007, 18(4), p. 225–235.

35. Huang Z., McWilliams A., Lur H., McLean D.I., Lam S., and Zeng H.,(2003) Near infrared Raman spectroscopy for optical diagnosis of lung cancer, International Journal Of Cancer, 107, p. 1047–1052.

36. Hjartåker A., Aagnes B., Robsahm T.E., Langseth H., Bray F., and Larsen I.K., (2013) Subsite-specific dietary risk factors for colorectal cancer: a review of cohort studies, Journal of Oncology, p. 703854.

37. Han Y., Xue X.F., Shen H.G., Guo X.B., Wang X., Yuan B., Guo X.P., Kuang Y.T., Zhi Q.M., and Zhao H., (2014) Prognostic significance of Beclin-1 expression in colorectal cancer: a meta-analysis, Asian Pacific Journal of Cancer Prevention, 15, p. 4583-4587.

38. Hanahan D., Weinberg R.A., (2000) The hallmarks of cancer. Cell, 100, p. 57–70.

39. Harvey D, Analytical chemistry 2.0, 2010.

40. Huang, Z.W., McWilliams, A., Lui, H., McLean, D.I., Lam, S., and Zeng, H.S., (2003) Nearinfrared Raman spectroscopy for optical diagnosis of lung cancer. International Journal Of Cancer, 107 (6), 10p. 47–1052.

41. Hanlon E., Manoharan R., Koo T.W., Shafer K., Motz J., Fitzmaurice M., Kramer J., Itzkan I., Dasari R., and Feld M., (2000) Prospects for in vivo Raman spectroscopy, Physics in medicine and biology, 45, p. 1-5.

42. Ii M., Yamamoto H., Adachi Y., Maruyama Y., Shinomura Y., (2006) Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Experimental Biology and Medicine (Maywood), 231, p. 20–7

43. J. Lin, Y. Zeng, J. Lin, J. Wang, L. Li, Z. Huang, B. Li, H. Zeng, R. Chen, (2014) Erythrocyte membrane analysis for type II diabetes detection using Raman spectroscopy in high-wavenumber region, Applied Physics Letters.

44. J.A., Menendez, L. Vellon, R. Colomer, (2005) Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically

enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu gene amplification.Annals Of Oncology, p. 359–371.

45. Llewellyn C.D., Jhonson L.W., and Warnakulasuriya K. A., (2004). Risk factors for oral cancer in newly diagnosed patients aged 45 years and younger: a case control study in Southern England. Journal of Oral Pathology and Medicine, 33(9), p. 525-532.
46. Jemal A., Tiwari R. C., Murray T., Ghafoor A., and Samuels A., (2004) Cancer statistics. CA: a cancer journal for clinicians, 54, p. 8–29.
47. José R. F. C., Érika C. P., Francisco C. Q., Francisco A. M. N., Cláudia A. R., and Silvia R. R., (2006) BMC Cancer, 6(48), p. 1471-2407.
48. Jonah D. K., and Jennifer R,G., (2010) The molecular pathogenesis of head and neck cancer. Cancer Biology and Therapy, 9(1), p. 1-7.
49. Jemal A., Siegel R., Xu J., Ward E., (2010) A Cancer Journal for Clinicians, 61(2), p. 133-4.
50. J.R. Ferraro, K. Nakamoto and C.W. Brown, (2003) Introductory Raman Spectroscopy (2nd ed., Academic Press, Boston.
51. Khuri F.R., Nemunaitis J., Ganly I., Arseneau J., Tannock I.F., Romel L., (2000) A controlled trial of intratumoral ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nature Medicine, 6. p. 879-85.
52. Keereweer S., Kerrebijn J.D.F., and van Driel P.B.A.A., (2011) Optical image-guided surgery-where do we stand? Molecular Imaging and Biology, 13(2), p. 199–207.
53. Kamal M.M., Arjune D.G., and Kulkarni H.R., (2002) Comparative study of fine needle aspiration and fine needle capillary sampling of thyroid lesions. Acta cytological, 46, p. 30-34.
54. King A.D., Vlantis A.C., Bhatia K.S., Zee B.C., Woo J.K., Tse G.M., Chan A.T., Ahuja A.T., (2011) Primary nasopharyngeal carcinoma: diagnostic accuracy of MR imaging versus that of endoscopy and endoscopic biopsy, 258(2), p. 531-7.
55. Kneipp K., Kneipp H., Itznak I., Dasari R.R., and Field M.S., (1999) Ultrasensitive chemical analysis by Raman spectroscopy, Chemical reviews, 99, p. 2957-2976

56. Kraft C., Knetschke T., Siegner A., Funk R.H.W., Salzer R., (2003) Vibrational Spectroscopy, 32, p. 75–83.

57. Krebs H.A., (1972) The Pasteur effect and the relations between respiration and fermentation. Essays in Biochemistry, 8, p. 1–34.

58. Liu Y., Borchert G.L., Surazynski A., Hu C.A., Phang J.M., (2006) Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling. Oncogene, 25, p. 5640–7.

59. Liu Y., Borchert G.L., Donald S.P., Diwan B.A., Anver M., Phang J.M., (2009) Proline oxidase functions as a mitochondrial tumor suppressor in human cancers. Cancer Research, 69, p. 6414–22.

60. Liu W., Zabirnyk O., Wang H., Shiao Y.H., Nickerson M.L., Khalil S., (2010) miR-23b targets proline oxidase, a novel tumor suppressor protein in renal cancer. Oncogene, 29, p. 4914–24.

61. Liu W., Le A., Hancock C., Lane A.N., Dang C.V., Fan T.W., (2012) Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. Proceedings Of The National Academy Of Sciences, 109, p. 8983–8.

62. Laviano A., Cascino A., Muscaritoli M., Fanfarillo F., and Rossi F., (2003). Tumor-induced changes in host metabolism: a possible role for free tryptophan as a marker of neoplastic disease. Advances In Experimental and Medical Biology, 527, p. 363-6.

63. Masood N., Malik F. A., and Kayani M. A., (2012) Unusual intronic variant in GSTP1 in head and neck cancer in Pakistan. Asian Pacific Journal of Cancer Prevention, 13(4), p. 1683-1686.

64. McLoughlin R.M., O'Morain C.A., (2006) Colorectal cancer screening. World Gastroenterol, 12(42), p. 6747-6750.

65. Marcu L.G., and Yeoh E., (2009) A review of risk factors and genetic alterations in head and neck carcinogenesis and implications for current and future approaches to treatment. Journal of Cancer Research and Clinical Oncology, 135, p. 1303–1314.

66. Merlo L.M., Pepper J.W., Reid B.J., (2006) Cancer as an evolutionary and ecological process. Nature Reviews Cancer, 6, p. 924-935.

67. Motz J.T., Gandhi S.J., Scepanovic O.R., Haka A.S., Kramer J.R., Dasari R.R., and Feld M.S., (2005) Real time Raman system for in vivo disease diagnosis, Journal Of Biomedical Optics, 10, p. 1-10.

68. M. Gasco, S. Shami and T. Crook, (2002) "The p53 pathway in breast cancer," Breast Cancer Research. 4, p. 70-76.

69. Molnar J., Robinson G. B., and Winzler, R. J., (1964) The Journal Of Biological Chemistry, 239, p. 3157.

70. Molnar J., Teegarden, D. W., and Winzler, R. J., (1965) Cancer Research., 25, p. 1860.

71. Mikuriya K., Kuramitsu Y., Ryozawa S., (2007) Expression of glycolytic enzymes is increased in pancreatic cancerous tissues as evidenced by proteomic profiling by two-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry. International Journal of Oncology, 30, p. 849–855.

72. Negri E., Franceschi S., Bosetti C., Levi F., Conti E., and Parpinel M., (2000) Selected micronutrients and oral and pharyngeal cancer. International Journal Of Cancer, 86, p. 122-7.

73. N.R. Simonsen, N.J. Fernandez-Crehuet, J.M. Martin-Moreno, (1998) Tissue stores of individual monounsaturated fatty acids and breast cancer: the EURAMIC study. European Community Multicenter Study on antioxidants, myocardial infarction, and breast cancer,The American Journal Of Clincal Nutrition, 68, p. 134–141.

74. Neugebauer U., Trenkmann S., Bocklitz T., Schmerler D., Kiehntopf M., and Popp J., (2014) Fast differentiation of SIRS and sepsis from blood plasma of ICU patients using Raman spectroscopy, Journal Of Biophotonics, 7, p. 232–240.

75. Osborne C., Wilson P., Tripathy D., (2004) Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. The Oncologist, 9, p. 361–377.

76. Pories S., Moses A., Lotz M., (2009). Cancer.

77. Perz J.F., Armstrong G.L., Farrington L.A,. (2006) The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. Journal Of Hepatology, 45, p. 529–38

78. Polyak K., Xia Y., Zweier J.L., Kinzler K.W., Vogelstein B. A., (1997) model for p53- induced apoptosis. Nature, 389, p. 300–5.

79. Pandhare J., Donald S.P., Cooper S.K., Phang J.M., (2009) Regulation and function of proline oxidase under nutrient stress. Journal Of Cellular Biochemistry, 107, p. 759–68

80. Pandhare J., Cooper S.K., Phang J.M., (2006) Proline oxidase, a proapoptotic gene, is induced by troglitazone: evidence for both peroxisome proliferator-activated receptor gamma-dependent and -independent mechanisms. The Journal Of Biological Chemistry, 281, p. 2044–52.

81. Ping H. C., Tien Y. S., Pei S. H., Chi C. T., Yi H. Y., Ying, C. L., Min-Shan K., Pei-Chien T., Shang-Lun C., Hung-Pin T., and Ying-Chin K., (2007). Prognostic factors associated with the survival of oral and pharyngeal carcinoma in Taiwan. BMC Cancer, (7), p. 101.

82. Qi D., and Berger A.J., (2007) Chemical concentration measurement in blood serum and urine samples using liquid-core optical fiber Raman spectroscopy, Applied Optics, 46, p. 1726–1734.

83. Rodier F., Campisi J., and Bhaumik D., (2007) Two faces of p53: aging and tumor suppression. Nucleic Acids Research, 35(22), p. 7475–7484.

84. Robsahm T.E., Aagnes B., Hjartåker A., Langseth H., Bray F.I., and Larsen I.K., (2013) Body mass index, physical activity, and colorectal cancer by anatomical subsites: a systematic review and meta-analysis of cohort studies, European Journal of Cancer Prevention, 22, p. 492-505.

85. Rath, T., Roth, E., Keidl, R. and Meissl, G. (1987) Phenylalanine – Total Amino-Acid ratio in 45 burn patients. Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery, 21, p. 297-300.

86. Roth E., Zoch G., Schulz F., Karner J., Muhlbacher F., Hamilton G., Mauritz W., Sporn P., and Funovics J., (1985) Amino-Acid concentrations in plasma and skeletal-

muscle of patients with acute hemorrhagic necrotizing pancreatitis. Clinical Chemistry, 31, p. 1305-1309.

87. R. L. Pecsok L. D. Shields, Modern Methods of Chemical Analysis (Wiley, New York, 1968); and A.T. Schwartz et al., Chemistry in Context (American Chemical Society, Washington, DC 1994.
88. Racker E., and Wu R., (1958) Limiting factors in glycolysis of ascites tumour cells and the pasteur effect. Regulation of Cell Metabolism, p. 205–229.
89. Sulli G., Micco R., and Fagagna F., (2012) Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. Nature Reviews Cancer, 12, p. 709-720.
90. Shackney S.E., and Silverman J.F., (2003) Molecular evolutionary patterns in breast cancer. Advances in Anatomic Pathology, 10, p. 278–290.
91. Toefil L., Oana C.T., Horea A. A., and Ovidiu M., (2007). Head and neck cancer, epidemiology and histological aspects – Part 1: A decade's results 1993- 2002. Journal of Cranio-Maxillofacial Surgery, 35(2), p. 120-125.
92. Saif M.W., (2008) 18F-FDG positron emission tomography CT (FDG PET-CT) in the management of pancreatic cancer: initial experience in 12 patients. Journal Of Gastrointestinal and Liver Diseases, 17(2), p. 173–8.
93. Singh R., and Riess F., (1998) Raman and the story of the Nobel Prize. Current Science, 75 (9), p. 965–971.
94. Sikirzhytski V., Virkler K., and Lednev., (2010) Discriminant Analysis of Raman Spectra for Body Fluid Identification for Forensic Purposes. Sensors, 10, p. 2869-2884.
95. Sacré P.Y., Lebrun P., Chavez P.F., Bleye C.D., Netchacovitch L., Rozet E., Klinkenberg R., Streel B., Hubert P., and Ziemons E., (2014) A new criterion to assess distributional homogeneity in hyperspectral images of solid pharmaceutical dosage forms, Analytica Chimica Acta, 818, p. 7–14.
96. Shao X., Zheng W., and Huang Z., (2011) In vivo diagnosis of colonic precancer and cancer using near-infrared autofluorescence spectroscopy and biochemical modeling, Journal Of Biomedical Optics, 16(6), p. 1-8.

97. Stone N., Kendall C., Smith J., Crow P., Barr H., (2004) Raman spectroscopy for identification of epithelial cancers, 126141–157, p. 169–83

98. Turati F., Edefonti V., Bosetti C., Ferraroni M., Malvezzi M., Franceschi S., Talamini R., Montella M., Levi F., Dal-Maso L., Serraino D., Polesel J., Negri E., Decarli A., and La-Vecchia C., (2013) Family history of cancer and the risk of cancer: a network of case-control studies, Annuals of Oncology, 24, p. 2651-2656.

99. Tangka F.K., Trogdo J.G., and Richardson L.C., (2010) Cancer treatment cost in the United States: has the burden shiftedover time? Cancer, 116(14), p. 3477–3484.

100. Tankiewicz A., Dziemiańczyk D., Buczko P., Szarmach I. J., Grabowska S. Z. & PAWLAK D., (2006) Tryptophan and its metabolites in patients with oral squamous cell carcinoma: preliminary study. Advances in medical sciences, 51 Suppl 1, p. 221-224.

101. T. Vo-Dinh: Biomedical Photonics Handbook (CRC Press, Boca Raton, 2003).

102. Utzinger U., Heintzelman D., Mahadevan-Jansen A., Malpica A., Follen M., and Richards-Kortum R., (2001) Near-infrared Raman spectroscopy for in vivo detection of cervical precancers, Applied Spectroscopy, 55(8), p. 955–959.

103. Uskokovic-Markovic, Jelikic-Stanko S.M., I-Holclajtner-Antunovic, and Durdevic P. (2013) , Raman spectroscopy as a new biochemical diagnostic tool, Journal of Medical Biochemistry, 32, p. 96-103.

104. World Health Organization. World cancer report 2008. Lyon (France): IARC; 2008.

105. Wechalekar K., Sharma B., and Cook G., (2005) PET/CT in oncology--a major advance. Clinical Radiology, 60(11), p. 1143–5.

106. William LJ. and Abdi H. (2010) Principal Component Analysis, Wiley Interdisciplinary Review: Computational Statistics, 1, p. 1-47.

107. Warburg O. Uber den Stoffwechsel der Tumoren. ¨ London, U.K.: Constable; 1930.

108. Yıgit M.V., Zhu L., and Ifediba M.A., (2013) Noninvasive MRI-SERS imaging in living mice using an innately bimodal nanomaterial. ACS Nano, 5, p. 1056–1066

109. Ye J., Janardan R., and Li Q., (2005). Two dimensional linear Discriminant Analysis, Advances in Neural Information Processing Systems, 17, p. 1569-1576.

PROFORMA

Raman Spectroscopy: Non-Invasive Technique for Cancer Detection

Informed Consent:

I am informed that I am participating in a study which may or may not benefit me directly, but will provide new knowledge which could benefit other patients with similar conditions to mine in the future. I understand that I will give a blood sample about 5 ml which will be used in this study. I am donating blood for research purpose only and not for commercial use. Giving this blood sample will not adversely affect health of the participants of the study in any way.

مجھے مطلع کیا گیا ہےکہ میں اپنا خون اس ریسرچ کیلے دونگی\ دونگا تو اس سے میرے جیسےمریضوں کو فایدہ
پہنچے گا اور یہ کہ مجھے اس سے کوئ نقصان نہیں ہو گا۔ اس خون کی مقدار
10ملی لیٹر ہو گی .
میں خون کا عطیہ صرف تحقیقی مقاصد کے لئے کر رہا ہوں.

Patient: **Date:**

Guardian:

Sponser:

Detection

Patient Information

Name:		
Age:	Sex: M F	
Marital status:	Spouse's name:	No. of Children:
City:	Phone #	
Hospital:	Patient's PRN number:	

Type of Cancer	Lung Cancer	Head & Neck Cancer	Colorectal Cancer
Stage/Grade			
Charaterization/ Subtypes of Cancers	○ Non-Small Cell Lung Cancer (NSCLC) 1. Adenocarcinoma 2. Squamous Cell Carcinoma (Epidermoid Carcinoma) ○ Small Cell Lung Cancer (SCLC)	○ Oral Cavity ○ Larynx ○ Trachea ○ Oropharynx ○ Nasopharynx ○ Hypopharynx	○ Adenocarcinoma ○ Lymphomas ○ Gastrointestinal stromal Tumors (GSTs) ○ Carcinoid Tumors ○ Sarcomas

1) **Any experience of working in Plastic /dye/ chemicals/ asbestos/ radiation based industry?**

(Yes / No)

2) **Case status:** Newly diagnosed / old case

3) **Does the person smoke?** (Yes / No)

If yes, then specify duration of smoking _____ years

Specify type: Cigarettes (packs/day)\Hookah\Cigar\Naswar\Biri\Pan\Gutka

4) **Does person have any history of HBV\ HCV\ HPV\ EBV viral infection?**

(HPV\HBV \ HCV \ EBV)

5) **Does patient have any other health problem like diabetes, high/low blood pressure?** (Yes / No)

6) **Does the patient have any other type of cancer? (Yes/ No)**

If yes, then specify cancer type _____

7) **Age of onset of disease _____ years.**

8) **Does patient have family history of cancer? (Yes/No)**

26. MCV: _____

27. MCH: _____

28. MCHC: _____

29. Platelet count: _____

30. Reticulocyte count: _____

Turnitin Originality Report

Raman Spectroscopy Non invasive Technique for cancer detection by Samina Javaid

From Samina Javaid (MS)

Similarity Index	Similarity by Source	
9%	Internet Sources	8%
	Publications	7%
	Student Papers	1%

Processed on 13-Jul-2016 13 37 PKT
ID 689382939
Word Count 10219

sources:

1 2% match (publications)
Pence, Isaac. "Raman Spectroscopy: From Benchtop to Bedside." Biomedical Photonics Handbook, Second Edition, 2014

2 2% match (Internet from 13-Apr-2016)
http://etheses.whiterose.ac.uk/8375/1/Thesis%20Salman%20Mian%202.pdf

3 2% match (Internet from 11-Dec-2009)
http://www.utd.edu/~herve/abdi-wireCS-PCA2010-inpress.pdf

4 2% match (Internet from 07-Oct-2014)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3295905/

5 2% match (Internet from 20-Nov-2013)