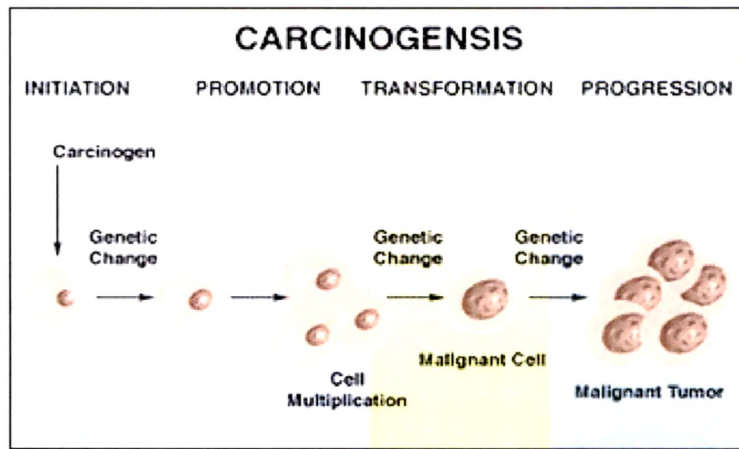


Prologue

1.1 Overview:

The term, cancer is derived from the Latin word *cancer* and the Greek word *karkinos* which means "*the arm of a crab*". Cancer is a multistep, multifactorial, multifaceted, and multimechanistic and recently classified as a proteomic disease (Petricoin et al, 2004). Therefore, the in-depth study of protein changes in cancer is very essential phenomenon. Carcinogenesis follows a multistep process involving distinct stages i.e. initiation, promotion, transformation and progression (**figure: 1**).

Figure-1: Multistep process of cancer development



Adapted from www.livercancer.com

Most cancers are thought to originate from a single cell that has undergone a somatic mutation. But, the progeny of this cell must undergo further changes, probably requiring several additional mutations before they become cancerous. This phenomenon of tumor progression, which usually takes many years, reflects the operation of evolution by mutation and natural selection among somatic cells. The rate of the process is accelerated both by mutagenic agents (tumor initiators) and certain non mutagenic agents (tumor promoters) that affect gene expression, stimulate cell proliferation, and alter the ecological balance of mutant and non mutant cells.

Cancer is a global health problem taking millions of lives every year. The international agency for research on cancer (IARC) estimated 10.9 million new

cases, 6.7 million deaths and 24.6 million persons living with cancer in the year 2002 (Parkin et al, 2005). Among various malignancies, myeloma constitutes 0.8% of all cancers worldwide (86,000 new cases). Incidence rates vary from 0.4 to 5 per 100,000; and it is very rare in person under 40 years of age (Parkin et al, 2005). A gradual increase in incidence and mortality of myeloma is observed in most regions of the world, the reason of which is unknown. In India, incidence of multiple myeloma (MM) varies from 0.3 to 1.9 per 100,000 and 0.4 to 1.3 per 100,000 in men and women, respectively (Kumar et al, 2006). More than 100 cases of MM are reported every year at The Gujarat Cancer and Research Institute (GCRI), Ahmedabad (Annual Report, 2004).

No specific etiological agent for the MM has been found. The predisposing factors like radiation exposure, chronic antigen stimulation, environmental exposure and human herpes virus 8 (HHV-8) are reported. MM is a malignant tumour of the plasma cells in bone marrow. These malignant cells accumulate in bone marrow and produce a monoclonal protein (immunoglobulin), usually IgG or IgA, often referred to as M protein or multiple myeloma protein, which can cause end organ damage. The understanding of hematological malignancies like MM at the M-protein level is important as the development of targeted treatments must be based on knowledge of the molecular pathogenesis of the tumours, inherited genetic variation and the mode of action of drugs. Till date, there are limited number of studies in MM highlighting the importance of various biochemical alterations in the discovery of novel protein markers, proteins resulting in drug resistance and identification of tumour markers which may facilitate the development of a rapid diagnostic test that can be easily applicable in the clinical settings.

Cervical cancer is a leading female malignancy with an estimated global incidence of 493,000 new cases and 274,000 deaths in 2002 (Parkin et al, 2005). In developed countries, cervical cancer accounts for only 3.6% of new cancers. It is a leading cause of cancer mortality among women in developing country. India has one third of the world's population but two third of the world's cervical cancer burden (National cancer control programme, 2008). At

our institute, around 1200 new cervical cancers cases are registered and diagnosed every year (Annual Report, 2004). Several risk factors are associated with cervical cancer including human papillomavirus (HPV), lack of regular screening tests, depressed immune system etc. Cancer of the cervix occurs most often in women over 40 years of age, with history of smoking, chronic use of birth control pills and having multiple pregnancies. The progressive histologic and cytologic changes that occur during the multistep process of cervical carcinogenesis can be divided into multiple stages. Early lesions are known as cervical intraepithelial neoplasias (CIN)-1 or low grade squamous intraepithelial lesions (SILs) and high grade lesions as CIN-2, CIN-3 or high grade SIL. The natural history of these cervical precancerous lesions is difficult to study because they usually need surgical biopsy for detection and usually treated as soon as detected. Papanicolaou (Pap) smear screening and HPV serology is currently being investigated as an adjunct to cytology in cervical cancer. While studies to date, support the conclusion that a woman with a negative HPV test is very unlikely to have cervical cancer. The clinical interpretation of positive result is not clear (Unger et al, 2004). Persistent viral infection with strong, constitutive expression of the viral oncogenes *E6* and *E7* is a decisive step for malignant transformation (Bae et al, 2005). In majority cases, HPV infections are benign in nature and are cleared by the host immune system, but approximately 3% to 10% of women cannot clear their HPV infection. Attempts to search for specific proteins in cervical cancer are needed especially to establish their role in the disease progression. Large scale analysis of the protein profiling in normal and diseased conditions offers the opportunity to identify potential diagnostic/prognostic markers and proteins applicable to very small lesions and limited number of cells. New biomarkers specific for advanced stage lesions can be directly sought and correlated with malignant phenotype. Such studies can provide the basis for developing new protein biomarkers for early diagnosis and treatment.

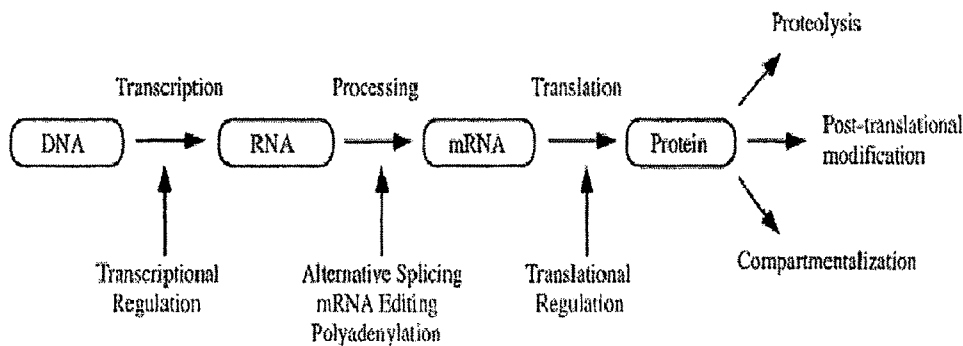
The biology of an organism is largely governed by the structure and function of the products encoded by genes. The most functional part is proteins and its

alterations may reside in the cell membrane embedded with different transmembrane proteins. Therefore, altered gene expression patterns lead to modification of protein structures and functions. There are several other compelling reasons for focusing the analysis of protein changes (Mueller et al, 2007):

- i. The level of mRNA expression frequently does not represent the amount of active protein in a cell.
- ii. The gene sequence does not describe post-translational modifications (e.g. glycosylation, phosphorylation and proteolytic cleavage) and isoforms, which may be essential for protein function, activity, turnover, localization and molecular interactions.
- iii. The genomic studies do not describe dynamic cellular processes.

1.2 Recent Developments in Protein Analysis: With completion of human genome sequencing, the biomedical science is focused to the functional part of genes i.e. proteins. Although DNA is the information archive, proteins do this entire work of the cell. More than one RNA can result from one gene through differential splicing. Additionally, there are more than 200 post-translational modifications that proteins could undergo which can affect function, protein-protein interaction, stability, targeting and half life (Srinivas et al, 2001; Wu et al, 2002). The protein expression and function are subject to modulation through transcription as well as through post-transcriptional and translational events (**figure: 2**).

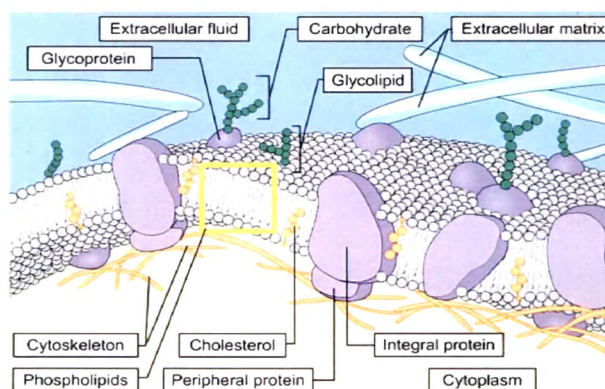
Figure- 2: The protein production pathway



Distinct changes, ranging from altered expression, differential protein modification and changes in specific activity to aberrant localization occur in the protein level during transformation of healthy biological counterparts at different stages of disease from preneoplasia to neoplasia. A pathogenic effect is selected during somatic genetic progression because the defect ultimately alters the protein network to generate a selective advantage for the cancer cell. The deranged, hyperactive, or dominating signal pathways may drive cancer survival, growth, invasion and metastasis (Geho et al, 2004). Therefore, the study of cancer protein aims not only to identify, catalogue and characterize relevant proteins; but also to understand how they interact to affect the overall initiation and metastatic progression. Due to extensive local invasion and metastasis, management of MM and cervical cancer is still a foremost dilemma. This approach can also help the understanding of the biology of the disease leading to additional discoveries. Additionally, it may provide a molecular characterization of cancers leading to discovery of individualized molecular therapy and the early detection of cancers like MM and cervical cancer.

1.3 Protein Glycosylation and Cancer:

Figure–3: Major components of cell membrane glycoproteins



Glycosylation is one of the most frequently occurring post-translational modifications of proteins. Glycoprotein is widely distributed in proteins with diverse functions that contain one or more covalently linked carbohydrate

chains. The carbohydrate components of a glycoprotein range from 1% to more than 85% of its weight and may be simple or very complex in structure (Murray et al, 2006). The oligosaccharide chains of glycoproteins encode biological information. These chains are important for glycoproteins for modulating their solubility, viscosity and for protecting them against proteolysis and in cell-cell interactions (**figure: 3**).

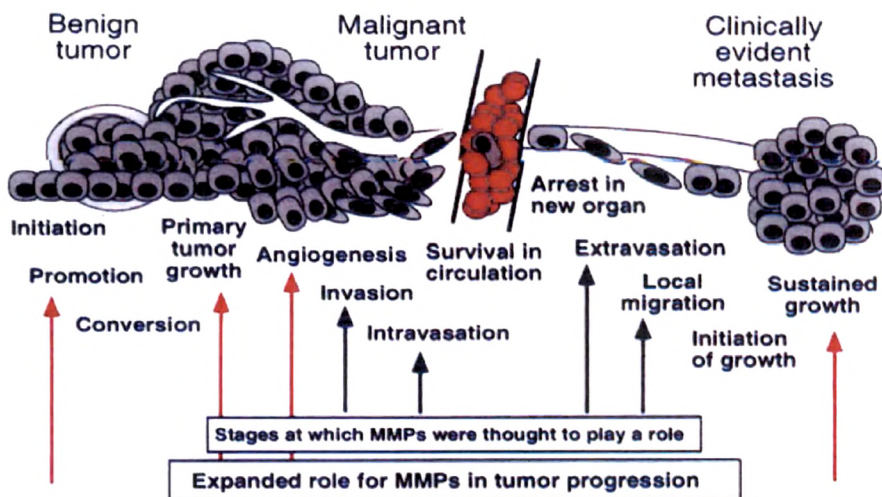
The cell surface factors are concerned with many physiological properties related to neoplastic transformation and metastatic spread of malignancy. These include cell shape, inflammation, growth, division, differentiation, cellular recognition, communication, adhesiveness, migration, and contact inhibition of growth, viral replication, parasitic infestations and immune defense. (Geyer and Geyer, 2006). Glycoproteins are the key molecules involved in the innate and adaptive immune responses. In cellular immune system, specific glycoforms are involved in folding, quality control, assembly of peptide, major histocompatibility complex antigens and T cell receptor complex. Although some glycopeptide antigens are presented by histocompatibility complex, generation of peptide antigens from glycoproteins require enzymatic removal of sugars before the protein can be cleaved. In the humoral immune system, all the immunoglobulins and most of the complement components are glycosylated. Although major function of sugars is to contribute stability of the proteins to which they are attached, specific glycoforms are involved in recognition of events (Rudd et al, 2001). Neoplastic changes in cell surface glycoconjugates and enzymes are expressed in or mediated through the cell membrane leading to abnormal growth and behavior of malignant cells. Being the major constituents of cell membrane, various glycoprotein conjugates are markedly elevated during malignant process. There is evidence that being attached to the surface of tumour cells, sialoglycoproteins affect various important functions (Patel et al, 1996; 1997a, 1997b, Shah et al, 2008). Therefore, studies on biomarkers addressing underlying several glycoconjugate e.g. total sialic acid (TSA), fucose, seromucoid fractions ie. mucoid proteins (MP) and hexoses and

alterations in different proteins and glycoproteins may play important role in MM and cervical cancer patients. The study of immunoglobulins in correlation to glycoprotein in MM, which is a plasma cell neoplasm accumulating in the bone marrow and producing a monoclonal M-protein (immunoglobulin) may have significant clinical usefulness in MM patients.

1.4 Matrixmetalloproteinases (MMPs) and Tissue inhibitors of Metalloproteinases (TIMPs)

It is well documented that tumour-host interactions influence the phenotypic expression of neoplastic changes. Tumour microenvironment also plays crucial role in progression of malignant diseases. Many proteinases are capable of degrading extra cellular matrix (ECM) components and basement membrane, the most important being serine proteases, cathepsins and MMPs. Among these, MMPs and their inhibitors TIMPs, are particularly important for matrix degradation which are known to be associated with angiogenic, invasive and metastatic behavior of cancer (Woessner, 2002) (**figure: 4**). Currently, the MMPs comprise a large family of over 20 secreting or transmembrane proteins that together can degrade or proteolysis major components of the ECM and basement membrane. This subgroup has two distinct members, known as gelatinase A (MMP-2) and gelatinase B (MMP-9).

Figure-4: Important role of MMPs in cancer



Adapted from Chamber A & Matrisian L, *J. Natl. Cancer Inst* 1997; 89:1260-70.

The activity of MMPs is regulated in three ways: gene transcription, proenzyme activation and by their natural inhibitors TIMPs (21 to 29 KDa). There are four members of the mammalian TIMP family, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMP-1 and TIMP-2 can preferentially block the pro forms of MMP-9 and MMP-2, respectively. The inhibitory activity of TIMPs might be important in inhibiting tumorigenesis and subsequent malignant transformation. MMPs are important in the late stage of tumor progression leading to metastasis; but the anti-apoptotic effects of some TIMPs support the tumor growth during the onset and early primary growth of tumour. Gelatinases (MMP-2 and MMP-9) and TIMPs (TIMP-1 and TIMP-2) are known to be closely associated with the metastatic potentials of tumor cells (Hoekstra et al, 2001). The important prerequisite for neoplastic cell invasion during tumorigenic processes is the remodeling events that occur within the stroma or ECM. Excess matrix degradation is one of the hallmarks of cancer and is an important component of the tumor progression. MM and cervical cancer are associated with extensive local invasion and metastasis. Therefore, studies on biomarkers addressing significance of MMPs and TIMPs can help to predict metastatic potentials and prognosis of MM and cervical cancer. On this basis, the study of proteins from complex biological sample containing information on biological process in cancer cells, cancer tissue microenvironment, and cancer cell-host interaction are important. Cancer cells release protein biomarkers into the extracellular fluid through secretion of intact or cleaved peptides (Alaoui-Jamali et al, 2006). In addition, cancer-associated markers can be contributed by the tumor microenvironment e.g. surrounding host cells such as fibroblasts and macrophages. Some of these products can end up in the blood stream. Hence, serum has been the traditional and potential sample for studies of biomarkers.

With these considerations, the protein changes and profiling using various parameters (total protein, TSA, fucose, MP and hexoses) were studied by highly sensitive and specific spectrophotometric methods in MM and cervical cancer patients. Immunoglobulins were studied using radial immunodiffusion

Total Sialic acid

method. Expression of MMP-2 and MMP-9 were studied using gelatin zymography method. The levels of total MMP-2 and MMP-9 (gelatinases) and their inhibitors were studied using sandwich based ELISA (Enzyme Linked Immunosorbant Assay) kits. The protein fractions and protein profiling were studied by different electrophoretic techniques. M-protein was isolated using electro-transferred on nitrocellulose membrane and then studied using 2 Dimensional Polyacrylamide Gel Electrophoretic (2D-PAGE) approach. The 2D-PAGE consisted of iso-electric focusing (IEF) ((first dimension) and sodium dodecyl sulfate, SDS-PAGE (second dimension). The 2D maps were scanned using gel documentation system and quantified using appropriate software.

The major aims of the study were to: (i) explore the role of protein/ glycoprotein profiling as cancer signatures and underlying significance of protein changes in MM and cervical cancer patients, (ii) study profiling of differentially expressed proteins which may support the development of valuable biomarkers through 2D-PAGE and (iii) assess the importance of proteomics and glycomics approach for MM and cervical cancer patients in cancer glycobiology. The study subjects were divided in 3 groups viz. controls, MM patients and cervical cancer patients. The MM patients were further classified into **Group-I** (MM patients with presence of M-protein) and **Group-II** (MM patients with absence of M-protein). The major **OBJECTIVES** of the present study were to evaluate:

- ❖ Role of protein profiling.
- ❖ Immunoprofiling (*in MM patients only*)
- ❖ Post-translational modifications in terms of alterations in various glyco-conjugates including TSA, fucose, MP and hexoses.
- ❖ Glycoprotein profiling.
- ❖ Alterations in MMP-2, MMP-9, TIMP-1 and TIMP-2.
- ❖ M-protein profiling through 2D-PAGE (*in MM patients only*)

The above objectives were explored to assess their clinical significance in MM and cervical cancer.