IDENTIFICATION OF POTENTIAL BIOMARKERS IN CERVICAL CANCER

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CERTIFICATE

This is to certify that the work titled "**Identification of potential biomarkers in cervical cancer**" submitted by "**Mishti Chaudhary** and **Shweta Kaundal**" in partial fulfillment for the award of degree of 5 Year Dual Degree Program B.Tech-M.Tech of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Date

SUMMARY

Biomarkers of cancer are becoming a key need in the diagnosis and prognosis of the disease for the better survivability of the patients..Mass spectral-based proteomic technologies are ideally suited for the discovery of protein biomarkers in the absence of any prior knowledge of quantitative changes in protein levels. The success of any biomarker discovery effort will depend upon the quality of samples analysed, the ability to generate quantitative information on relative protein levels and the ability to readily interpret the data generated. Advances in protein analytical technologies are finding broad application in drug discovery programmes. One area of intense interest is in the area of biomarker discovery. Biomarkers are indicators of a biological process and may be genes, proteins, small molecules or metabolites. Numerous strategies have been applied in the area of biomarker discovery. Mass spectral technologies have been widely used in concert with separations technologies such as two-dimensional polyacrylamide gels and chromatographic techniques. Mass spectrometry has also been used to identify and quantify proteins in complex mixtures without prior separations. While quite powerful, protein changes identified by these strategies will still only provide candidate biomarkers. Validation of these candidates will require sophisticated bioinformatics technologies to analyse the data and assign statistical significance and confidence to protein measurements. Validation will ultimately provide tools by which experts in the biology of a particular system may visualise the data and draw meaningful conclusions as to the context of a biomarker or panel of biomarkers. This report will focus on strategies for the identification of protein biomarker candidates, with emphasis on the issues surrounding the adaptation of separations and identification technologies to high throughput analysis of complex biological fluids.

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Chapter 1

Introduction

Despite major advances in diagnostic imaging technology, surgical management, and therapeutic strategies, cancer remains a major cause of mortality. Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008 (1). Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 (2). The race to obtain control over the disease process is gaining speed and focus. Advances in the fields of genomics and proteomics are hoped to provide insights into the molecular complexity of the disease process and thus enable the development of tools to help in treatment as well as in detection and prevention. Genomics-based approaches to biomarker development include the measurements of expression of full sets of mRNA, such as differential display (3,4), serial analysis of gene expression (5,6), and large-scale gene expression arrays. However, interpreting the best data and adapting the results to a particular application remain challenging. Although studies of differential mRNA expression are informative, they do not always correlate with protein concentrations (7,8). Proteins are often subject to proteolytic cleavage or posttranslational modifications, such as phosphorylation or glycosylation. Cancer biomarker discovery strategies that target expressed proteins are becoming increasingly popular because proteomic approaches characterize the proteins, modified or unmodified, involved in cancer progression. Unlike genomics, however, acquiring proteomic data faces technical challenges, particularly with regard to lack of standardized methodologies, sensitivity and reproducibility. Nevertheless, these obstacles can be overcome with the current research efforts to develop high-resolution proteomic instrumentation for high-throughput monitoring of protein changes that occur in cancer. (9,10).

Among the important tools critical to detection, diagnosis, treatment, monitoring, and prognosis are biomarkers. Biomarkers are biological molecules that are indicators of physiologic state and also of change during a disease process (11). The utility of a biomarker lies in its ability to provide an early indication of the disease, to monitor disease progression, to provide ease of detection, and to provide a factor measurable across populations. Ultimately, biomarkers offer the promise of more efficient discovery and development of novel therapies as well as improved and more individualized disease prevention and treatment. And one of the most valuable classes of biomarker over the past century has been protein biomarkers. Although a number of protein

biomarkers have found their way into clinical practice, very few of these have been discovered by proteomics approaches. Reasons for this situation include the significant time and cost required to validate, or invalidate, the markers suggested by proteomics experiments. The availability of fast, cost-effective, mass spectrometry-based validation approaches would change that situation.

Current progress in proteomics has been largely due to recent developments in mass spectrometry (MS)-based technologies (12). Particularly, new techniques for the ionization of proteins and peptides, such as matrix-assisted laser desorption-ionization (MALDI) and electrospray ionization (ESI) combined with time-of-flight (TOF), as well as new hybrid mass spectrometers, are now becoming the tools of choice for protein characterization.

Chapter 2

Review of literature

Cervical cancer is a malignant neoplasm arising from cells originating in the cervix uteri. One of the most common symptoms of cervical cancer is abnormal vaginal bleeding, but in some cases there may be no obvious symptoms until the cancer has progressed to an advanced stage. Treatment usually consists of surgery (including *local excision*) in early stages, and chemotherapy and/or radiotherapy in more advanced stages of the disease

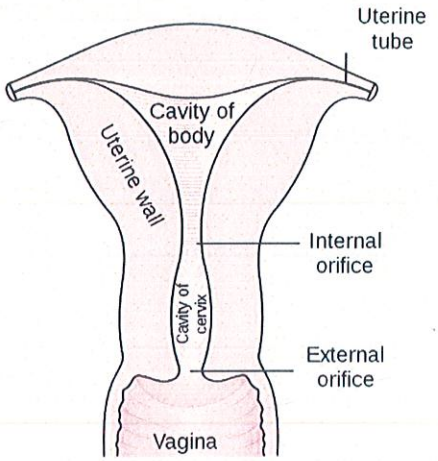


Fig. 1

Cancer screening using the Pap smear can identify precancerous and potentially precancerous changes in cervical cells and tissue. Treatment of high-grade changes can prevent the development of cancer in many victims. In developed countries, the widespread use of cervical screening programs has dramatically reduced the incidence of invasive cervical cancer.

Human papilloma virus (HPV) infection appears to be a necessary factor in the development of almost all cases (90+%) of cervical cancer. HPV vaccines effective against the two strains of this large family of viruses that currently cause approximately 70% of cases of cervical cancer have been licensed in the U.S, Canada, Australia, and the EU. Since the vaccines only cover some of the cancer-causing ("high-risk") types of HPV, women should seek regular Pap smear screening, even after vaccination.

The cervix is the narrow portion of the uterus where it joins with the top of the vagina. Most cervical cancers are squamous cell carcinomas, arising in the squamous (flattened) epithelial

cells that line the cervix. Adenocarcinoma, arising in glandular epithelial cells is the second most common type. Very rarely, cancer can arise in other types of cells in the cervix.

Signs and symptoms

The early stages of cervical cancer may be completely asymptomatic. Vaginal bleeding, contact bleeding, or (rarely) a vaginal mass may indicate the presence of malignancy. Also, moderate pain during sexual intercourse and vaginal discharge are symptoms of cervical cancer. In advanced disease, metastases may be present in the abdomen, lungs or elsewhere.

Symptoms of advanced cervical cancer may include: loss of appetite, weight loss, fatigue, pelvic pain, back pain, leg pain, and swollen legs, heavy bleeding from the vagina, bone fractures, and/or (rarely) leakage of urine or faeces from the vagina.

Causes and risk factors

Infection with some types of human papilloma virus (HPV) is the greatest risk factor for cervical cancer, followed by smoking. Other risk factors include human immunodeficiency virus. Not all of the causes of cervical cancer are known, however, and several other contributing factors have been implicated.

Human papillomavirus

Human papillomavirus type 16 and 18 are the cause of 70% of cervical cancer globally while 31 and 45 are the cause of another 10%[1]. Women who have many sexual partners (or who have sex with men who have had many other partners) have a greater risk. Of the 150-200 types of HPV known, 15 are classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 as probable high-risk (26, 53, and 66), and 12 as low-risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108).

Genital warts, which are a form of benign tumor of epithelial cells, are also caused by various strains of HPV. However, these serotypes are usually not related to cervical cancer. It is common to have multiple strains at the same time, including those that can cause cervical cancer along with those that cause warts. The medically accepted paradigm, officially endorsed by the American Cancer Society and other organizations, is that a patient must have been infected with HPV to develop cervical cancer, and is hence viewed as a sexually transmitted disease (although many dispute that, technically, it is the causative agent, not the cancer, that is a sexually transmitted disease), but most women infected with high risk HPV will not develop cervical cancer. Use

of condoms reduces, but does not always prevent transmission. Likewise, HPV can be transmitted by skin-to-skin-contact with infected areas. In males, there is no commercially available test for HPV, although HPV is thought to grow preferentially in the epithelium of the glans penis, and cleaning of this area may be preventative.

Smoking

Smoking has also been linked to the development of cervical cancer. There are a few different ways that smoking can increase the risk of cervical cancer in women which can be by direct and indirect methods of inducing cervical cancer. A direct way of contracting this cancer is a female smoker has a higher chance of CIN3 occurring which has the potential of forming cervical cancer. When CIN3 lesions lead to cancer, most of them have the assistance of the HPV virus, but that is not always the case which is why it can be considered a direct link to cervical cancer. An indirect means of developing this cancer by smoking is that it can lead to human papillomavirus which can result in cervical cancer. Heavy smoking and long term smoking seem to have more of a risk of getting the CIN3 lesions than lighter smoking or not smoking at all. Although smoking has been linked to cervical cancer, it aids in the development of HPV which is the leading cause of this type of cancer. Also, not only does it aid in the development of HPV, but if the woman is already HPV-positive she is at an even greater likelihood of contracting cervical cancer.

Diagnosis

Biopsy

While the pap smear is an effective screening test, confirmation of the diagnosis of cervical cancer or pre-cancer requires a biopsy of the cervix. This is often done through colposcopy, a magnified visual inspection of the cervix aided by using a dilute acetic acid (e.g. vinegar) solution to highlight abnormal cells on the surface of the cervix.[1] Medical devices used for biopsy of the cervix include punch forceps, Spira Brush CX, Soft Biopsy. Colposcopic impression, the estimate of disease severity based on the visual inspection, forms part of the diagnosis. Further diagnostic and treatment procedures are loop electrical excision procedure (LEEP) and conization, in which the inner lining of the cervix is removed to be examined pathologically. These are carried out if the biopsy confirms severe cervical intraepithelial neoplasia.

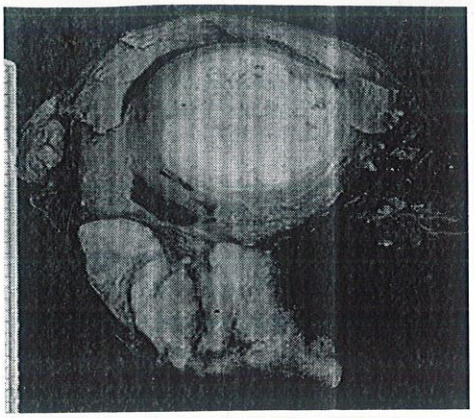


Fig. 2

Precancerous lesions

Cervical intraepithelial neoplasia, the potential precursor to cervical cancer, is often diagnosed on examination of cervical biopsies by a pathologist. For premalignant dysplastic changes, the CIN (cervical intraepithelial neoplasia) grading is used.

The naming and histologic classification of cervical carcinoma percursor lesions has changed many times over the 20th century. The World Health Organization classification system was descriptive of the lesions, naming them mild, moderate or severe dysplasia or carcinoma in situ (CIS). The term, Cervical Intraepithelial Neoplasia (CIN) was developed to place emphasis on the spectrum of abnormality in these lesions, and to help standardise treatment. It classifies mild dysplasia as CIN1, moderate dysplasia as CIN2, and severe dysplasia and CIS as CIN3. More recently, CIN2 and CIN3 have been combined into CIN2/3. These results are what a pathologist might report from a biopsy.

These should not be confused with the Bethesda System terms for Pap smear (cytopathology) results. Among the Bethesda results: Low-grade Squamous Intraepithelial Lesion (LSIL) and High-grade Squamous Intraepithelial Lesion (HSIL). An LSIL Pap may correspond to CIN1, and HSIL may correspond to CIN2 and CIN3, however they are results of different tests, and the Pap smear results need not match the histologic findings.

Cancer subtypes

Histologic subtypes of invasive cervical carcinoma include the following: Though squamous cell carcinoma is the cervical cancer with the most incidence, the incidence of adenocarcinoma of the cervix has been increasing in recent decades.

- squamous cell carcinoma (about 80-85%)
- adenocarcinoma (about 15% of cervical cancers in the UK)
- adenosquamous carcinoma

- small cell carcinoma
- neuroendocrine tumour
- glassy cell carcinoma
- villoglandular adenocarcinoma

Non-carcinoma malignancies which can rarely occur in the cervix include

- melanoma
- lymphoma

Prevention

Screening

The widespread introduction of cervical screening by the Papanicolaou test, or *Pap smear* for cervical cancer has been credited with dramatically reducing the incidence and mortality of cervical cancer in developed countries. Pap smear screening every 3–5 years with appropriate follow-up can reduce cervical cancer incidence by up to 80%. Abnormal results may suggest the presence of pre cancerous changes allowing examination and possible preventive treatment. If precancerous disease or cervical cancer is detected early, it can be monitored or treated relatively noninvasively, with little impairment of fertility.

Cervical cancer screening is typically recommended starting at age 21. Recommendations for how often a Pap smear should be done vary from once a year to once every five years, in the absence of abnormal results.Guidelines vary on how long to continue screening, but well screened women who have not had abnormal smears can stop screening about age 60 to 70.

Liquid-based cytology is another potential screening method. Although it was probably intended to improve on the accuracy of the Pap test, its main advantage has been to reduce the number of inadequate smears from around 9% to around 1%. This reduces the need to recall women for a further smear. The United States Preventive Services Task Force supports screening every 5 years in those who are between 30 and 65 years when cytology is used in combination with HPV testing.

Vaccination

There are two HPV vaccines (Gardasil and Cervarix) which reduce the risk of cancerous or precancerous changes of the cervix and perineumby about 93%. HPV vaccines are typically given to women age 9 to 26 as the vaccine is only effective if given before infection occurs.

Vaccines have been shown to be effective for at least 4 to 6years, and it is believed they will be effective for longer; however, the duration of effectiveness and whether a booster will be needed is

unknown. The high cost of this vaccine has been a cause for concern. Several countries have considered (or are considering) programs to fund HPV vaccination.

Treatment

The treatment of cervical cancer vairies worldwide, largely due to large variances in disease burden in developed and developing nations, access to surgeons skilled in radical pelvic surgery, and the emergence of "fertility sparing therapy" in developed nations. Because cervical cancers are radiosensitive, radiation may be used in all stages where surgical options do not exist.

Microinvasive cancer (stage IA) may be treated by hysterectomy (removal of the whole uterus including part of the vagina). For stage IA2, the lymph nodes are removed as well. Alternatives include local surgical procedures such as a loop electrical excision procedure (LEEP) or cone biopsy For 1A1 disease, a cone biopsy (aka *cervical conization*) is considered curative.

If a cone biopsy does not produce clear margins (findings on biopsy showing that the tumor is surrounded by cancer free tissue, suggesting all of the tumor is removed), one more possible treatment option for patients who want to preserve their fertility is atrachelectomy. This attempts to surgically remove the cancer while preserving the ovaries and uterus, providing for a more conservative operation than a hysterectomy. It is a viable option for those in stage I cervical cancer which has not spread; however, it is not yet considered a standard of care, as few doctors are skilled in this procedure. Even the most experienced surgeon cannot promise that a trachelectomy can be performed until after surgical microscopic examination, as the extent of the spread of cancer is unknown. If the surgeon is not able to microscopically confirm clear margins of cervical tissue once the patient is under general anesthesia in the operating room, a hysterectomy may still be needed. This can only be done during the same operation if the patient has given prior consent. Due to the possible risk of cancer spread to the lymph nodes in stage 1b cancers and some stage 1a cancers, the surgeon may also need to remove some lymph nodes from around the uterus for pathologic evaluation.

A radical trachelectomy can be performed abdominally or vaginally and there are conflicting opinions as to which is better. A radical abdominal trachelectomy with lymphadenectomy usually only requires a two to three day hospital stay, and most women recover very quickly (approximately six weeks). Complications are uncommon, although women who are able to conceive after surgery are susceptible to preterm labor and possible late miscarriage. It is generally recommended to wait at least one year before attempting to become pregnant after surgery. Recurrence in the residual cervix is very rare if the cancer has been cleared with the trachelectomy. Yet, it is recommended for patients to practice vigilant prevention and follow up care including pap screenings/colposcopy,

with biopsies of the remaining lower uterine segment as needed (every 3–4 months for at least 5 years) to monitor for any recurrence in addition to minimizing any new exposures to HPV through safe sex practices until one is actively trying to conceive.

Early stages (IB1 and IIA less than 4 cm) can be treated with radical hysterectomy with removal of the lymph nodes or radiation therapy. Radiation therapy is given as external beam radiotherapy to the pelvis and brachytherapy (internal radiation). Patients treated with surgery who have high risk features found on pathologic examination are given radiation therapy with or without chemotherapy in order to reduce the risk of relapse.

Larger early stage tumors (IB2 and IIA more than 4 cm) may be treated with radiation therapy and cisplatin-based chemotherapy, hysterectomy (which then usually requires adjuvant radiation therapy), or cisplatin chemotherapy followed by hysterectomy.

Incidence rate

Every year cervical cancer is diagnosed in about 500,000 women globally and is responsible for more than 280,000 deaths annually. There is a wide variation in the incidence of cervical cancer across the globe. In the west, early detection through regular screening has aided to significantly control the prevalence of this disease, thereby, lowering its incidence. In the last 50 years in the United States, the Pap smear tests have reduced the deaths related to cervical cancer by three-quarters. At one time cervical cancer was one of the most dreaded cancer and the leading causes of of death in women in the US but now it is the eighth most common cancer there. 80% of the new cervical cancer cases occur in developing countries, like India, which reports approximately one fourth of the world's cases of cervical cancer each year.

There has been a regular campaign against cervical canal for 30 years in India, but this has had little impact on the morbidity and mortality from the disease, with India ranking fourth worldwide. The number of deaths due to cervical cancer is estimated to rise to 79,000 by the year 2010. The cancer mostly affects middle- aged women (between 40 and 55 years), especially those from the lower economic status who fail to carry out regular health check-ups due to financial inadequacy. In urban areas, cancer of the cervix account for over 40% of cancers while in rural areas it accounts for 65% of cancers as per the information from the cancer registry in Barshi. Eastern and South Africa, Central and South America and the Caribbeans too report very high incidence of cervical cancer.

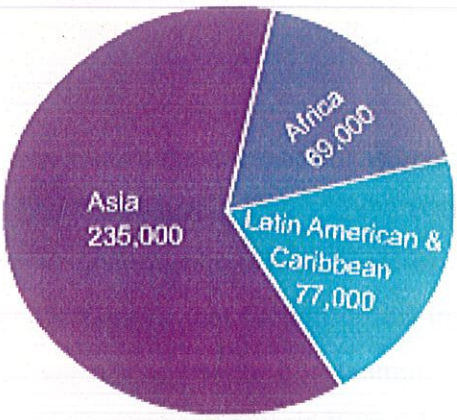


Figure. 3

Prognosis

Prognosis depends on the stage of the cancer. With treatment, the 5-year relative survival rate for the earliest stage of invasive cervical cancer is 92%, and the overall (all stages combined) 5-year survival rate is about 72%. These statistics may be improved when applied to women newly diagnosed, bearing in mind that these outcomes may be partly based on the state of treatment five years ago when the women studied were first diagnosed. With treatment, 80 to 90% of women with stage I cancer and 60 to 75% of those with stage II cancer are alive 5 years after diagnosis. Survival rates decrease to 30 to 40% for women with stage III cancer and 15% or fewer of those with stage IV cancer 5 years after diagnosis. According to the International Federation of Gynecology and Obstetrics, survival improves when radiotherapy is combined with cisplatin-based chemotherapy. As the cancer metastasizes to other parts of the body, prognosis drops dramatically because treatment of local lesions is generally more effective than whole body treatments such as chemotherapy.Interval evaluation of the patient after therapy is imperative. Recurrent cervical cancer detected at its earliest stages might be successfully treated with surgery, radiation, chemotherapy, or a combination of the three. Thirty-five percent of patients with invasive cervical cancer have persistent or recurrent disease after treatment. Average years of potential life lost from cervical cancer are 25.3 (SEER Cancer Statistics Review 1975-2000, National Cancer Institute (NCI)). Approximately 4,600 women were projected to die in 2001 in the US of cervical cancer (DSTD), and the annual incidence was 13,000 in 2002 in the US, as calculated by SEER. Thus the ratio of deaths to incidence is approximately 35.4%.Regular screening has meant that pre cancerous changes and early stage cervical cancers have been detected and treated early.

Biomarkers

A **biomarker**, or **biological marker**, is an indicator of a biological state. It is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In medicine, a biomarker can be a substance that is introduced into an organism as a means to examine organ function or other aspects of health. For example, rubidium chloride is used as a radioactive isotope to evaluate perfusion of heart muscle.It can also be a substance whose detection indicates a particular disease state, for example, the presence of an antibody may indicate an infection. More specifically, a biomarker indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment.

A biomarker can also be used to indicate exposure to various environmental substances in epidemiology and toxicology. In these cases, the biomarker may be the external substance itself (e.g. asbestos particles or NNK from tobacco), or a variant of the external substance processed by the body (a metabolite).

In cell biology, a biomarker is a molecule that allows for the detection and isolation of a particular cell type (for example, the protein Oct-4 is used as a biomarker to identifyembryonic stem cells).

In genetics, a biomarker (identified as genetic marker) is a DNA sequence that causes disease or is associated with susceptibility to disease.

Cervical cancer develops over a long time through precursor lesions that can be detected by cytological screening. Majority of these lesions regress spontaneously. Therefore, the challenge of cervical cancer screening is to detect the lesions that have a high risk of progression. Several promising biomarkers have been described that may improve screening of cervical cancer, but to date, new biomarkers have not been thoroughly validated in high-quality studies. The knowledge about human papillomavirus as a causative agent of cervical cancer has accumulated over the last decades has opened the possibility to improve the existing prevention strategies and screening practices. p16 has amply been applied on cytologic samples and has been shown to be a promising marker especially in identification of high-grade dysplasia. ProEx C, a replication marker, has also been recently shown to be a good marker for identification of high-grade dysplasia and has been used on cytologic samples. Proliferation markers such as MYC, Ki67, telomerase, MCM,

topoisomerase 2A and 3q amplification by in situ hybridization technique are other methods being employed in identification of high-grade dysplasia. However, currently available data on most of the biomarkers does not warrant their routine use yet.

Biomarker requirements

For chronic diseases, whose treatment may require patients to take medications for years, accurate diagnosis is particularly important, especially when strong side effects are expected from the treatment. In these cases, biomarkers are becoming more and more important, because they can confirm a difficult diagnosis or even make it possible in the first place. A number of diseases, such as Alzheimer's disease or rheumatoid arthritis, often begin with an early, symptom-free phase. In such symptom-free patients there may be more or less probability of actually developing symptoms. In these cases, biomarkers help to identify high-risk individuals reliably and in a timely manner so that they can either be treated before onset of the disease or as soon as possible thereafter.

In order to use a biomarker for diagnostics, the sample material must be as easy to obtain as possible. This may be a blood sample taken by a doctor, a urine or saliva sample, or a drop of blood like those diabetes patients extract from their own fingertips for regular blood-sugar monitoring.

For rapid initiation of treatment, the speed with which a result is obtained from the biomarker test is critical. A rapid test, which delivers a result after only a few minutes, is optimal. This makes it possible for the physician to discuss with the patient how to proceed and if necessary to start treatment immediately after the test.

Naturally, the detection method for a biomarker must be accurate and as easy to carry out as possible. The results from different laboratories may not differ significantly from each other, and the biomarker must naturally have proven its effectiveness for the diagnosis, prognosis, and risk assessment of the affected diseases in independent studies.

A biomarker for clinical use needs good Sensitivity and specificitsensitivity e.g. ≥ 0.9, and good specificity e.g. ≥ 0.9 although they should be chosen with the population in mind so positive predictive value and negative predictive value are more relevant.

Discovery of molecular biomarkers

Molecular biomarkers have been defined as biomarkers that can be discovered using basic and acceptable platforms such as genomics and proteomics.Many genomic and proteomics techniques are available for biomarker discovery and few recently using techniques are given below. Apart from genomics and proteomics platforms biomarker assay techniques, metabolomics, lipidomics, glycomics, and secretomics are the most commonly used as techniques in identification of biomarkers.

Genomic Approach

1.Northern blot

2.Gene expression

3.SAGE

4.DNA Microarray

Proteomic Approach

1.2D-PAGE

2.LC-MS

3.SELDI-TOF (or MALDI-TOF)

4.Ab Microarray

5.Tissue Microarray

Metabolomics Approach

The term metabolomics has been recently introduced to address the global analysis of all metabolites in a biological sample. A related term, metabonomics, was introduced to refer specifically to the analysis of metabolic responses to drugs or diseases. Metabonomics become a major area of research it is the complex system biological study,used as a to identify the biomarker for various disease. In general most of the disease case some of the metabolic pathway had been activate or deactivated,this parameter can be used as a marker for some diseases. Serotonin production pathway activated in alcoholic drinking person it can be metabolic marker of recent alcohol consumption

Lipidomics Approach

Lipidomics refers to the analysis of lipids. Since lipids have unique physical properties, they have been traditionally difficult to study. However, improvements in new analytical platforms have made it possible to identify and to quantify most of lipids metabolites from a single sample. Three key platforms used for lipid profiling include mass spectrometry, chromatography, and nuclear magnetic resonance. Mass spectrometry was used to delineate the relative concentration and composition of high-density lipoproteins (HDL) particles from lipid extracts isolated from coronary bypass patients and healthy volunteers. They found that HDL particles from coronary bypass patients contained significantly lesssphingomyelin relative to phosphadidylcholine and higher triglycerides relative to cholesterol esters. Lipidomic profiling was also used to study the effect of rosiglitazone, a PPARγ agonist, on lipid metabolism on mice. Rosiglitazone was observed to alter lipid composition in different organs. It increased triglycérides accumulation in the liver; altered free fatty acids in the heart, in the adipose tissue, and in the heart; and reduced triglyceride levels in plasma.

Potential disadvantages

Not all biomarkers should be used as surrogate endpoints to assess clinical outcomes. Biomarkers can be difficult to validate and require different levels of validation depending on their intended use. If a biomarker is to be used to measure the success of a therapeutic intervention, the biomarker should reflect a direct effect of that intervention.

Biomarkers in Cancer

Cancer biomarkers are present in tumor tissues or serum and encompass a wide variety of molecules, including DNA, mRNA, transcription factors, cell surface receptors, and secreted proteins. Cancer biomarkers can be used for prognosis: to predict the natural course of a tumor, indicating whether the outcome for the patient is likely to be good or poor (prognosis). They can also help doctors to decide which patients are likely to respond to a given drug (prediction) and at what dose it might be most effective (pharmacodynamics).

Importance of biomarkers in cervical cancer

Biomarkers help doctors know more about an individual person's tumor, allowing them to make better, more informed decisions about treatment options. Thus, the identification of biomarkers is the first step in personalizing cancer treatment. Biomarkers are grouped according to their type — protein and genetic — and function — screening, diagnostic aid, determining prognosis, guiding treatment, monitoring response to treatment and detecting recurrence or progression. Each function, except screening, has a role in personalizing cancer therapy.

In the recent years, knowledge about cancer biomarkers has increased tremendously providing great opportunities for improving the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment. Recent technological advancement has enabled the examination of many potential biomarkers and renewed interest in developing new biomarkers. Biomarkers of cancer could include a broad range of biochemical entities, such as nucleic acids, proteins, sugars, lipids, and small metabolites, cytogenetic and cytokinetic parameters as well as whole tumour cells found in the body fluid.

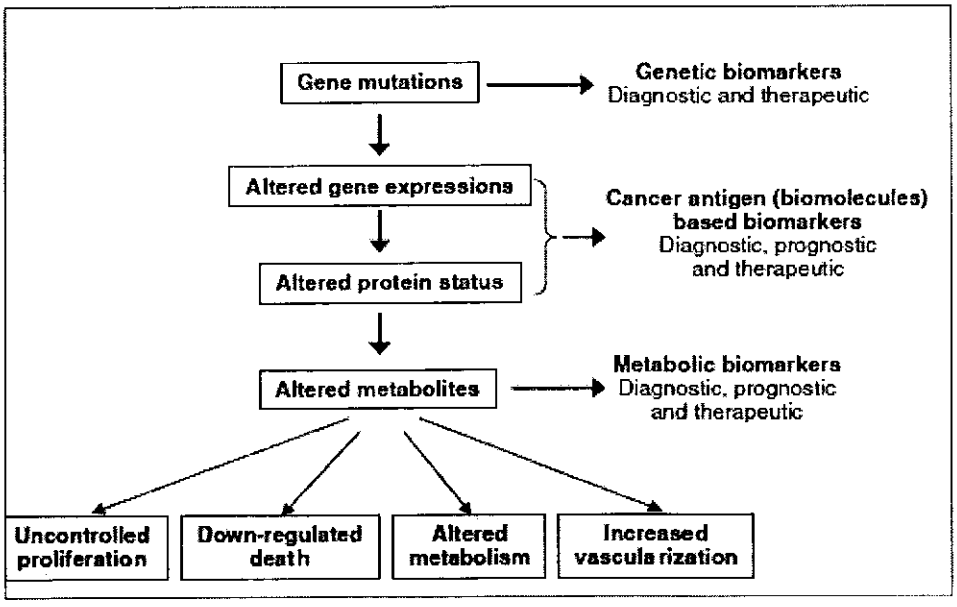


Figure. 4

Types of Biomarkers

Protein Biomarkers

Protein biomarkers include substances that are either produced by cancer cells themselves or by other cells in response to cancer. Most protein biomarkers related to cancer are used to monitor response and/or detect recurrence or progression during follow-up after treatment . Some biomarkers are used to predict the outcome, or prognosis.

Genetic Biomarkers

Several genetic alterations have become biomarkers because they are linked to the development of cancer. Genetic biomarkers offer the most benefit in guiding treatment decisions . A genetic biomarker is sometimes referred to as a pharmacogenetic marker because it affects the response or resistance to a drug. Again, one of the most well-known examples of a genetic biomarker is found in the setting of breast cancer: the HER2 gene.

Currently available markers for cervical cancer

Tumor markers that may be helpful in the management of patients with cervical cancer together with the phase of development for each marker as well as the level of evidence (LOE) for its clinical use . For squamous cell cervical cancer, squamous cell carcinoma antigen (SCC) is the marker of choice. Serum levels of SCC have been found to correlate with tumor stage, tumor size, residual tumor after treatment, recurrent or progressive disease, and survival in patients with squamous cell cervical cancer. Carcinoembryonic antigen (CEA) and CA125 in particular have demonstrated possible utility in patients with cervical adenocarcinoma. These guidelines focus on the use of SCC in squamous cell cervical cancer, because squamous cell cervical cancer is the most prevalent histologic type of cervical cancer and SCC seems the most promising marker. although SCC is not suitable for screening or diagnosis of cervical cancer, serum SCC levels correlate with tumor stage, tumor size, residual tumor after treatment, recurrent or progressive disease, and survival. Highly elevated pre-treatment SCC levels may indicate the presence of lymph node

metastases or extra-cervical spread, but a normal SCC level does not exclude the presence of lymph node metastases.

Pre-treatment SCC levels may be used to individualize treatment planning, in particular in patients with low stage squamous cell cervical cancer, but no randomised trials have yet been conducted to confirm this hypothesis. An elevated pre-treatment SCC level has been found to be an independent risk factor for poor survival in several studies. Whether pre-treatment SCC level is really useful in clinical practice remains uncertain. There is no evidence that more aggressive treatment improves pelvic control and survival in patients with elevated SCC levels. SCC shows a strong correlation with the clinical course and is suitable for monitoring disease after primary treatment, and may therefore be useful in the management of patients. However, there is as yet no evidence that earlier detection of recurrent disease influences treatment outcome or prognosis after primary treatment.

Cancer Marker	Proposed Use	Phase of Development	LOE
SCC	Pretreatment identification of high-risk group with lymph node metastases in squamous cell cervical cancer	Needs further evaluation for clinical usefulness	III
	Pretreatment prediction of prognosis in squamous cell cervical cancer	Independent prognostic value in several studies, not validated for individualizing treatment	III
	Prediction of response to treatment in squamous cell cervical cancer	Needs further evaluation	IV
	Monitoring disease and detecting recurrent disease in squamous cell cervical cancer	Strong correlation with course of disease, in clinical use in some centers	III
CA125	Pretreatment prediction of prognosis, in particular in cervical adenocarcinoma	Needs further evaluation	III–IV
	Preoperative prediction of the presence of lymph node metastases, in particular in cervical adenocarcinoma	Needs further evaluation	III–IV
	Monitoring disease, in particular in cervical adenocarcinoma	Needs further evaluation	IV
CEA	Pretreatment prediction of prognosis	Results conflicting, needs further evaluation	III–IV
	Preoperative prediction of the presence of lymph node metastases, in particular in cervical adenocarcinoma	Needs further evaluation	III–IV
	Pretreatment prediction of clinical response to neoadjuvant chemotherapy	Needs further evaluation	IV
Cytokeratins (TPA, TPS, cyfra 21-1)	Pretreatment prediction of prognosis	Needs further evaluation, results conflicting	III–IV
	Monitoring disease after primary treatment	Needs further evaluation, results conflicting	III-IV

Figure 6

Chapter 3

Materials and methods

Sample Collection

The cancer samples were collected from the pathology laboratory at Kanpur. There were 10 different cancer tissue samples in totality.

Homogenization of cancer tissue sample

Reagents used:

Table 1: Tissue Lysis Buffer

Reagents	Stock	100ml	Final conc.	Vol. for 10 ml
Tris HCl	0.5 M	10 ml	50 mM	1 ml
EDTA	0.5 M	400 µl	2 mM	0.04 ml
EGTA	0.5 M	200 µl	1 mM	0.02 ml
Glycerol	-	10 ml	10%	1 ml
Tween 80	-	100 µl	0.1%	0.01 ml
DTT	0.5 M	200 µl	1 mM	0.02 ml
PMSF	0.1 M	1 ml	1 mM	0.1 ml
PIC	-	200 µl	-	20 µl
NaCl	-	3.522 g	500 mM	0.2922 g

Protocol

1. Add 1ml whole cell lysis(WCL) extraction buffer per 100mg of tissue.
2. Homogenize tissues.
3. Transfer to 1.2ml eppendof tubes.
4. Spin at 13,000 rpm for 10 minutes at 4°C.
5. Remove the supernatant and save in another tube.

6. If necessary centrifuge supernatant again.

7. Measure protein concerntration with Lowry's method.

Lowry's method of protein estimation

Reagents used in Lowry's Method

A- 2% Sodium Carbonate in 0.1N NaOH

B- 1% Sodium-Potassium Tartrate in distilled water

C- 0.5% Copper(II) Sulphate in distilled water

D- 48ml of A, 1ml of B, 1ml of C

E- Phenol reagent: 1 part of Foline-Ciocalteau's (2N) : 1 part of distilled water

F- Bovine Serum Albumin (BSA) standard- 1mg/ml

Procedure

1. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ ml.

2. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well.

3. This solution is incubated at room temperature for 10 mins.

4. Then add 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660 nm.

5. Plot the absorbance against protein concentration to get a standard calibration curve.

Protein estimation by Bradford assay

Procedure

1) The protein of varying concentrations was added to different wells of the microtitre plate and the volume was raised to a final equal volume with distilled/miliQ water in each well. The concentration was made as per the following table-

Protien amount(µg)	1	2	4	6	8	10	12	14	16	18	20
BSA std.(2mg/ml) in µl	0	1	2	3	4	5	6	7	8	9	10
dH_2O(µl)	10	9	8	7	6	5	4	3	2	1	0

1) To each well, 200µl of Bradford reagent was added using a micropipette.
2) The plate was allowed to stand in an incubator at 37°C for 30 minutes.

The readings were then recorded in a colorimeter at 595nm and a standard curve was plotted

Protien estimation by TCA assay

Procedure

1. 1 volume of TCA was added to 4 volume of protien sample in vials.
2. The vials were incubated at 4°C for 10 minutes.
3. The samples were spinned at 14,000 rpm for 5 minutes.
4. Then the supernatant was removed and whitish fluffy pellet was observed.
5. The pellet was washed with 200µl cold acetone.
6. Again the tubes spinned at 14000 rpm for 14 minutes.

7. Steps 4-6 were repeated for total 2 acetone wash.
8. The pellet was dried at 95℃ in heat block for 5-10 minutes.
9. Then 2X or 4X sample buffer(lysis) was added.

SDS-PAGE

Materials :

Reagents used:

5x Sample Buffer

10% w/v	SDS
10 mM	beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

1x Running Buffer:

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

Resolving gel:

Table 2 : Resolving gel (12%)

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

Stacking gel:

Table 3: Stacking gel (5%)

	15 ml	**4 ml**	5 ml	10 ml
H_2O	10.2 ml	**2.66 ml**	3.4 ml	6.8 ml
30% acrylamide	24.9 ml	**0.67 ml**	0.83 ml	1.66 ml
1 M Tris(6.8)	1.89 ml	**0.5 ml**	0.63 ml	1.26 ml
SDS(10%)	150 µl	**0.04 ml**	0.05 ml	100 ml
APS(10%)	150 µl	**0.04 ml**	0.05 ml	100 ml
TEMED	15 µl	**0.004 ml**	0.005 ml	10 µl

Protocol :

1. Assembling the glass plates:

The glass plates were cleaned with detergent (tween-20) and water then with ethanol. They were then assembled with spacers. For the mini-gel, the glass plate assembly was attached to the apparatus and the whole assembly was then placed in a small, flat Tupperware container.

2. Casting of the gel:

The gels were cast beginning with the resolving gel followed by the stacking gel.

The degassed resolving gel was transferred to a beaker followed by addition of APS/TEMED and then mixed. Quickly the resolving gel solution was added with the help of pipette to the centre of the plates to a height of 4 cm from the top of the large plate. Iso-butanol is quickly added to the top till the plate's level and allowed to stand till the resolving gel polymerized. This prevents the gel's contact with air/oxygen that will react with the free radicals' generated by APS in the process and delay polymerization.

The stacking gel reagents were added to the flask and degassed for 10 minutes. Meanwhile, the iso-butanol was poured off and water was used several times to rinse it within the gel plate space. The space was then air-dried in an incubator at 37°C. To polymerize the stacking gel, APS/TEMED was added and mixed followed by pouring on top of the polymerized resolving gel. The comb was inserted straight down into the stacking gel while it was allowed to polymerize undisturbed for 20-30 minutes. Any bubbles in the gel must be removed carefully.

3.Loading the gel

Tris-glycine electrophoresis buffer was poured into the upper and lower chambers. The wells were flushed with syringe just before loading to get rid of any unpolymerized polyacrylamide that may have seeped in. When loading the gel, 1X loading buffer was loaded in every lane so the dye front will migrate more evenly.

4.Sample Preparation

The samples were prepared with BSA as standard. 15µl of BSA with 6µl of loading buffer was used as marker. 27µl of each sample alongwith 6µl of loading buffer was prepared. The samples and marker were heated at 100°C for 10-12 minutes in a water bath then vortexed for 10 seconds. The samples were then loaded onto the gel which was run at 50V till the stacking gel was crossed followed by 100V till the end.

5. Staining the gel

The gel was stained using coomassie blue. 12mg of Commassie blue was used from the stock of 60mg/litre and added to the staining solution. Staining solution was prepared using

10% acetic acid and 40% methanol in a total volume of 200 ml. The volume was raised using distilled water. The gel was then submerged in staining solution for 30 minutes.

6.Destaining the gel

The gel was then destained using destaining solution and the bands were observed. The destaining solution constituted of 50% Methanol, 10% Acetic Acid and the rest was distilled water in a total volume of 100ml. The gel was immersed in destaining solution till the solution turned blue. The step was repeated till clear bands became visible and the gel was washed with distilled water and stored.

1D profiling of proteins by Silver staining

Reagents :

10. Farmer's reagent: 0.3g Potassium ferricyanide
    0.7g Sodium thiosulphate
    0.1g Sodium carbonate
2. $AgNO_3$ solution: 0.2g in 200ml dH_2O
3. 2.5% Na_2CO_3
4. Developer: 2.5% $Na2CO_3$ in 270 µl formaldehyde
5. Stop solution: 2% Acetic acid and 10% C_2H_5OH, then raise the volume with distilled water till 200 ml.

Protocol

1. For direct silver staining after SDS- PAGE fix in 90% (100ml) methanol and 10% (25ml) acetic acid with gentle stirring (250ml) for 2 hours.
2. The gel was washed with 10% methanol for 10 minutes. The was repeated for 5 minutes.
3. The gel was washed with distilled water for 30 minutes changing the distilled water after every 5 minutes. This was done to wash off traces of ethanol.
4. The gel was then dipped in farmer's reagent for 50-60 seconds.

5. It was rinsed with distilled water for 5 minutes (thrice).

6. Silver Nitrate solution was used to immerse the gel for 1-5 minutes until it became black and then the solution was discarded. Light was prevented during weighing silver nitrate and while the reaction was going on.

7. The gel was rinsed with Sodium Carbonate for 5 minutes.

8. It was dipped in developer for 30 seconds to 2 minutes. As soon as bands appear the reaction was stopped with stop solution. 2% acetic acid can also be used for stopping the reaction.

9. Farmer's reagent was again used to dip the gel in order to remove background.

10. Farmer's reagent was removed by washing the gel thrice with distilled water.

Isolation and 1D profiling of proteins from BU25TK cell line

1. Cultured cells of BU25TK cell line were transferred to eppendorf.
2. Cells were centrifuged at 2000 rpm for 7-8 minutes at 4°C.
3. Lysis buffer(50 µl) was poured into the eppendorf.
4. Then added 2 µl of protease inhibitor and 2 µl of PMSF.
5. Left the eppendorfs for incubation at room temperature and kept vortexing after every 10 minutes.
6. After vortexing the vials were stored at -20°C.
7. Next day samples were taken and run on SDS-PAGE to observe bands.
8. 1.5 ml of RBC lysis buffer was added and kept on continuously inverting the

Protien profiling from human blood(match-control)

Reagents : RBC Lysis Buffer

Tris	2.42 g
NH_4Cl	7.5 g
Deionised water	1 lt

Protocol

1. 4ml of human blood was collected in Edta coated vials and kept in ice
2. 500µl was added in each vial. vials for 15 minutes.
3. The vials were centrifuged at 13000 rpm for 1 minute.

4. Supernatant was discarded and kept the pellet.
5. Pellets were rinsed with 1ml PBS.
6. Then 40µl cell lysis buffer was added to the pellet.
7. Then added 40µl PMSF to each vial.
8. Vortexing was done every 10 minute for half an hour till the pellet got suspended.
9. 5µl of loading dye was added to each vial.
10. Vials were kept at 95℃ for 12 minutes.
11. Then vials were centrifuged for 14 seconds at 2000rpm.

Chapter 4

Results and discussions

1. Lowry's method of protein estimation

The absorbance against protein concentration was plotted as follows to get a standard calibration.

Concentration of BSA(1mg/ml)	O.D at 750nm
25	0.0799
50	0.1781
100	0.3478
200	0.5231
400	0.8887
800	1.432

Table 3.1.1 Standardization of Lowry's protocol

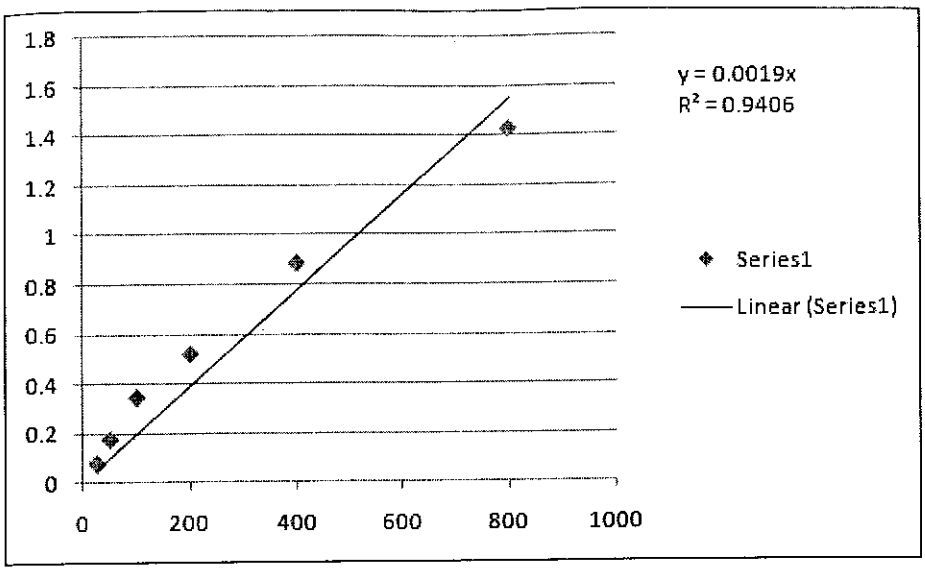


Table-3.1.2 Standard Curve for Lowry's Assay

Concentration of the samples

1. S1- 1.398mg/ml
2. S2- 1.858mg/ml

2.Protien estimation by Bradford assay

The readings recorded are as follows

Protien amount(µg)	0	2	4	6	8	10	12	14	16	18	20
Od	0	0.34	0.865	1.0215	1.1215	1.018	1.321	1.231	1.404	1.54	1.55

Table-3.2.1 Average Absorbance for Bradford's Standard Plot

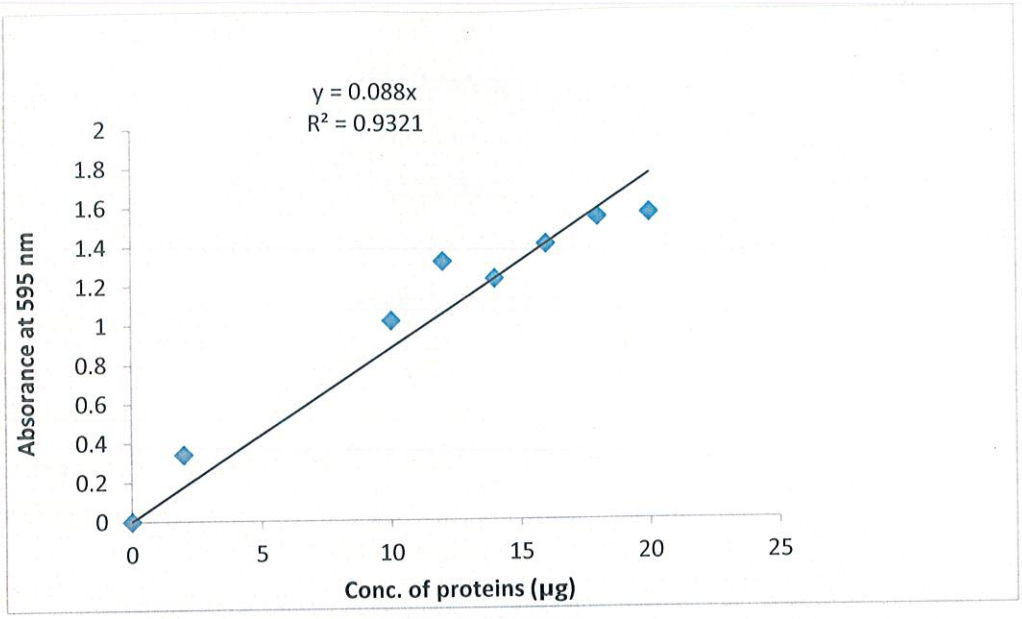


Table-3.2.2 Standard Plot for Bradford's Assay

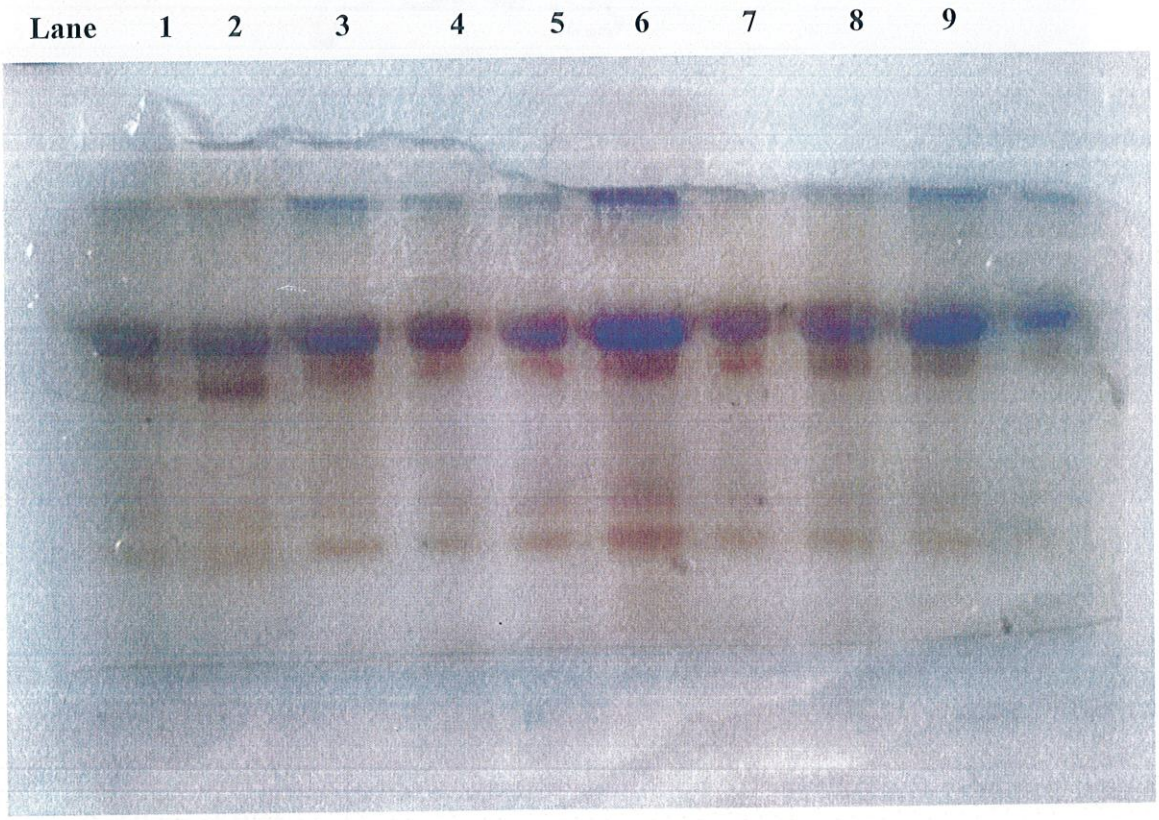
Recommended Standard proteins for Bradford Assay include bovine gamma globulin (IgG), lysozyme and ovalbumin because they have closer to the average or typical number of amino acids that bind the dye. Although BSA is often recommended in literature, but it is not a good choice for a standard for the Bradford assay, as it has a high content of reacting amino acid residues. The 595nm absorbance of BSA is about 2.1 times that of IgG.

The colour development in Bradford assay is complete within 2 minutes and the colour remains stable for upto an hour. With the micro-assay procedure, Bradford assay can be used to determine proteins in the range of 1 to 20µg. Bradford assay has few interfering components known to be Triton X-100 and Sodium Dodecyl Sulphate.

SDS- PAGE :

Initially, in SDS-PAGE no desired bands were observed due to low concentration of protein in samples. There is also a possibility that the protein was degraded or there might have been some problem with the reagents used. Therefore, standardization and optimization procedures were carried out for optimum results.

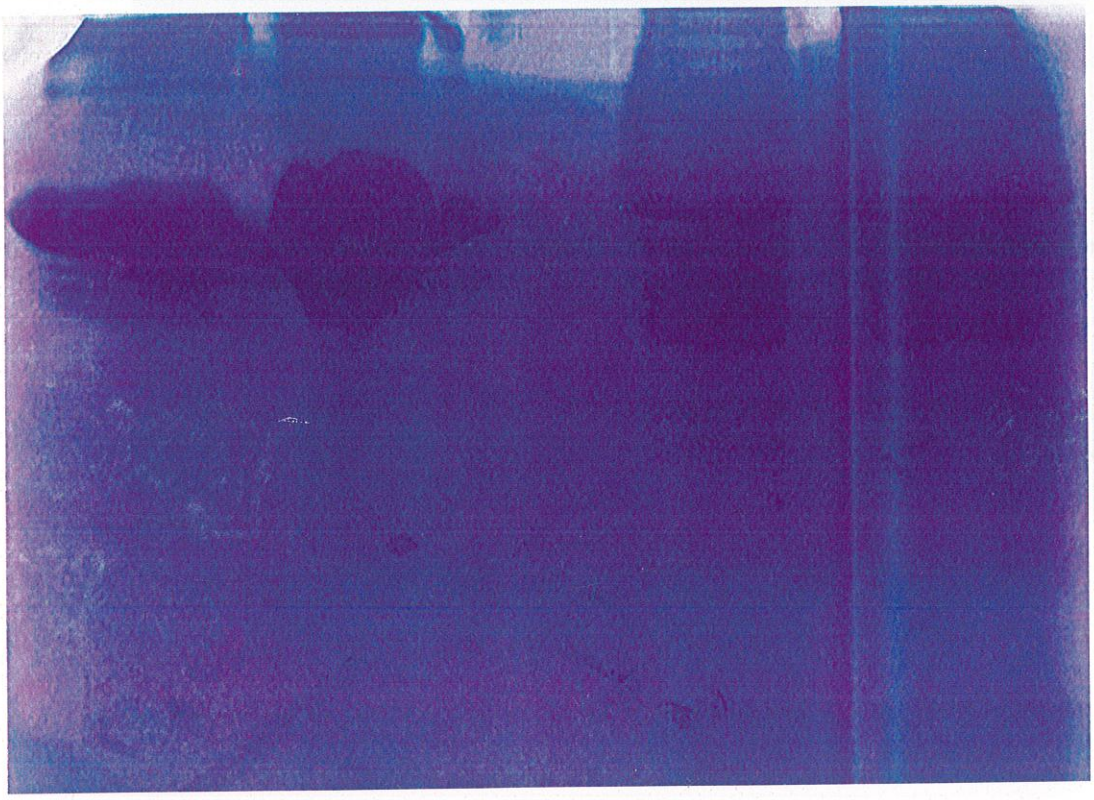
SDS- PAGE using BSA protein(2mg/ml)



20 micrograms of BSA was loaded in each well. Bands can be clearly seen after commassie blue staining followed by silver staining.

SDS-PAGE gel picture of proteins extracted from BU25TK cell line

Lane **1** **2** **3** **4** **5**



Lane 1- 25 µg of BSA

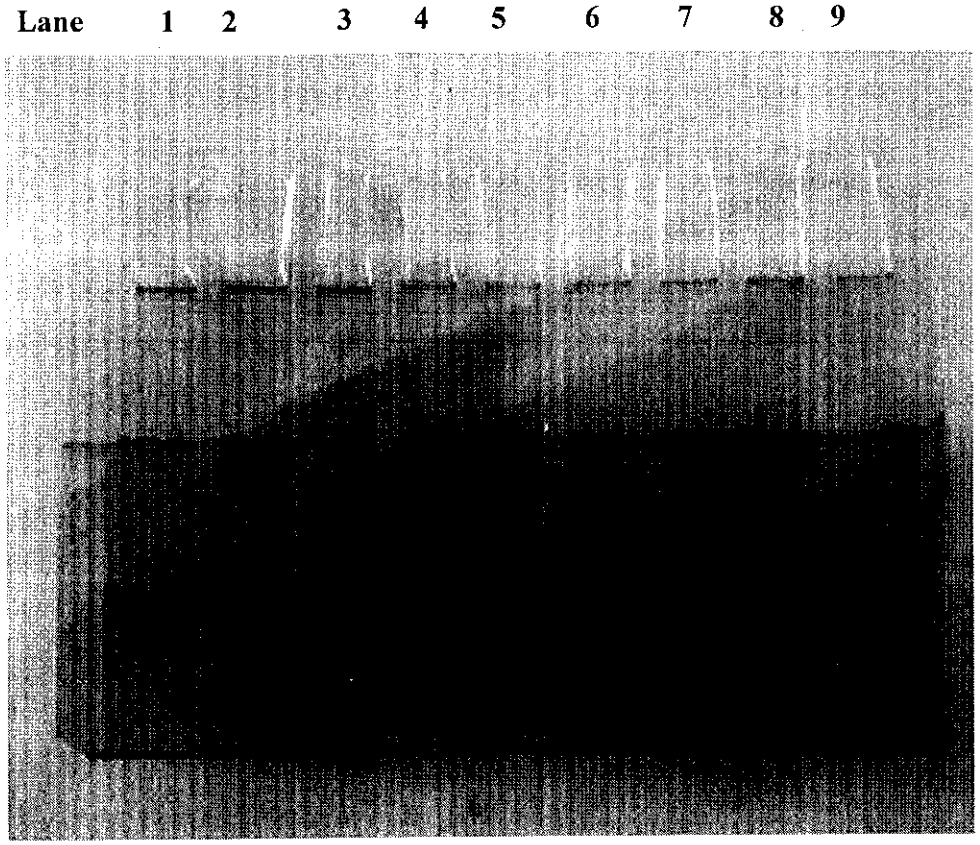
2- 50 µg of BSA

4- 25 µg of whole cell protein from BU25TK cell line

5- 50 µg of whole cell protein from BU25TK cell lines

Several protein bands can be observed in whole cell lysate of MCF-7 cell line after staining with Commassie blue.

2. **Result of 1D profiling of proteins extracted from human blood(match-control) using silver staining**



The most abundant protein present in serum is albumin and, in fact, 95% of the protein content of serum is made up of around 20 highly abundant proteins, which must be removed prior to proteomic analysis in order to prevent them from masking the expression of low abundant putative biomarkers.

Normal human blood serum after lysing RBCs was run on a 1-D gel and several distinct bands could be observed under white light. This was done with the purpose of comparison as matched control

CONCLUSION

Despite the lifetimes that have increased in breast cancers due to the the early screening programs and new therapeutic strategies, many cases still are being lost due to the metastatic relapses. For this reason, new approaches such as the proteomic techniques have currently become the prime objectives of breast cancer researches.

Various omic-based techniques have been applied with increasing success to the molecular characterisation of breast tumours, which have resulted in a more detailed classification scheme and have produced clinical diagnostic tests that have been applied to both the prognosis and the prediction of outcome to the treatment. Implementation of the proteomics-based techniques is also seen as crucial if systems biology approach is to be developed in the discovery of biomarkers for the early diagnosis, prognosis and prediction of the outcome of the breast cancer therapies. However, it has not yet been possible to define a proteomic predictive marker to predict either therapeutic response or the responses to cytotoxic treatment, whether of micro-metastatic carcinomas or of the whole body.

FUTURE ASPECTS OF THE PROJECT

- 2D-PAGE
- Mass Spectrometry
- Immunohistochemistry
- Co-immunoprecipitation
- Validation of Outcome

Appendices

The reagents were obtained from the following manufacturers:

SR.No	Reagents	Manufacturers
1.	SDS-PAGE assembly	Biorad
2.	Bradford Assembly	Biorad
3.	TEMED	Biorad
4.	Sodium Carbonate	Sisco Research Labs Pvt Ltd
5.	Potassium Ferricyanide	S.D.Fine chemical Ltd
6.	Potassium Thiosulphate	Merck
7.	Formaldehyde	Merck
8.	Ethanol	Changshu Yangyuan chemical,china
9.	Acetic Acid	Fisher Scientif
10.	Methanol	Merck
11.	Commassie RT50	Biorad
12.	Loading buffer	Biorad

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Brief description of the students

I am currently pursuing B.Tech- M.Tech dual degree in biotechnology and will be completing the degree in June 2014 from Jaypee University of Information Technology, Waknaghat, Solan (H.P.). My current CGPA is 7.2(75%) and my interest lies in the field of cancer biology. I am looking forward to pursue Ph.D in biotechnology after M.Tech. My objective is to utilize my technical and social skills to secure a promising professional carrier with ample opportunities and challenges for advancement, while continuously building on my knowledge skills.

Mishti Chaudhary

I am currently pursuing B.Tech- M.Tech dual degree in biotechnology and will be completing the degree in June 2014 from Jaypee University of Information Technology, Waknaghat, Solan (H.P.). I would like to obtain a position that utilizes and further develops each of my skills and will allow me to work my way up into a position of more responsibility and side by side gaining experience in the field of cancer biology.my extra co-curricular includes as follow:

- Participated in Miss.himachal pageant 2011,and crowned as miss perfect figure 2011.
- Particitates in college co-curricular activities like ramp walk.
- Took part in relay race and awarded silver medal.
- Participated in slogan writing completion and was given anticipation award.

Shweta Kaundal