Synopsis

Proteomics-based identification of serum biomarkers: Role of secreted MCSF and CRP in glioma pathogenesis

Gliomas are the neoplasia of glial cells present in the brain and comprises of more than 70% of all the neoplasms of the central nervous system. They consist of a family of primary brain tumors that are categorized based on the cell type of origin. Astrocytoma, a tumor of astrocytic glial cells has the highest frequency of occurrence as compared to other glioma types and often referred to as glioma. Based on the malignancy of the tumor, World Health Organization (WHO) has classified astrocytoma into Grade I/Pilocytic Astrocytoma (PA), Grade II/ Diffuse Astrocytoma (DA), Grade III/Anaplastic Astrocytoma (AA) and Grade IV/Glioblastoma (GBM). GBM is the most frequent and malignant primary brain tumor in adults. The standard treatment for GBM includes surgical resection of the tumor followed by radiation and temozolomide therapy. In spite of such mutlimodal treatment protocol followed, the median survival of the GBM remains as low as 15 months. While multiple markers with potential utility in diagnosis, prognosis and therapy of GBM have been reported based on profiling of gene expression, protein expression, miRNA and methylation pattern using tumor tissue, serum-based markers are scanty. Serum biomarkers have great potential in clinical decision making and management of glioma. Serum is an attractive source for biomarker mining since it can be obtained easily by less invasive method. Further, they have wide range of application which includes diagnosis, disease classification, prognosis, predicting risk and outcome of the disease. More importantly, serum can be obtained easily during follow-up of disease which could make it useful to detect tumor recurrence. This is particularly beneficial because glioma diagnosis and monitoring tumor recurrence are carried out by Magnetic Resonance Imaging (MRI)

and Computed Tomography (CT) scan, which is time consuming and less cost effective. The current study was designed to identify serum biomarkers of glioma by using proteomic approaches and to understand the functional role of selected biomarkers in glioma pathogenesis. This study has been divided into three parts. In part I, we profiled cytokines using bead array method and by applying various statistical analyses we derived an 18-cytokine signature. In part II, Macrophage Colony Stimulating Factor (MCSF), one of the proteins elevated in GBM sera as identified by bead array method was investigated for its regulation and function in glioma. In part III, serum profiling by antibody microarray was performed and developed a serum based three-marker panel for distinguishing GBM from normal samples. Further, the role of C-Reactive Protein (CRP), a highest abundant serum protein in GBM, was investigated in detail.

PART I: Serum cytokine profiling and identification of 18-cytokine signature for glioma diagnosis

Cytokines are implicated in various tumor development and progression. They are deregulated in many cancers including glioma. In this part, serum samples from normal (n= 26), DA (n= 24), AA (n= 22) and GBM (n= 148) were profiled for 48 cytokines using bead array proteomic method. The cytokine profiling resulted in the identification of 33 cytokines that are present in differential abundance in GBM sera when compared to normal sera. Further, with the interest to identify minimum number of cytokines that could efficiently discriminate GBM sera from normal sera, we carried out Prediction Analysis for Microarrays (PAM). First, we randomly divided the normal and GBM samples into two equal halves namely training set and test set. Next, by performing PAM in training set, we derived an 18-serum cytokine signature that could distinguish GBM from normal samples with a diagnostic accuracy of 95.40% and minimum error of 0.046.

The robustness of this signature was confirmed by internal validation using random subset analysis and Support Vector Machine (SVM). Further, we applied 18-cytokine signature in the test set which resulted in discrimination of GBM sera from normal sera with diagnostic accuracy of 96.55% and an overall error rate of 0.034. The signature was also tested on DA vs normal and AA vs normal samples and resulted in diagnostic accuracy of 96% and 95.83% respectively. Further, network analysis using 18 cytokines of the signature resulted in the enrichment of two pathways with high significance: cytokine-cytokine receptor interaction and JAK-STAT pathways. In conclusion, we identified 18-cytokine signature that could distinguish normal and glioma samples with high accuracy and also demonstrated the importance of their differential abundance in glioma biology. This finding suggests that 18-cytokine signature have the potential to be used for the development of blood-based diagnostic tests for glioma.

PART II: Regulation of MCSF by SYK-P13K-NF-κB pathway and identification of IGFBP1 in the microglial secretome as a novel mediator of MCSF-induced angiogenesis in glioma

MCSF, one of the elevated cytokines in GBM sera, identified by bead array method, was investigated for its role in glioma pathogenesis. In this part, we analyzed MCSF transcript and protein expression and found to be elevated in glioma tumor tissue and cell lines. Next, we demonstrate that MCSF is regulated by SYK-PI3K-NFkB pathway in glioma. Further, we also found MCSF to be an independent indicator of poor prognosis in GBM. MCSF is a secreted protein, hence we hypothesized that it may function in both autocrine and paracrine fashion. The modulation of MCSF in glioma cell lines, either by overexpression or silencing revealed that, there is no significant autocrine role for MCSF

in glioma. MCSF is known to mainly function through its receptor Macrophage Colony Stimulating Factor Receptor (MCSFR). Macrophages/microglial cells express MCSFR and hence are the targets for MCSF action. Based on the stimuli received, macrophages/microglial cells can be either M1 phenotype which is anti-tumorigenic or M2 phenotype which is pro-tumorigenic. By immunostaining, we observed numerous macrophages/microglia infiltrations in glioma tumor tissue compared to normal brain and these cells belong to M2 phenotype. We carried out macrophages/microglia polarization studies and found that glioma cell condition media (GCM) induces M2 phenotype. However, our results demonstrate that MCSF was not essential for M2 polarization of macrophages/microglial cells. Further investigation identified that MCSF functions in a paracrine manner via macrophages/microglial cells and induced these cells to secrete certain factors which subsequently increased angiogenesis *in vitro* and *in vivo*. In order to identify the MCSF-regulated secreted factors in microglia, that are responsible for angiogenesis, we utilized Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC), a quantitative proteomic approach. We subjected the secretome of microglial cells, which was either treated with control GCM or GCM derived from MCSF silenced condition, to SILAC. The analysis of SILAC data yielded 71 proteins to be significantly regulated by MCSF, which included VEGF, a well-known angiogenic factor. Further, network analysis using MCSF-regulated proteins resulted in the enrichment of several pathways relevant to endothelial function and angiogenesis. Insulin-like Growth Factor-Binding Protein 1 (IGFBP1), one of the MCSF-regulated proteins identified by SILAC, was further validated both at transcript and secreted protein levels. To examine the role of IGFBP1 in MCSF-induced angiogenesis, we performed angiogenesis assay using supernatants derived from microglia by two approaches- neutralizing IGFBP1 in the GCM treated-microglial supernatant using specific antibody or silencing IGFBP1

expression by siRNA in microglial cells prior to GCM treatment. Our results found IGFBP1 to be the novel mediator of MCSF-induced angiogenesis. Collectively, this study demonstrated requirement of SYK-PI3K-NFkB pathway for MCSF regulation. Further, we identified that glioma-secreted MCSF promotes angiogenesis via macrophages/microglial cells which is mediated by IGFBP1. This study suggests IGFBP1 could be a potential candidate for developing a targeted therapy for GBM.

PART III: Identification of microglia-secreted Interleukin 1β as the novel mediator of CRP-induced endothelial cell survival

In this part, serum samples from normal healthy individuals (n= 27) and GBM (n= 28) were subjected to antibody microarray analysis. The antibody microarray used in this study targeted 724 cancer-associated proteins. This analysis identified 69 proteins to be present in differential abundance in GBM sera when compared to normal sera. Further, five differentially abundant serum proteins- Interleukin-12 subunit alpha (IL12A), L-selectin (LYAM1), Basic helix-loop-helix protein 40 (BHE40), C-reactive protein (CRP) and Somatostatin receptor type 4 (SSR4) with high significance were subjected to Receiver Operating Characteristics (ROC) curve analysis. This analysis found that each of these proteins could discriminate GBM serum samples from normal serum samples with accuracy of $\geq 70\%$. In order to improve the discriminatory power, we combined markers which had an Area Under Curve (AUC) of above 0.75 and performed ROC curve analysis. This analysis resulted in a panel consisting of three serum proteins- CRP, LYAM1 and BHE40 which could discriminate GBM sera from that of normal with a superior accuracy of 89.7%.

CRP was the highest abundant protein in GBM sera when compared to nor-

mal sera as identified by antibody microarray. First, we validated CRP levels by ELISA in an independent set of sera samples. We found serum CRP levels to be an indicator of poor prognosis in GBM patients. Next, we analyzed CRP transcript levels in glioma tumor tissue as well as in cell lines and found that the expression was similar to that of normal brain tissue, suggesting a non-tumoral origin of serum CRP. Interestingly, immunostaining results revealed high protein expression of CRP and its receptor FcγRIII in the glioma tumor tissue compared to normal brain tissue particularly in microglial cells, suggesting an important role of CRP in glioma development/progression through FcγRIII. Further, we demonstrate that IL6 secreted by glioma cells act on hepatocytes to secrete CRP mediated by JAK/STAT pathway. We found that CRP does not have direct effect on tumor cells, microglial cells and endothelial cells by performing various *in vitro* experiments using recombinant CRP (rCRP). However, we found that CRP acts on microglial cells via FcγRIII and induces these cells to secrete certain factors which in turn increased endothelial cell survival under nutrient-deprivation condition. In order to identify the CRP-regulated secretory proteins in microglia, that are responsible for increased endothelial cell survival, we performed transcriptome analysis of CRP-treated microglial cells. This analysis identified a total of 134 genes to be regulated by CRP. Interleukin 1 beta (IL1β) was one of the upregulated genes in CRP-treated microglia and was further validated both at transcript and secreted protein levels. To assess the role of IL1β in CRP-induced endothelial cell survival, we performed endothelial cell survival experiments using microglial supernatant derived from two approaches- neutralizing IL1β in the CRP-treated microglial supernatant using specific antibody or silencing IL1β expression by siRNA in microglial cells prior to CRP treatment. The results of these two approaches demonstrated important role of IL1β in CRP-induced endothelial cell survival under nutrient-deprivation condition. In conclusion, our study identified a

serum based three-marker panel for distinguishing GBM sera from that of healthy individuals with high accuracy. We found serum CRP levels to be poor prognostic indicator in GBM. Further, we demonstrate glioma-secreted IL6 induces CRP expression in hepatocytes by JAK-STAT pathway. We also found microglia-secreted IL1β as the key mediator of CRP-induced endothelial cell survival with a potential of developing a targeted therapy.