Abstract

Gliomas are primary brain tumors in adults that are believed to originate from different types of glial cells. Central nervous system gliomas pose particularly difficult problems because of their tendency towards malignancy, rate of tumor spread, and the lack of effective therapy. Glioblastoma (GBM/ grade IV glioma) is one of the most aggressive types of gliomas. Despite the advancement in treatment modalities including surgery, chemotherapy and radiotherapy, the overall median survival of a GBM patient remains at 14.6 months. This dismal overall picture of survival is further aggravated by the grim quality of life of the GBM patients.

Advances in high-throughput technologies have enabled us to mine the molecular pathways contributing to pathogenesis and resistance of GBM. Integrated genomic and epigenomic screens have revealed several molecular markers and has tremendously improved the classification of these heterogeneous tumors. These finding have also given key insights into the various genetic and epigenetic derailments that contribute towards gliomagenesis. Broadly speaking, genomics and transcriptomics for several decades has kept the focus of research on mutation, copy number variation, chromatin remodellers and transcriptional regulation as major determinants of gene expression alterations in cancer. Recent advances in these fields have paved the way to gain a better understanding of the regulation imposed at the post-transcriptional level. Non-coding RNAs and RNA binding proteins (RBPs) are emerging as important post-transcriptional modulators of gene expression. Our study illustrates the various aspects of RBP biology in GBM, where our focus ranges from surfacing the RBP landscape of GBM, delineating the magnitude of regulation of a particular RBP, IMP3 (*I*GF2 *m*RNA binding *p*rotein 3) and also determine one of the targets of this protein which contributes towards glioma stem-like cell (GSC) maintenance in GBM. The thesis is divided in three work-related chapters: chapter 3, chapter 4 and chapter 5. In chapter 3, we have performed extensive bioinformatics analyses to get an idea about the altered RBPome of GBM and the probable mechanisms leading to this mis-regulation. In chapter 4, we exemplify IMP3 to study the extent of multi-level post-transcriptional regulation imposed by RBPs in glioma cells. Further, in chapter 5, we conclude that IMP3 is a critical RBP for GSC maintenance. Moreover, we establish p65, a subunit of NF-κB pathway as a translationally activated target

of IMP3. p65 also acts as a downstream mediator of IMP3 in maintaining glioma stem-like cell survival and proliferation.

CHAPTER 3: Elucidation of the genetic and epigenetic landscape alterations in RNA binding proteins in glioblastoma

RNA binding proteins (RBPs) are global regulators which participate in various steps of RNA metabolism. Though, recent studies have indicated their importance in diseases including cancer, the molecular mechanisms regulated by these proteins still remains elusive.

In this study, we have carried out an integrated bioinformatics analysis of the status of RBPs (n = 1756) in various datasets (n = 11) to identify several genetic and epigenetically altered events among RBPs in GBM. Alterations in RBPs which included mutation, InDels (Insertion and Deletions) and expression level changes, which may lead to aberrant activity of these global regulators were assessed in our analyses. Whole exome sequencing (WES) data from The Cancer Genome Atlas (TCGA) was analysed to identify mutated RBPs in GBM. We identified 13 RBPs to be mutated in minimum of 2% of GBM samples. Further, correlation of mutations in *AHNAK* predicted poor prognosis. Integrated analyses of transcriptome, Copy Number Variation (CNV) data, DNA methylome and miRnome were carried out to identify differentially regulated RBPs. There were 472 differentially regulated RBPs. Additional analysis revealed that a significant proportion of differential regulation is due to CNV (9%), DNA methylation (9.5%) and miRNA targeting (37%). To obtain insights into transformation and aggressiveness related RBPs in GBM, we analysed transcript levels of RBPs in grade II and grade IV (GBM) samples. Differentially regulated RBPs in grade II astrocytoma when compared to control brain samples, which had similar expression in GBM were identified as transformation related RBPs. On the other hand, RBPs that showed differential regulation only in GBM when compared to grade II and control brain samples were identified as aggressiveness related RBPs. These two sets of genes may be implicated in initial astrocytic transformation and glioma progression respectively. We also compared the transcriptome data of neural stem cells (NSC), glioma stem-like cells (GSC) and differentiated glioma cells (DGC) to identify GSC specific RBPs signature, which was further subjected to survival analysis and gene set enrichment analysis. These analyses led to the identification of a unique set of differentially regulated RBPs (n = 34) specifically in GSCs compared to NSC and DGC.

RBP risk score derived from four prognostic RBPs (*NOL3, SUCLG1, HERC5* and *AFF3*) is demonstrated to be an independent poor prognostic indicator in GBM. RBP risk score also stratified GBM patients into low-risk and high-risk groups with a significant survival difference. Gene set enrichment analysis (GSEA) of differentially regulated genes between high-risk and low-risk identified positive enrichment of NF-κB, inflammatory response, epithelial mesenchymal transition and hypoxia pathways in high-risk GBM. Thus, our study provides a comprehensive overview of genetic and epigenetic regulation of RBPs in glioma development and progression.

CHAPTER 4: Transcriptome and translatome regulation by IMP3 in glioma cells

IMP3 in the previous section was found to be an upregulated and aggressiveness related RBP in GBM. Previous work from our laboratory had established IMP3 as an RBP which contributes to proliferation, migration, invasion, chemoresistance and angiogenesis of glioma cell lines. Moreover, it was shown to enhance *IGF2* translation, without altering its transcript levels. This regulation was attributed to the increased activation of pro-survival pathways like PI3K and MAPK in IMP3 overexpressing cells. Enormous volume of literature demonstrates that RBPs are bestowed with the ability to bind multiple targets and regulate their fate at various levels including their degradation, localization and translation.

Recent research has laid emphasis on RBPs as global regulators of RNA metabolism and translation. Hence, we were intrigued to understand the effect of IMP3 on transcriptome and translatome of glioma cells. To get insights in IMP3 modulated transcriptome, we performed a microarray based global gene profiling of total cellular RNA of IMP3 silenced U251 cells. We identified 2788 differentially regulated genes at the transcript level. Further, these differentially regulated genes were classified as direct and indirect targets depending on the presence of IMP3 binding sites. We speculate that the RNA stability of these direct targets may be regulated by IMP3. Our observations suggest that, IMP3 may act as a bimodular regulator of RNA stability of the identified direct targets. This implies that few of the targets may be stabilized while others may be destabilized by IMP3 binding. Correlation of the expression of the differentially regulated targets with *IMP3* transcript in GBM tumors (TCGA data is used) revealed a list of genes which is more likely to be regulated by IMP3 in GBM

tumors. Biological processes enrichment analysis of these direct and correlated targets suggested that IMP3 regulates cell cycle progression by regulating these genes.

Next, we were interested in identifying the genes getting differentially regulated exclusively at the level of translation. Polysome analysis was performed on IMP3 silenced cells, and RNA from the pooled heavy polysome fractions was subjected to microarray. Differentially regulated genes in heavy polysome fractions whose expression remain unaltered in total RNA were selected for further investigation. Similar trend of bimodular regulation was also observed at the level of translation. Direct and indirect targets were identified on the basis of presence of IMP3 binding sites. Interestingly, we found that several direct translation targets of IMP3 were associated with apoptosis and cell death related pathways.

We have also directed our efforts in unravelling few of the possible mechanisms which may contribute in regulating the indirect targets, at transcriptome and translatome level. Transcription factors (TFs) and RBPs which are direct targets of IMP3, and which may influence the gene expression of indirect targets of IMP3 were identified. Taken together, we have unravelled the IMP3 regulons in glioma cells and their role in cell cycle progression and apoptosis.

CHAPTER 5: IMP3 contributes to glioma stem-like cell maintenance and chemoresistance by promoting the translation of *RelA/p65*

Therapy resistance presents a severe challenge in battling GBM. One of the culprits for chemo- and radioresistance identified in GBM tumors is a small proportion of slow-dividing glioma stem-like cells (GSCs) which are refractory to current treatment modalities. These cells are thus spared by the treatment and then repopulate to give rise to an even more belligerent recurrent tumor. Identification of molecules specifically expressed in GSCs, but not in their normal counterparts (adult human neural stem cells-ahNSC) may provide potential lucrative targets for therapy.

Interestingly, using a publically available microarray data for GSC and ahNSC, we found that IMP3 was the most upregulated RBP in GSCs as compared to ahNSC. Our Gene Set Enrichment Analysis results indicated that GSC signature genes were also closely linked to IMP3 expression in GBM tumors. Experiments carried out in IMP3 silenced conditions

revealed that IMP3 is required for GSC maintenance and imparts chemoresistance to glioma cells and GSCs. These experiments provide compelling evidence that expression of IMP3 is imperative for GSC survival and their chemoresistance.

Owing to the promiscuous binding of RNA binding proteins, we were intrigued to identify a direct target regulated by IMP3 which is also necessary for GSC maintenance by IMP3. We were keen to identify the transcription factors which are regulated by IMP3 at the translation level. Thus, we focussed on the transcription factors harbouring IMP3 binding sites and which may be unregulated at the transcript level by IMP3. Integrated bioinformatic analysis using published datasets revealed *RELA* (*p65*) as a potential target which fulfilled all the aforementioned criteria. Ectopic overexpression and silencing of IMP3 in glioma cell lines confirmed that IMP3 increases the NF-κB pathway activity. Moreover, as expected, *p65* transcript levels did not change under IMP3 modulated conditions, while significant protein level changes were observed. IMP3 overexpression led to increased p65 protein levels, while its silencing reduced p65 protein levels significantly in the glioma cell lines. Furthermore, the reduced concentration of p65 protein in IMP3 depleted cells was due to decreased translation of *p65* transcript in these conditions, with no effect on its protein stability upon IMP3 modulation. We also establish that IMP3 directly binds to three sites present at the 3'UTR of *p65* transcript. Furthermore, alleviation of decrease in neurosphere numbers was observed upon exogenous overexpression of p65 in IMP3 silenced GSCs. We also establish IMP3 as a transcriptional target of p65.

Taken together, this study establishes p65 as a novel and bonafide target of IMP3 and as a mediator of IMP3 in GSC maintenance. It also underscores the significance of IMP3 as a therapeutic target, which can indirectly be used to target NF-κB pathway in glioblastoma.