

Discussion

5.1 Significance of The Present Study:

MM is a poorly understood, devastating and uniformly fatal malignancy of B lymphocytes characterized by the slow proliferation of malignant plasma cells within the bone marrow. Malignant cells accumulate in the bone marrow and produce single clone of monoclonal protein (immunoglobulin, usually IgG or IgA), often referred to as M-protein. MM comprises 10% of all haematological malignancies. Cervical cancer is the second most common malignancy in women worldwide after breast cancer (Parkin et al, 2005). It is seventh in overall cancer frequency worldwide and the incidence is increasing day by day. Despite advances in diagnostic technology and therapeutic modalities, it remains a major cause of morbidity and mortality worldwide. In India also an increasing trend of MM and cervical cancer is observed (Kumar et al, 2006). In post-genomic era, the research efforts are focused on understanding of functional biology in various stages of cancer development i.e. initiation, promotion, progression, and metastasis of MM as well as cervical cancer.

The progress in this regard has been slow and frustrating due to complex multifactorial nature and heterogeneity of the cancer. The dilemma in oncology practice is due to large number of patients presenting with macro or micro-metastasis at primary presentation. Clearly, there is urgent need to unravel novel protein biomarkers for early detection of cancer and its metastasis. On the other hand, genes exert their effect through proteins after transcription and translation. Thus, genomics do not readily predict protein abundance or provide information about protein function at the biochemical level. It is estimated that 45,000 human genes generate approximately 250,000 spliced variants of RNA which are translated into over 1.5 million proteins as a result of post-translational modifications and processing (Ozier et al, 2003). Most of the functional information of the cancer-associated genes resides in proteome. The later is an exceptionally complex biological system involving several proteins that function through post-translational modifications and dynamic intermolecular collisions. These protein complexes can be regulated by signals emanating from cancer cells, their surrounding

tissue microenvironment and/or from the host. The proteins are secreted and/or cleaved into the extracellular milieu and may represent valuable serum biomarkers for diagnostic and management purpose. Such complexity clearly highlights the need for assembling the protein circuits that are activated in cancer cells and their surrounding microenvironment to achieve suggestive clinical prediction of the disease for robust quantitative protein measurements and data acquisition.

The understanding of MM at the M-protein level is important as the development of targeted treatments must be based on knowledge regarding the molecular pathogenesis of the tumour, inherited genetic variations and the mode of action of drugs. Although limited number of studies to date performed on MM, highlight the potential future impact of these technologies in the discovery of novel protein markers associated with drug resistance and the tumour markers which may facilitate the development of a rapid diagnostic test for better applicability in clinical setting. Rapid large scale analysis of the protein profiling in normal pathways and disease states offers the opportunity of identification of potential diagnostic as well as prognostic markers and proteins associated with the malignant phenotype. The intra epithelial lesions and cervical squamous cell SCC have high risk of progression depending on the presence of unfavorable biology of the disease. It has been known that genes related to cellular proliferation, extracellular matrix interactions (Non-HPV), cell adhesion molecules, protease and cytokines are related to the disease progression. Therefore, intensive screening to search for new proteins that play a vital role in causation and progression of cervical cancer.

Considering its importance, changes in circulating proteins, the final products manufactured in the living cells according to the 'blue print' contained in the genome and protein changes at post-translational modification (e.g. glycosylation) were evaluated in the current study. The study was aimed to protein changes between controls and MM patients. MM patients were further classified into the two groups: group-I and group-II MM patients. Present

investigation analysed circulating levels of glycoconjugates (TSA, fucose, MP and hexoses) which are expressed at or mediated through the cell membrane leading to abnormal growth and behavior of malignant cells. Metastatic process consists of a series of tumour-host interactions that involve multiple extracellular matrix degrading enzymes including serine proteases, cysteine proteases and MMPs. First step in metastasis involves degradation of underlying basement membrane which mainly consists of type IV collagen (Nelson et al, 2000). The degradation of the extracellular matrix is intrinsic to the invasion and propagation of cancer. MMPs are the terminal effectors of the proteolytic cascade in matrix degradation and their natural inhibitors, TIMPs are part of a family of homologous proteins that potentially act against metastasis. These arrays of proteins and enzymes constitute vital biomolecules that interact with each other leading to a series of sequential events such as angiogenesis, invasion and metastasis that are critical for maintaining the proliferation of cancer cells. Gelatinases (MMP-2 and MMP-9) and their natural inhibitors (TIMP-1 and TIMP-2) were also studied to evaluate their clinical usefulness. Thus, the foremost aim of this study was to evaluate the role of different proteins and glycoprotein profiles as cancer signatures and underlying significance of the protein changes in MM and cervical cancer patients. An attempt was made for profiling of differentially expressed proteins through isolation and characterization of M-protein with the help of 2D-PAGE approach.

5.2 Agar Gel Electrophoretic Patterns in MM patients:

Ivanova et al (2002) have compared capillary zone electrophoresis and high-resolution agarose gel electrophoresis to detect protein components in serum and cerebrospinal fluid of patients. Both electrophoretic methods were proved to be useful for detection of protein abnormalities (e.g. mono and oligoclonal bands) in biological fluids. The results were proved to be valuable in diagnosis and monitoring of blood dyscrasias in clinical practice. Rawal et al (2003) have reported clinical significance of serum protein fractions in controls and MM patients. In the present study, comparison of serum protein

fractions by agarose gel electrophoresis delineated five major protein zones viz. albumin, alpha-1, alpha-2, beta and gamma globulins. Serum total protein levels, alpha-2 as well as gamma globulin fractions were elevated significantly in MM patients as compared to the controls. Whereas serum A:G ratio was decreased significantly in MM patients as compared to the controls. Serum total protein levels, gamma globulin fractions and A:G ratio were significantly elevated in group-I as compared to group-II MM patients. The ROC curve is defined as a plot test of sensitivity versus its 1-specificity. It is an effective method to assess the qualitative performance of diagnostic test. ROC plots provide a pure manifestation of accuracy by demonstrating the limits of a test's ability to discriminate between two groups. AUC is a measure of the overall performance of a diagnostic test and is interpreted as the average value of sensitivity for all possible values of specificity. Its value can be between 0 and 1. If AUC is closer to 1, the overall diagnostic performance of the test is better and a test with an AUC value of 1 is the one that is perfectly accurate. The interpretation lower limit for the AUC of a diagnostic test is 0.5. The data from ROC curve analysis for serum protein fractions suggested that serum total protein levels, alpha-2, beta and gamma globulin fractions and A:G ratio could significantly discriminate between the controls and MM patients. Serum total protein levels, gamma globulin fractions and A:G ratio could significantly discriminate between group-I and group-II MM patients. There is no report which shows the ROC curve analysis and its clinical utility to discriminate between controls and MM as well as group-I and group-II MM patients.

5.3 Study of Immunoglobulin Profiling:

Various immunoprecipitation techniques are used for immunoglobulin profiling. In the current study, we used radial immunodiffusion technique to quantify immunoglobulins. Comparison of the immunoglobulin levels suggested that serum IgG and IgM were significantly elevated in MM patients

as compared to the controls whereas serum IgA level was comparable among these groups. Earlier investigations, have reported M-protein in 82% of the MM patients (Fentress et al, 2006). Rawal et al (2003) also have documented that M-protein was present in 69% of MM cases. The qualitative and quantitative estimation of monoclonal protein band showed that majority of the M-proteins were of IgG subclass. Kyle et al (2003) have reported similar results and found IgG as monoclonal band in more than 50% of MM patients. The present study also found presence of M-protein in 58% of MM patients. The M-protein content ranged between 3-6 gm/dl in 45% of the patients. Higher concentration of M-protein suggested that high rate of B lymphocytes proliferation to malignant plasma cells within the bone marrow. These malignant cells accumulated in the bone marrow and produced single clone of monoclonal protein.

5.4 PAGE Patterns in MM and Cervical Cancer Patients:

Previous reports have documented significant clinical usefulness of protein fractions in various malignancies. It is reported that pretreatment serum albumin level is a significant prognostic factor, which should be evaluated along with other well-defined prognostic factors in deciding therapy for gastric carcinoma (Onate-Ocana et al, 2007). Measurement of albumin levels in bronchial washings could be a useful additional diagnostic tool to differentiate malignant from non-malignant lung diseases (Charokopos et al, 2004). Moreover, combined measurement of carcinoembryogenic antigen and serum albumin in bronchial washing fluid can be helpful in follow-up studies of lung cancer. Kanoh et al (2001) have documented that measurement of serum alpha-2 microglobulin levels may be useful in the diagnosis and follow-up study of bone metastasis in prostate cancer. Increased globulin levels and decreased albumin levels have been observed in advanced malignant diseases which were found to be normalized upon successful treatment (Krecicki, Leluk; 1992). Serum contains a number of proteins which can be separated electrophoretically. In the present study, serum protein electrophoresis showed multiple bands for each sample which were categorized into six major

groups namely UnLMW, prealbumin, albumin, alpha, beta and gamma globulins. As compared to agarose gel electrophoresis, the native-PAGE has property to separate more number of polypeptide chains. Serum total protein levels, UnLMW, prealbumin, albumin, alpha, and gamma globulin values were found to be significantly higher in MM patients as compared to the controls. Mean serum total protein levels, albumin and gamma globulin were higher in group-II as compared to group-I MM patients. Serum total protein levels, UnLMW, prealbumin, alpha and beta globulins were found to be significantly elevated in cervical cancer patients as compared to the controls. ROC curves analysis of native-PAGE results showed that serum prealbumin could significantly discriminate between controls and MM patients. serum total protein levels and gamma globulin could significantly discriminate between group-I and group-II MM patients. Serum albumin could significantly discriminate between controls and cervical cancer patients. Thus, the ROC curve analysis for the electrogram fractions revealed significant information on protein changes in group-I and group-II MM patients, which is not reported in earlier.

Serum total protein profiling ratio were calculated to determine relative changes in the protein levels. Serum total protein levels, UnLMW:gamma and prealbumin:gamma globulin values were significantly higher in MM patients as compared to the controls. Mean serum total protein levels, UnLMW:gamma and prealbumin:gamma values were significantly elevated in group-I as compared to the group-II MM patients. Elevations of serum UnLMW:gamma, prealbumin:gamma, albumin:gamma, alpha:gamma and beta:gamma globulin ratios were found to be significant in cervical cancer patients as compared to the controls. Ratio of UnLMW:gamma could significantly discriminate between controls and MM patients. Serum total protein levels and UnLMW:gamma and prealbumin:gamma could significantly discriminate between group-I and group-II MM patients. Serum total protein levels, UnLMW:gamma, prealbumin:gamma, albumin:gamma alpha:gamma and beta:gamma could significantly discriminate cervical cancer patients. Along with the native-PAGE,

SDS-PAGE results were compared between control and cervical cancer patients. 10-15 unknown protein bands were separated on the basis of their molecular weight, which ranged between 14 kDa to 100 kDa.

The multivariate analysis provides regression analysis and analysis of variance for multiple dependent variables by one or more variables or covariates. The variables divide the population into groups. Multivariate analysis was carried out to correlate serum total protein profiles, serum total protein profiles ratio and clinicopathological parameters like age, histopathology, early vs advanced stage of the disease and pathological tumour differentiation in cervical cancer patients. Multivariate analysis of total protein levels, UnLMW, prealbumin, albumin, alpha, beta and gamma globulin and various clinicopathological parameters revealed that the alterations in prealbumin were significantly associated with early vs advanced stage and gamma globulin was significantly associated with pathological tumour differentiation. UnLMW:gamma, prealbumin:gamma, alpha:gamma and beta:gamma ratios were significantly associated with pathological tumour differentiation.

5.5 Glycosylation Changes in MM and Cervical Cancer Patients:

Previous reports have documented significant clinical usefulness of glycosylation changes in various cancers. Cylwik et al (2005) reported that serum TSA concentrations were significantly elevated in different types of cancers as compared to healthy individuals and patients with nonmalignant disease. Circulatory levels of TSA were reported to be significantly elevated in untreated oral cancer patients compared to healthy individuals as well as patients with oral precancerous conditions (Rajpura et al, 2005). Aranganathan et al (2005) found markedly elevated levels of plasma TSA, protein-bound hexoses, hexosamine and fucose in ovarian cancer patients. Roy and Chakraborty (2005) assessed sialic acid values in cervical tissue and serum. Their results showed slightly increased sialic acid in benign inflammatory lesions, moderate increase in severe dysplasia and preinvasive carcinoma and marked increase in invasive cervical cancer. Both cervical tissue and serum values increased gradually with the advanced grade of

malignancy. Liang et al (2003) have assessed the diagnostic significance and clinical effect of the detection of serum TSA in three kinds of malignant tumours (lung cancer, leukemia and rectal carcinoma) and reported that the TSA levels of the malignant tumour groups before treatment were significantly higher as compared to controls. High levels of TSA have been reported in cholangiocarcinoma patients using the periodate thiobarbituric acid method (Wongkham et al, 2003). Gokmen et al (2004) documented that (i) serum TSA was elevated in lung cancer patients of different histological types with or without metastasis (ii) serum TSA was found to be significantly higher in lung cancer patients with metastases than those without metastasis and (iii) TSA was not found to be a suitable marker for distinguishing lung cancer patients with extrapulmonary metastasis from those without extrapulmonary metastasis. Rawal et al (1999) have reported that serum levels of sialic acid forms and seromucoid fraction were significantly elevated in untreated head and neck cancer patients as compared with the controls. Seromucoid fraction was measured in terms of MP and hexose content from serum of healthy women, women with benign breast diseases, untreated patients with breast cancer, and follow-up samples collected from the same breast cancer patients. MP and hexose levels were found to be significantly elevated in untreated patients with breast cancer compared with the healthy participants and patients with benign breast diseases (Patel et al, 1998). Higher values of glycoprotein constituents were reported to be associated with higher proliferative activity in ovarian cancer (Goldhirsch et al, 1998). Fernandez-Rodriguez et al (1997) determined fucose levels in normal and tumour-derived tissues from patients with colorectal adenocarcinoma. Free and bound fucose levels were significantly higher in tumour tissue. A previous report from our laboratory analysed blood-based biochemical profile for diagnosis and management of human leukemias (Patel et al, 1994). Serum TSA/TP, lipid bound sialic acid, fucose/TP and seromucoid fraction levels were measured by highly specific spectrophotometric methods. All the biomarkers were significantly elevated in anemia patients (pathological controls) and untreated leukemia patients as compared to the controls. Furthermore,

significantly elevated levels of the markers were observed in untreated leukemia patients compared to anemia patients. The levels of the biomarkers in patients with persistent leukemic activity/accelerated leukemic phase were significantly higher than those in patients in remission. The values were comparable to those with pretreatment levels. In accordance with these reports, serum levels of glycoconjugates and protein ratio of glycoconjugates, serum TSA, TSA/TP, fucose/TP, MP and hexoses levels were found to be significantly higher in MM patients as compared to the controls in the present study. Significantly higher serum TSA/TP and fucose/TP levels were found in group-II as compared to group-I MM patients. Serum TSA, TSA/TP, fucose/TP and hexoses levels were significantly higher in cervical cancer patients as compared to the controls. Mean values of MP and fucose levels were higher in controls than cervical cancer patients. Cylwik et al (2007) have studied usefulness of serum TSA for discrimination of malignant and nonmalignant jaundice. Serum TSA and the ratio of TSA/TP were significantly higher in malignant than in nonmalignant jaundice. However, sialic acid levels and sialic acid/TP could not discriminate between these types of jaundice by ROC curve analysis. The diagnostic sensitivity of sialic acid and sialic acid/TP in both types of jaundice was observed up to 95.8%. Patel et al (1997a) have documented that ROC curves analysis clearly showed that glycoconjugates (TSA, lipid bound sialic acid, MP and hexose) levels serve as diagnostic indicators of cancer. The current study revealed that serum levels of glycoconjugates and protein ratio of glycoconjugates, TSA, TSA/TP, fucose/TP, MP and hexoses levels could significantly discriminate between controls and MM patients. Serum TSA/TP, fucose/TP, MP and hexoses could significantly discriminate between group-I and group-II MM patients. Serum TSA, TSA/TP, fucose and fucose/TP could significantly discriminate between controls and cervical cancer patients. Glycoconjugates and protein ratio of glycoconjugates were studied to normalize any variations caused by alterations in total protein (Raval et al, 2003). Correlation coefficient analysis that serum TSA, fucose and hexoses showed significant positive association with TSA/TP, fucose/TP and MP in MM, group-I and group-II and cervical

cancer patients. Wongkham et al (2003) have reported that serum sialic acid concentration was not associated with clinicopathological features or tumour burden of the cholangiocarcinoma patients. Increased levels of the serum glycoproteins correlated well with the clinical staging of the head and neck cancer patients (Bathi et al, 2001). Fernandez-Rodriguez et al (1997) could not establish a clear association between fucose content and clinical staging according to the Dukes' classification in colorectal adenocarcinoma. In the current study, serum levels of glycoconjugates, protein ratio of glycoconjugates and clinicopathological parameters suggested that alterations in MP and hexoses were significantly associated with early vs advanced stage of the disease.

5.6 Serum Glycoprotein Profilinig by native-PAGE:

Serum contains number of glycoproteins which can be separated electrophoretically. Various glycoproteins have been claimed to clinical usefulness for cancer. Patel et al (1997b) reported that mean values of albumin and alpha globulin were significantly lower in tobacco users and patients with oral precancerous conditions compared to nontobacco users. Gamma globulins were significantly elevated in patients with oral precancerous conditions and untreated cancer patients compared to nontobacco users. Albumin was significantly lower, whereas gamma globulin was significantly elevated in non responders compared to their pretreatment levels. The glycoprotein values in complete responders were comparable with nontobacco users. Patel et al (1996) documented decreasing number of complete responders and increasing non responders with elevated albumin fraction glycoproteins (compared to pretreatment levels). Gamma region glycoproteins showed the reverse pattern to that of albumin region glycoproteins. Alpha and beta region glycoproteins revealed an increasing number of complete responders having higher values with increase duration of follow-up. In comparison with their pretreatment values, complete responders showed significantly increased values of albumin, alpha and beta globulin and decreased gamma globulin. The albumin, alpha, beta and

gamma region glycoprotein levels were comparable between non responders and untreated breast cancer patients. The albumin fraction contains tryptophan rich protein, small amounts of carbohydrates and hexosamines. The alpha glycoproteins contain alpha-1 acid glycoprotein (orosomucoid), haptoglobin, alpha-2 macroglobulin and ceruloplasmin. The beta glycoproteins contain somewhat less amount of carbohydrate than alpha glycoproteins. The beta fraction includes beta-2 microglobulin and transferrin. Gamma globulins contain an appreciable amount of carbohydrates with higher amounts of sialic acid and fucose. Salmon, Cassady (1993) have documented that the increased carbohydrate content accompanies elevations in gamma globulin. This has significant diagnostic value in MM. There are numerous other reports indicating that alpha-1 acid glycoprotein, beta-2 microglobulin, sialic acid, fucose etc. have great value as tumour markers (Xing et al, 1994; Chakravarty et al, 1994; Johnson et al, 1993). In accordance with these reports, our study found multiple glycoprotein bands on native-PAGE. The glycoprotein electrophoretic patterns for each sample was categorized into five groups: prealbumin, albumin, alpha, beta and gamma globulins. Significantly higher serum total glycoprotein values, prealbumin, albumin, alpha, beta and gamma glycoprotein fractions were found in MM patients as compared to the controls. Serum total glycoprotein values, alpha, beta and gamma glycoprotein fractions were found to be significantly increased in group-II as compared to the group-I MM patients. Serum total glycoprotein values, prealbumin, albumin, alpha, beta and gamma glycoprotein fractions were also significantly higher in cervical cancer patients. ROC curve analysis supported with the data and revealed that serum total glycoprotein values, prealbumin and albumin could significantly discriminate between controls and MM patients. Serum albumin, beta and gamma globulin fractions could significantly discriminate between group-I and group-II MM patients. Serum total glycoprotein values, prealbumin, albumin, alpha, beta and gamma globulin fractions could significantly discriminate between controls and cervical cancer patients. Previously, ROC curve analysis for the electrogram analysis parameters is not reported in previous studies.

Patel et al (1997b) have documented decreased albumin:gamma, alpha:gamma and beta:gamma globulin values in patients with oral precancerous conditions as well as untreated oral cancer patients as compared to nontobacco users. Albumin:gamma, alpha:gamma and beta:gamma ratios were lower in non responders as compared to their pretreatment values. Our results suggested that mean serum levels of total glycoprotein values and prealbumin:gamma were significantly higher in MM patients as compared to the controls. Serum total glycoprotein values were found to be significantly higher in group-II as compared to group-I MM patients. Serum total glycoprotein value and prealbumin:gamma were found to be significantly elevated in cervical cancer patients as compared to the controls. ROC curve analysis revealed that serum total glycoprotein values, alpha:gamma and beta:gamma could significantly discriminate between controls and MM patients. Serum total glycoprotein values could significantly discriminate between group-I and group-II MM patients. Serum prealbumin:gamma albumin:gamma, alpha:gamma and beta:gamma could significantly discriminate between controls and cervical cancer patients. ROC curve analysis revealed better clinical utility of these parameters. The analysis of serum total glycoprotein profiles, protein ratio and clinicopathological parameters showed that alterations in total glycoprotein values and gamma were significantly associated with histopathology. Serum total glycoprotein values, albumin:gamma and alpha:gamma were significantly associated with histopathology, while beta:gamma was significantly associated with early vs advanced stage of the disease.

5.7 MMPs and TIMPs in MM and Cervical Cancer Patients:

Metastatic spread of tumours continues to be a major obstacle to successful treatment of cancer. Approximately, 30% of those patients diagnosed with a solid tumour have clinically detectable metastasis. Metastasis are formed continuously throughout the life of the tumour in the remaining 70%. In addition, various growth factors, hormones, oncogenes and tumour promoters are thought to play an important role in the regulation of MMP gene

transcription. After translation, the MMPs are secreted in a latent form. Activated MMPs are formed following proteolytic cleavage of the -NH₂ terminal pro-domain by other MMPs or other proteases. MMPs are a family of zinc dependent endopeptidases with proteolytic activity against wide range of components in the basement membrane and ECM. These enzymes have been implicated in physiologic turnover of ECM and bone remodeling as observed in wound healing, angiogenesis, bone resorption and several other pathological conditions (Barille et al, 1997). Inhibition of MMPs occurs through protease inhibitors such as alpha-2-macroglobulin and by a group of specific TIMPs. All MMPs are synthesized as inactive proenzymes requiring the removal of 80-amino acid terminal for activation. Latent or active MMPs interact with their specific inhibitors (TIMPs). The extracellular activity of MMPs is controlled at the level of gene expression, enzyme activation, and interaction with TIMPs. TIMPs inhibit the catalytic activity of MMPs and they act as growth factors. Finally, the balance of MMPs and TIMPs is critical for control of proteolysis by MMPs. The balance between activated MMPs and free TIMPs determines the net MMP activity. Role of MMP-2, MMP-9, TIMP-1 and TIMP-2 is studied extensively in various malignancies including oral cancer (Patel et al, 2005; 2007), colorectal cancer (Mook et al, 2004), lung cancer (Aljada et al, 2004; Simi et al, 2004), prostate cancer (Ross et al, 2003), cervical cancer (sheu et al; Nair et al, 2003) and renal cell carcinoma (Lein et al, 2000).

Expression of MMPs can be determined by various techniques i.e. Immunohistochemistry, real time polymerase chain reaction, ELISA, western blot, zymography. Among these, gelatin zymography is widely used. This identifies MMPs by the degradation of their preferential substrate and by their molecular weight. This technique also determines different forms of MMPs i.e. Pro(latent) and active forms of MMPs. This technique is widely used as it is simple, sensitive, experimental and functional assay to analyze MMPs from biological samples. In this zymographic assay SDS, used for electrophoresis, dissociates most of the enzyme substrate complex. The various authors have mentioned that gelatin zymography is the main quantitative tool to measure

gelatinases in blood (Wass et al, 2002, 2003, 2005; de Hingh et al, 2004; Dragutinovic et al, 2006). In the present study, gelatinases were analysed from the blood samples by gelatin zymography as well as ELISA. The results of MMP-2 and MMP-9 estimated by zymography and ELISA were comparable. Thus, the zymography is a better alternative to ELISA.

There are few studies showing circulating levels of MMP-2 and MMP-9 using zymography. Sliwowska and Kopczynski (2007) have performed zymography and mentioned it is a simple, sensitive, and functional assay for analysing proteolytic activity of gelatinase A and B. Measuring their activity in serum breast cancer indicates clinical conditions of patients. A good correlation between serum gelatinases activity and disease prognosis suggest its usefulness as marker both for the follow-up and prognosis of breast cancer patients. Dragutinovic (2006) concluded that gelatinase B in serum played an important role in the progression of gastric cancer. ProMMP-9 can be used as a marker for invasiveness of gastric cancer. Study from our laboratory showed that active and latent forms of MMP-2 and MMP-9 had increased gelatinolytic activity in oral SCC malignant tissue compared to their adjacent normal tissue (Patel et al, 2005). Lopata et al (2003) studied 95 women treated for endometrial cancer and 98 women with other gynecological conditions. Gelatin zymography revealed elevated levels of latent and active forms of MMP-2 and MMP-9 in patients with endometrial cancer. For each patient, individual bands of gelatinase activity were given a score from 0 to 5. Total score was summed to provide total MMP score for further analysis. The mean MMP score in uterine washings of patients with endometrial cancer was 10.0 (range 1 to 22) compared to 0.8 (range 0 to 15) in the group without cancer. Sheu et al (2003) has mentioned about the cancer-derived MMPs and proposed them to be essential for stromal invasion and subsequent cancer metastasis as well as progression. They examined the expression of various MMPs and TIMPs in precancerous and cancerous lesions of the uterine cervix. Immunohistochemistry studies demonstrated that MMP-2 and MMP-9 were expressed in more than 90% of SCCs and 83–100% of high-grade SILs. But

they were less frequently expressed in low grade SILs and normal squamous epithelium (13%). MMP-1, MMP-14, and MMP-15 were detected in 55–81% of SCC cases; whereas MMP-1 was detected in 39% of high-grade SILs. TIMPs were weakly expressed in SCC (10–61%). By direct analysis of enzymatic activities in micro dissected specimens, they found that the gelatinolytic activity of MMP-9 was significantly higher in high-grade SILs and SCC than in normal cervix. Levels of active form of MMP-2 increased progressively from high-grade SILs to SCC of stage I and more advanced stages. Gelatinolytic activity of MMP-9 and active form of MMP-2 in SCC were strongly correlated with lymphovascular permeation and subsequent lymph node metastasis. Lengyel et al (2001) suggested that proMMP-9 gelatinolytic activity, but not active MMP-2 or MMP-9 serves as a useful statistically independent prognostic factor in ovarian cancer (FIGO stage III), thus helping in identifying aggressive forms of ovarian cancer. Most of studies in haematological malignancies concerning MMPs are *in vitro* studies. In MM, increased activity of MMPs can cause excessive bone marrow resorption and result in spreading of the myeloma cells. Using *in situ* hybridization and zymography techniques have also shown in MM and mycosis fungoides that enhancement of both MMP-2 and MMP-9 expression is linked to more aggressive and advanced disease state and more extensive neovascularization (Vacca et al 1997, 1999, 2000). In accordance with earlier data, present study analyzed pro-, active and total MMP-2 and MMP-9 activity and TIMP-1 and TIMP-2 levels from serum sample in controls and patients with MM and cervical cancer using gelatin zymography and ELISA. By gelatin zymography all four forms of MMP-2 and MMP-9 (i.e. proMMP-2, activeMMP-2, proMMP-9 and activeMMP-9) were visibly separated. Serum proMMP-2, proMMP-9, activeMMP-2, total MMP-2 and total MMP-9 were significantly higher in MM patients as compared to the controls. Serum activeMMP-2 (7 fold) and activeMMP-9 (7 fold) were significantly higher in cervical cancer patients as compared to the controls. Higher activity of activeMMP-2 and activeMMP-9 may lead to increased invasion and metastasis in MM and cervical cancer patients. Activation ratio of MMP-2 and MMP-9 is worthwhile to study activation of MMP-2 and MMP-9. It

is more meaningful way to express gelatinolytic activity as latent and active forms of MMP-2 and MMP-9. Very few studies have used activation ratio of MMP-2 and MMP-9 to compare relative activity of activeMMP-2 and activeMMP-9. Recent reports from our laboratory documented higher activation ratio of MMP-2 and MMP-9 in oral SCC compared to adjacent normal tissue (Patel et al, 2005). In the present study, activation ratio of MMP-2 and MMP-9 were significantly higher in MM and cervical cancer patients as compared to the controls. The activation ratio of serum MMP-2 levels was significantly higher in group-I as compared to group-II MM patients. Activation is important step in degradation of ECM. Higher the activation ratio, more capacity to degrade active forms of gelatinase.

Plasma proMMP-2 levels have been reported as potential diagnostic marker in primary colorectal carcinoma. This diagnostic potential was absent in liver metastasis of colorectal carcinoma (Wass et al, 2005). Lopata et al (2003) have observed that ROC curve analysis with MMP cutoff score of 3 gave 98% sensitivity and 91% specificity in detection of endometrial cancer. ROC curve for MMP-9 was reported to significantly discriminate between controls and head and neck SCC patients (Ranuncolo et al, 2002). The current study suggested that serum proMMP-2 could significantly discriminate between control and MM patients. Serum proMMP-2, proMMP-9 and activeMMP-9 could significantly discriminate between controls and cervical cancer patients. The ROC curve analysis could reveal good clinical relevance of alterations in MMP-2 and MMP-9.

Pearson's correlation was studied for pro- and active forms of MMP-2 and MMP-9 because gelatinases (MMP-2 and MMP-9) are secreted as inactive (latent) form known as zymogen or a proMMP. These latent zymogens require activation before they can cleave protein components *in vitro* and *in vivo*. The current data suggested that serum proMMP-2 showed significant negative association with serum activeMMP-2 and proMMP-9. Similarly, proMMP-9 was also found negatively and significantly associated with activeMMP-2 in MM patients. The alterations in proMMP-2 was revealed significant positive

correlation with serum activeMMP-2 and proMMP-9 in group-I and group-II MM patients. Correlation analysis for pro-, active and total forms showed serum proMMP-2 were correlated negatively and positively with serum proMMP-9 and serum total MMP-9 respectively in cervical cancer patients. Serum proMMP-2 were showed negative as well as positive and significant correlation with serum activeMMP-2 and total MMP-2, respectively. Serum proMMP-9 showed significant positive association with total MMP-9. Serum proMMP-9 were obtained from data positive and significantly associated with serum proMMP-2 and total MMP-2. Serum ProMMP-9 were showed negative and significant association with serum activeMMP-2. MMP-2 and MMP-9 can also activate each other since the active forms of MMP-2 and MMP-9 is the member of same family. The current study exhibited negative and significant association of serum activeMMP-2 with serum activeMMP-9. In our present study, activation of activeMMP-2 and activeMMP-9 were found in 28% and 56% in cervical cancer patients, respectively.

Lopata et al (2003) reported no significant association between MMP score and histological grade of tumour, vascular invasion or depth of myometrial invasion. In an immunohistochemistry study by Aglund et al (2004) for gelatinase A and B in endometrial cancer revealed that 52% of cases were positive for MMP-9 and 72% for MMP-2. Both, MMP-2 and MMP-9 were correlated with histologic grade of cancer. MMP-9 correlated with clinical stage of the disease whereas MMP-2 did not. However, authors did not found any association with either depth of the invasion, menopausal status or the age of the patient. In accordance with this report, multivariate analysis of serum MMP-2 and MMP-9 (pro-, active, total and activation ratio) and various clinicopathological parameters showed alterations in activation ratio of MMP-2 was significantly associated with age of the patients, whereas proMMP-9 was significantly associated with pathological tumour differentiation in the current study. Total MMP-9 was found significantly correlated with pathological tumour differentiation and early vs advanced stage of the disease in cervical cancer patients.

Beside their ability to inhibit MMP activities as the name suggests, the TIMPs have multidimensional activities. Traditionally TIMPs have been associated with homeostasis of ECM by regulating the activity of MMPs. TIMPs are well-known for their ability to inhibit MMP activity, thereby having a major role in inhibiting growth and metastasis of tumours (Hayakawa et al, 1992; Denhardt et al, 1993; Eccles et al, 1996; Gomez et al 1997). Monitoring the levels of gelatinases and their tissue inhibitors by measuring the immunoreactive protein levels in the serum of patients with cancer is a fascinating possibility. Circulating markers are easy to detect from the serum of cancer patients. This is less invasive compared to taking biopsy from tumour. Several different ELISA tests have been described for the measurement of these circulating proteins. Circulating gelatinases as prognostic markers in solid tumours has been under evaluation during the last few years. Several studies have reported higher levels of circulating gelatinases in patients as compared with healthy controls (Sheen-Chen et al, 2001; Rocca et al, 2004; Staack et al, 2006). Kopczynska et al (2007) have studied serum concentration of MMP-2 and 9 in non-small cell lung cancer patients and concluded that serum MMP-9 may be a marker of metastasis in non-small cell lung cancer. Somiari et al (2006) suggested that plasma gelatinase concentration could be used to distinguish patients with benign breast disease from those who have high risk of developing breast cancer. Elevated levels of circulating TIMP-1 have previously been shown to be associated with poor prognosis in lung, ovarian, transitional cell and colorectal carcinoma (Ylisirnio et al, 2000; Manenti et al, 2003; Aljada et al, 2004; Holten-Andersen et al, 2004). The present study carried out total MMP-2, total MMP-9, TIMP-1 and TIMP-2 by ELISA and zymography from serum samples obtained from controls and MM and cervical cancer patients. Our results showed that serum levels of TIMP-1 were significantly higher in MM patients as compared to the controls. The mean values of serum total MMP-2, total MMP-9 and TIMP-2 were also higher in MM patients as compared to the controls. Serum total MMP-9 levels were significantly higher in cervical cancer patients as compared to the controls.

Jinga et al (2006) found that MMP-9:TIMP-1 and MMP-2:TIMP-2 ratio values were obtained significantly different in malignant tumours compared to benign tumours. Abnormal MMP-9:TIMP-1 balance plays a role in the configuration of breast invasive carcinoma of no special type and also in tumour growth, while altered MMP-2:TIMP-2 ratio value could be associated with lymph node invasion and used as a prognostic marker in correlation with Nottingham prognostic index. Li et al (2005) have demonstrated that the balance between MMP-2 and TIMP-2 plays a crucial role in invasion and metastasis of colorectal carcinoma. Therefore, concurrent study of MMPs and TIMPs is important in order to achieve a better understanding of their role in cancer.

TIMP-1 is a potent inhibitor of MMP-9 and thus, it plays very important role in regulation of MMP-9. Likewise TIMP-2 is a potent inhibitor of MMP-2. Pearson's correlation coefficients of serum total MMP-2 showed significant and positive correlation with serum TIMP-2 in MM, group-I and group-II MM and cervical cancer patients. In current study there was a positive trend, between serum level of TIMP-1 and MMP-9 in controls and MM and cervical cancer patients.

Somiari et al (2006) have reported significant correlation between serum gelatinases and clinicopathological parameters in breast cancer. Turpeenniemi-Hujanen (2005) has assessed the over expression of gelatinases and their tissue inhibitors and found them to correlate with the grade and stage of several solid cancers. Rocca et al (2004) have documented that preoperative serum samples indicated a correlation between serum MMP-2 and MMP-9 and c-erbB2 expression of the tumour, and an inverse correlation with estrogen receptor and nuclear grade of the tumour. Kuropkat et al (2002) found no correlation between TIMP-1 over expression and grade, stage or metastatic potential among head and neck SCC patients. Iki et al (2002) observed that significant correlation between tumour growth and serum MMP levels. Our present study as well as other studies on that serum total MMP-2, MMP-9, TIMP-1, TIMP-2 and various clinicopathological

parameters showed that alterations in serum TIMP-1 was significantly associated with age. A positive correlation relation between TIMP-1 and age in colorectal carcinoma as well as in healthy controls has been reported earlier (Wass et al, 2006; Holeten Anderson et al, 2000, 1999) here, its significance and specifically relation with regard to malignancies are not known. The balance between MMP-2:TIMP-2 complex was found to be significantly associated with early vs. advanced stage of the disease.

5.8 M-Protein Isolation and Its Proteome Analysis:

In our study, M-protein was isolated from serum of 58 year old man diagnosed with MM based on clinical, biochemical [elevated gamma globulin (7.5 gm/dl), decreased A:G ratio (0.48 gm/dl) and M-protein with IgA (10,250 mg/dl)], haematological, radiological, biopsy and histopathological evidences. SDS-PAGE revealed polypeptide chains of different subunits of unknown M-protein. Isolated M-protein were further separated depending on their molecular mass to identify and correlate the unknown protein with isolated M-protein in MM patients. The isolated M-protein exhibited seven different bands separated on the basis of electrophoretic pattern by the SDS treatment. The nature of unknown protein bands was determined with the help of standard protein molecular weight marker. Different molecular weight of proteins such as ~11.48, ~17.78, ~24.54, ~34.67, ~45.70, ~69.18, ~100 kDa were identified from isolated M-protein which was further analyzed for more precise study by proteome approach. The present study also correlated with other published reports mainly in these studies, 2D-PAGE has been used to study heterogeneity of protein expression and provide detailed information for protein perspective in both ways qualitative and quantitative. Huang et al, 2007 have reported that identification of differentially expressed proteins in esophageal cancer and normal tissue will be helpful for screening biomarker for early diagnosis. Herzog et al (2004) have attempted to identify protein markers proteomically that are involved in initiation of apoptotic cell death in preneoplastic colonocytes. These may help to develop new strategies for cancer prevention. Dwek and Alaiya (2003) have analyzed the proteome of

normal and pathological breast specimens removed surgically to identify proteins to differentiate between normal, benign and malignant breast diseases. They have used hierarchical cluster analysis enabled discrimination according to protein expression profiles among these breast tissue specimens. Zhang et al (2003) have reported that loss of clustrin both in serum and tissue correlated with the tumourigenesis of esophageal SCC through proteomic approach. Ryu et al (2003) have observed the over and under expressed proteins in human gastric cancer tissues by proteomic approach. Using proteomics based approaches, Brichory et al (2001) have suggested that protein gene product, 9.5 antigen and or antibodies in serum may have utility in lung cancer screening and diagnosis. In the present study, isolated M protein estimation was performed using 2D-PAGE approach and quantified with PDQuest, the discovery series™ software. Our current study highlighted seven protein spots. In addition to diagnosis of MM, 2D-PAGE appeared to be very suitable technique for in-depth study of microheterogeneity of monoclonal immunoglobulins chain which may be used in clinical laboratory practice. The present study also included that glycosylation changes in group-I and group-II MM patients. The results revealed that TSA, fucose and hexose were higher in group-I as compared to group-II MM patients. The isolated M-protein also showed higher glycosylation. The discovery of new protein in the context of MM and elucidation of the exact protein linkages of the network will be of critical importance for drug development, correct target selection and successful clinical therapy.

In conclusion, the present investigations indicated that incidence of MM were higher in males in age group of 40-70 years (mean age 54 years). While the age range of occurrence of cervical cancer was 20-70 years (mean age 46 years). Histologically confirmed SCC were found in more than 80% of cervical cancers. Less number of ECC and unknown variety were also documented. Majority of cervical cancer patients were diagnosed in the advanced stage. Moderate tumour differentiation was observed in 58% of cervical cancer patients. In addition, current study highlighted the role of protein and their

post-translational modifications in cancer and several important points were noted. Serum total protein levels and fractions were higher in MM as well as group-I and group-II MM patients. Serum IgG and IgM were elevated significantly in MM patients as compared to the controls. Serum total protein levels, gamma fractions and A:G ratio were significantly elevated in group-I as compared to group-II MM patients. M-Protein were present in 58% of MM patients. M-protein estimation can be a guide to malignant plasma cell load and decreased A:G ratio could be helpful in monitoring cancer progression. Monoclonal protein showed that majority of the M-protein was of IgG type (79.4%). Higher serum protein profiles and ratio may be considered as potential tumour markers for diagnosis in MM, group-I and group-II and cervical cancer patients. Higher levels of glycoconjugates, profiling patterns, protein ratio of glycoconjugates and glycosylation patterns may correlate with increased malignant transformation of cells which may serve as early indicator of malignancy. The protein ratios may be useful in establishing an index which can identify high risk group of cancer. Higher levels of gelatinases and their tissue inhibitors showed that increased the breakdown of basement membrane and ECM that leads to invasion and metastasis. Seven different bands of isolated M-protein revealed by 2D-PAGE approach and its glycosylation pattern could be used as possible novel protein for therapeutic target for MM.