

Synopsis

Malignancy of glial cells is termed as glioma. Gliomas comprise of thirty percent of all tumors of the central nervous system (CNS) and eighty percent of malignant brain tumors (Goodenberger and Jenkins, 2012). Astrocytoma, a type of glioma that arise from astrocytes, is the most common and lethal type of intracranial tumor. It is divided into four groups according to WHO (2007) classification based on histopathology. Grade I or pilocytic astrocytoma is benign in nature; however, the other three grades are progressively more malignant. Grade II or diffused astrocytoma is less aggressive and have a median survival of 5-8 years (Tove *et al.*, 2012); while Grade III or anaplastic astrocytoma and glioblastoma (GBM) are classified under high grade gliomas with median survival of 2-3 years and 12-15 months respectively (Nuño *et al.*, 2013; Arvold *et al.*, 2014). GBM tumor is fast growing, highly infiltrative, and treatment refractory. It can be divided into two categories on the basis of their origin - primary GBM that accounts for 90 percent of GBM cases, manifest *de novo* without prior evidence of a pre-existing tumor of lower grade; while secondary GBMs develop through malignant progression of lower grade astrocytomas. Secondary GBMs typically exhibit genetic aberrations like TP53 mutation, IDH1 mutation, PTEN mutation, loss of chromosomal arm 10q etc. Primary GBMs show a whole host of genetic aberrations including EGFR amplification, PDGFR amplification, mutation in TP53, PTEN, NF1, RB etc., amplification of MDM2 and MDM4, loss of chromosomal arms 10p and 10q, CDKN2A and CDKN2B loss (Furnari *et al.*, 2007). With the current treatment modality of GBM that includes surgical resection followed by radiotherapy and temozolomide chemotherapy, the median survival achieved till date is only 15 months and in almost all cases, the tumor recurs (Stupp *et al.*, 2009). The aggressive and recurrent nature of GBM demands further insights into the molecular pathways deregulated in GBM. The changes that happen within a cell that lead to malignancy include both genetic and epigenetic alterations. Our objective is to delineate deregulated pathways in GBM progression and development through screening of genes via next generation sequencing (NGS) and methylome array. We further strive to characterize the importance of identified altered molecules and deregulated pathways in the context of GBM pathogenesis.

Part A. Unraveling the genetic landscape of glioma using next generation sequencing

NGS based techniques help us to look at the genome of cells on a large-scale, yet comprehensive manner. Once we obtain the entire genetic alteration profile of GBM, it helps us in identifying particular molecules or pathways that has strong implications in GBM pathogenesis. This paves the path for identification of novel molecules that undergo changes in GBM tumor cells, thus opening up avenues for targeted therapy.

Part A.1. Genetic landscape of glioma reveals that mutated inactivated neuroactive ligand-receptor interaction signaling pathway predicts poor survival of GBM patients

The first objective of this study includes grade specific analysis of the mutation spectrum of malignant astrocytoma patients in an Indian cohort (our patient set). Towards this, we have carried out whole exome sequencing (WES) of 43 astrocytoma patients (10 grade II, 13 grade III and 20 GBM) along with matched peripheral blood tissue derived DNA. Using Mutect tool, tissue derived DNA was compared with matched blood-derived DNA to find out tumor-specific mutations. Comparison of the mutational landscape of our patient set with that of WES data from The Cancer Genome Atlas (TCGA) revealed tumors from both datasets to behave similarly.

Our next objective was to find out mutated pathways in GBM that could predict survival thus contributing to tumor aggressiveness. For this purpose, we also analyzed WES data of a larger cohort of astrocytoma patients (63 grade II, 131 grade III and 291 GBM) from TCGA. Using both datasets (our patient set and TCGA dataset), we have carried out integrative genomic study to determine novel altered molecules for possible therapeutic interventions in GBM. Top mutated genes from both datasets were subjected to survival analysis and the significant genes were used for KEGG pathway enrichment. Of the various pathways enriched, neuroactive ligand-receptor interaction pathway inactivation by mutation was found to decrease the survival of GBM patients drastically. This pathway contains 165

G-protein coupled receptors (GPCRs) that were identified to be involved in neuronal signaling, thus influencing animal behavior.

The highest mutated gene present in this pathway was found to be calcitonin receptor (CTR). We further looked into the function of this gene through *in vitro* cell-line based experiments. The RNA level of CTR was found to be down-regulated in GBM tumor tissue as well as GBM cell lines as compared to control samples. This led us to hypothesize that CTR possibly functions as a tumor suppressor in GBM. Indeed, activation of this pathway by its ligand, calcitonin (CT), in a GBM cell line which expresses higher levels of CTR, was found to decrease proliferation, migration and anchorage-independent growth capacity of GBM cells with a concomitant decrease in the phosphorylation levels of Erk, Akt and Jnk signaling molecules. Further, we observed that CT inefficiently inhibited the proliferation and migration in a GBM cell line where a CTR level is low. In fact, in the CTR-low cell line, exogenous expression of CTR and addition of CT led to a significant decrease in proliferation and migration, suggesting that activation of CTR by its ligand leads to inhibition of pro-proliferative and pro-migratory pathways in GBM cells. We next introduced GBM patient-derived mutations in the cDNA of the CTR gene. We tested for the effects of two such mutations of CTR in GBM cells. Indeed, one of the above two mutations led to a significant abrogation of the tumor suppressive function of CTR, i.e., addition of CT in CTR-mutant over-expressing GBM cell line failed to inhibit proliferation and migration of the cells. Our future direction involves, understanding the effects of all the mutations found in CTR gene and studying the efficacy of CTR as a novel therapeutic target in GBM treatment through *in vivo* experiments. Thus, we found that CTR behaves as a tumor suppressor in GBM and its mutational inactivation possibly leads to GBM aggressiveness.

Part A.2. Elucidating the cancer-specific genetic alteration spectrum of glioblastoma derived cell lines from whole exome and RNA sequencing

Cell lines are cells derived from tumor tissues and cultured *in vitro* for long period of time. Over decades, they have been used as a valuable system for the study of gene regulation and cancer development in human. Comprehensive characterization of the genetic background of cell lines could provide important clues on novel genes responsible for carcinogenesis and help in choosing cell lines for particular studies. In this study, we have

carried out WES and RNA-sequencing of commonly used GBM cell lines (U87, T98G, LN229, U343, U373 and LN18) to unearth single nucleotide variations (SNVs), indels, differential gene expression profile, gene fusions and RNA editing events.

Here, we particularly focus on the genetic alterations in cell lines which have imminent roles in carcinogenesis. From the total SNV data, we have filtered out those variants which are present in normal individuals (ESP6500, 1000g and dbSNP datasets) to unearth cancer-specific alterations. The most frequent alterations were observed in TP53, PTEN, TCHH and MLL3 genes. Further, mutations in EGFR, NF1 and PDGFRA were found in some of the cell lines. No mutation in IDH1 was observed in any of the cell lines. The mutation status of hTERT promoter was looked at using Sanger sequencing. Five out of six cell lines were mutant for hTERT promoter and this was accompanied by a concomitant increase in hTERT gene expression levels. Comparison of the transcriptome of the cell lines with GBM tumor tissue microarray data from TCGA revealed a concordance of ~73% between GBM cell lines and tumor tissue suggesting that the transcriptomic make up of these cell lines closely resemble that of the tumor. Gene fusion analysis showed five significant fusion events of which NUP93-CYB5B fusion was validated using Sanger sequencing. The fusion protein was hypothesized to bind to CREB-binding protein and bring it onto the outer mitochondrial membrane. This may lead to acetylation of outer mitochondrial membrane proteins thus causing a change in the energy status of cancer cells. Additionally, an average of 18,949 RNA editing events was also obtained. Thus we have generated a comprehensive catalogue of genetic alterations for six GBM cell lines.

Part B. Methylation silencing of GNG4 is essential for SDF1 α /CXCR4 signaling and cell migration in mesenchymal glioblastoma

With current advancement of our understanding, it is evident that epigenetics has an equally important role in carcinogenesis. Global DNA methylation profiling of GBM was carried out previously, and this identified Guanine Nucleotide binding-protein Gamma subunit 4 (GNG4) to be one of the most hyper methylated and down regulated genes in GBM (Shukla *et al.*, 2013). G-protein trimers, consisting of alpha, beta and gamma subunits, are responsible for mediating signals from GPCRs to the inside of the cell. The alpha subunit generally activates effector molecules post GPCR activation and the beta-gamma

heterodimers behave as regulators of the signal. GNG4 is one of the fourteen gamma subunits encoded by the human genome.

Since, GNG4 is down-regulated in GBM by methylation, we hypothesized that it is a tumor suppressor gene. The importance of GNG4 as a potential tumor suppressor was evaluated in GBM cell lines where we found that ectopic over-expression of the gene leads to inhibition of proliferation and migration capacity of the cells. Furthermore, exogenous over expression of GNG4 in immortalized normal human astrocytes led to a decrease in the transformation of these cells by RAS oncogene.

Integrative analysis was carried out to find out potential GPCRs regulated by GNG4 which revealed chemokine receptors to be the potential targets. Next, we selected chemokine receptor CXCR4 for further experiments because it is highly up-regulated in GBM and it is known to play important role in GBM cell proliferation and migration. Indeed, addition of SDF1 α to CXCR4-expressing GBM cells showed increase in migratory capacity with a concomitant increase in phospho-Erk1/2 and phospho-Jnk levels. However, addition of SDF1 α where GNG4 is over-expressed, failed to increase migration as well as phosphorylation of Erk1/2 and Jnk. The inhibitory association between GNG4 and SDF1 α /CXCR4 was more evident in mesenchymal subtype of GBM which is characterized by more infiltrative and aggressive tumor cells. Thus, this study identifies GNG4 as an inhibitor of SDF1 α /CXCR4-dependent signaling and emphasizes the significance of epigenetic inactivation of GNG4 in glioblastoma, especially in mesenchymal subtype.