Differential gene expression of Grade II and Grade IV human astrocytoma and anti-cancer drugs targeting glioma *in vitro* and *in vivo*

Thesis submitted to the University of Hyderabad for the award of Ph.D. degree in
Department of Biotechnology and Bioinformatics

By

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(A central university established in 1974 by act of parliament)

Hyderabad-500046, INDIA

DECLARATION

I **Ch. Ramulu** hereby declare that thesis entitled **"Differential gene expression of Grade II and Grade IV human astrocytoma and anti-cancer drug(s) targeting glioma *in vitro* and *in vivo*"** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University for the award of any degree or diploma.

Date:

Name: Ch. Ramulu

Signature of the Student

Regd. No. 08LTPH04

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(A Central University established in 1974 by act of parliament)
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CERTIFICATE

This is to certify that thesis entitled **"Differential gene expression of Grade II and Grade IV human astrocytoma and anti-cancer drug(s) targeting glioma *in vitro* and *in vivo*"** is a record of bonafide work done by **Ch. Ramulu** research scholar for Ph.D. programme in Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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Ch. Ramulu

INDEX

ABBREVIATIONS

ALP: Alkaline peroxidase

ASR: Age Standardized incidence or mortality Rate

APES: Amino-Propyl tri-ethoxy-Silane

BBB: Blood Brain Barrier

BCIP: 5-Bromo-4-chloro-3'-indoylphosphate p- toluidine salt

Bcl-2: B-cell lymphoma protein-2

Bcl-xl: B-cell lymphoma-extra large

BCNU: Bis-chloro-ethylnitro-sourea

BCPC: Brain Cancer–Propagating Cells

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CMIC benzyl: (1-benzyl-H-1,2,3-triazol-4-yl) methyl 3-(2,6-dichloro phenyl)-5-methyl isoxazole-4-carboxylate

CT: Computed Tomography.

DR: death Receptor

DcR: Decoy Receptor

ECM: Extra Cellular Matrix

EDTA: Ethylene diamine tetra acetate

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ENU: n-Ethyl n-nitrosourea

ERK1/2: Extracellular signal regulated kinase ½

FGF: Fibroblast growth factor

GBM: Glioblastoma multiforme

GFAP: Glial fibrillary acidic protein

GSK3β: Glycogen synthase kinase 3β

IARC: International Agency for Research on Cancer

IDH1: Isocitrate dehydrogenase 1 (NADP+), soluble

IHC: Immuno-histochemistry

HIF: hypoxia-inducible factor

MAPK: Mitogen activated protein kinase

MDM2: Mouse double minute-2 protein

MEF: Mouse embryo fibroblasts

MIR-85: Micro RNA 185

MMP: Matrix Metallo Proteinase

MNU: n-Methyl n-nitrosourea

MRI: Magnetic resonance imaging

NBT: Nitro-blue tetrazolium chloride

NF1: Neurofibromin 1

NF-*κ*B: Nuclear factor *κ*B

NPC: Neuronal precursor cells

PARP: Poly (ADP-ribosyl) polymerase

PET: Positron Emission Tomography

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

PDGFR: Platelet derived growth factor receptor

PFA: Para Formaldehyde

PI3K: Phospho-Inositol-3-Kinase

PMSF: Phenyl methyl sulphonyl fluoride

PTEN: Phosphatase tensin homologue

Rb: Retinoblastoma

RIPA: Radioimmunoprecipitation assay

RTK: Receptor tyrosine kinase

SIX1: Sine oculis homeobox homolog 1

SNP: Single Nucleotide Polymorphism

SPECT: Single Photon Emission Tomography

SVZ: Sub-ventricular zone

TBS: Tris-buffered saline

TBST: Tris-buffered saline Tween 20

TEP1: TEnsin-like phosphatase

TFs: Transcription factors

TICs: Tumor–Initiating Cells

TMZ: Temozolomide

TNFα: Tumor necrosis factor α

TRAIL: Tumor Necrosis Factor- related apoptosis inducing ligand

WHO: World health organization

ZNF24: Zinc Finger Protein 24

CHAPTER 1

General introduction

1.1. INTRODUCTION

The brain, together with the spinal cord, makes up the central nervous system (CNS). This vital organ coordinates the body's functions. Brain consists of nerve cells or neurons that are involved in communication among themselves and with other organs of the body through nerve impulses. These neurons are supported by glial cells, which are differentiated into astrocytes, oligodendrocytes and ependymal cells.

In our body many cell types progressively become older and undergo natural cell death. These dead cells are replaced with new cells by a process called cell division. Cell division is generally a well regulated controlled phenomenon. Any deregulation in this cell division process leads to uncontrolled proliferation resulting in cellular lump or mass formation which is called tumor. Different types of tumors originate from varied precursors and are classified based on their origin.

The word glioma encompasses all tumors that originate from glial cells. Gliomas occupy nearly 80% of malignant tumors of the brain and it includes astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas based on their cell type. Among these gliomas astrocytomas account for 76% oligodendroglioma 6.5% and ependymomas 5.95% (CBTRUS 2011).

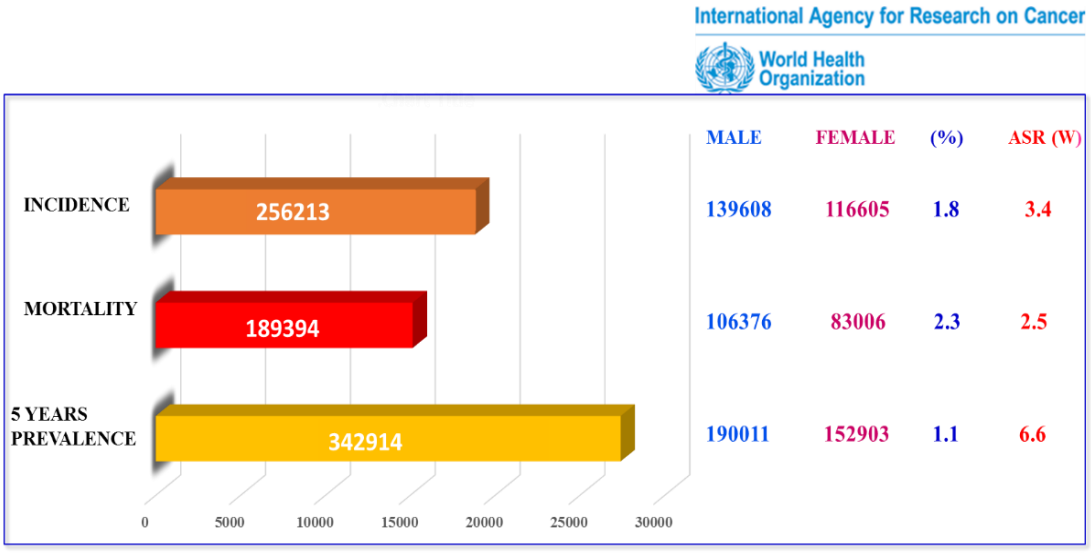
1.1.World Health Organization (WHO) Classification of Gliomas

WHO classified glioma into four grades based on nuclear atypia, mitosis, and microvascular proliferation and necrosis. According to above criteria astrocytoma is divided into four grades. They are:

Grade I: Pilocytic astrocytoma (WHO grade I) is more frequent in children benign tumors. These tumors do not progress to grade II and grade III and their genetic alterations differ with grade II & grade III. Typically have a good prognosis.

Figure 1: BRAIN AND NERVOUS SYSTEM CANCERS

Estimated Incidence. Mortality and Prevalence Worldwide in 2012



Estimated Incidence, Mortality and Prevalence in INDIA in 2012

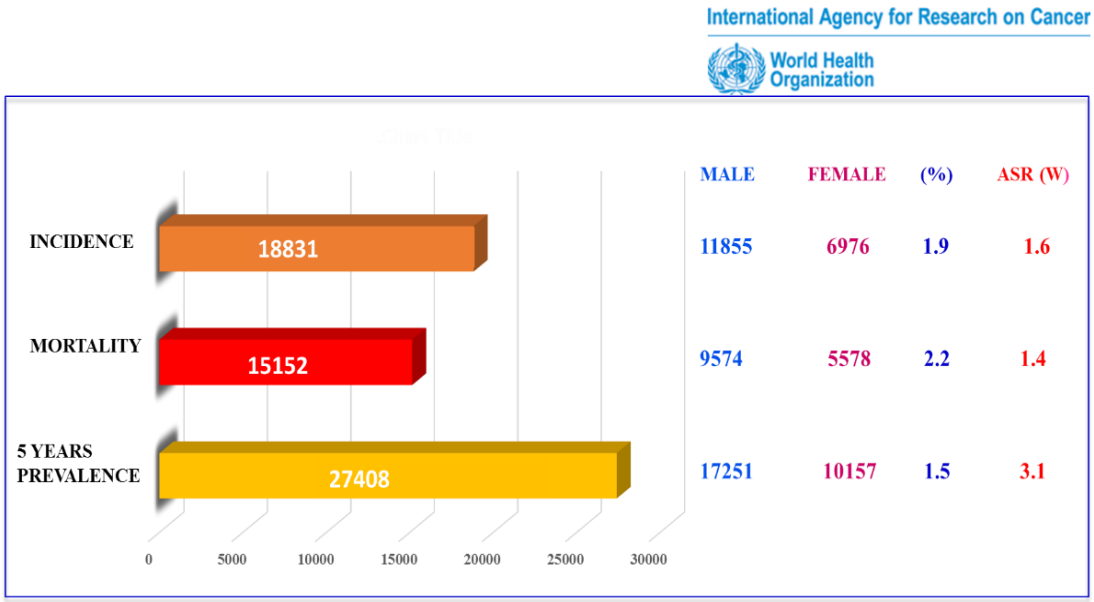


Figure 1. Data taken from IARC GLOBOCON 2012. http://globocan.iarc.fr/Default.aspx ; (%) = percentage of Brain and Nervous system cancers among all cancers; Incidence and mortality data for all ages. 5-year prevalence for adult population only. ASR (W) and proportions per 100,000; ASR (W) Age-standardized incidence or mortality rate (world). It is also expressed per 100,000.

Grade II: Diffuse astrocytoma (WHO grade II) tumors are characterized on histological examination by hyper-cellularity and infiltrating tumors. They account for 1.6% among all gliomas (CBTRUS 2011). Grade II is strongly associated with amplification of PDGFRα and/or PDGFRβ and their ligands PDGF-A, B or C and D and loss of *P53* (Lokker, Sullivan et al. 2002; Rao, Uhm et al. 2003; Louis 2006; Shih and Holland 2006; Liang, Ma et al. 2008; Mason and Cairncross 2008; Sathornsumetee and Rich 2008). Grade II tumors may progress to grade III and grade IV. Median survival of grade II astrocytomas is 5-8 years.

Grade III: Anaplastic astrocytomas (WHO Grade III) are malignant tumors characterized on histologic examination by presence of hyper cellularity, nuclear atypia as well as mitotic features. Most of the patients with this tumor progress to grade IV. Genetic alterations observed in anaplastic astrocytomas are loss of Rb (Retinoblastoma) gene expression, which is involved in cell cycle regulation. Down regulation or mutations are present in tumor-suppressor gene p16INK4A/CDKN2A and *p53*. Amplification of MDM2 gene which is an inhibitor of *p53* is also seen (Reifenberger, Liu et al. 1993; Rao, Uhm et al. 2003; Soni, King et al. 2005; Louis 2006; Mason and Cairncross 2008; Sathornsumetee and Rich 2008). Patients with grade III tumors have a poor prognosis having 5 year survival rate (CBTRUS 2011).

Grade IV: Grade IV astrocytomas are also known as Gliobastoma multiforme (GBM). It is highly malignant and heterogeneous tumor. Characteristic features of GBM include hyper cellularity, nuclear atypia, mitotic figures and presence of angiogenesis and necrosis. It accounts for 53.7% of all gliomas and they occupy nearly 15% of primary and central nerve system tumors (CBTRUS 2011). GBM can be classified as primary or secondary tumors based on their clinical presentation, patient's age and genetic

alterations, nearly 90% GBMs are primary type. These tumors present *de nova* in older patients (>60 years) without a previous history of lower grade tumors. Secondary GBM tumors arise from lower grades, grade II and grade III astrocytomas or from mixed oligoastrocytoma (Rao, Uhm et al. 2003; Louis 2006; Mason and Cairncross 2008; Sathornsumetee and Rich 2008). GBMs show frequent gains of chromosomes 7 and 19, losses of chromosomes 10 and 13, EGFR amplification (including amplification of EGFR variants), PTEN mutation, CDKN2A/B deletion, TP53 mutation, NF1 mutation, PDGFRA1 mutation, and MDM2 amplification (Network-CGA 2008). Recent studies showed IDH1 and IDH2 mutations were found at a low, but real, prevalence in glioblastomas (Network-CGA 2008; Parsons, Jones et al. 2008; Yan, Parsons et al. 2009; Verhaak, Hoadley et al. 2010). IDH1 or IDH2 mutations were strongly associated with clinical features of secondary GBMs (<10% of primary GBMs have IDH mutations) (Goodenberger and Jenkins 2012), thus, glioblastomas remains one of the most lethal cancers. Over the past 30 years of major research efforts for development of therapies for glioblastoma, only two medical treatments (radiotherapy and temozolomide based cytotoxic chemotherapy) have demonstrated significant efficacy in patients with newly diagnosed GBM. Despite these aggressive therapies, GBM patients die within one year from the diagnosis and only 5% of the patients survive more than 5 years (CBTRUS 2011).

Figure 1.2. Origin of GBM

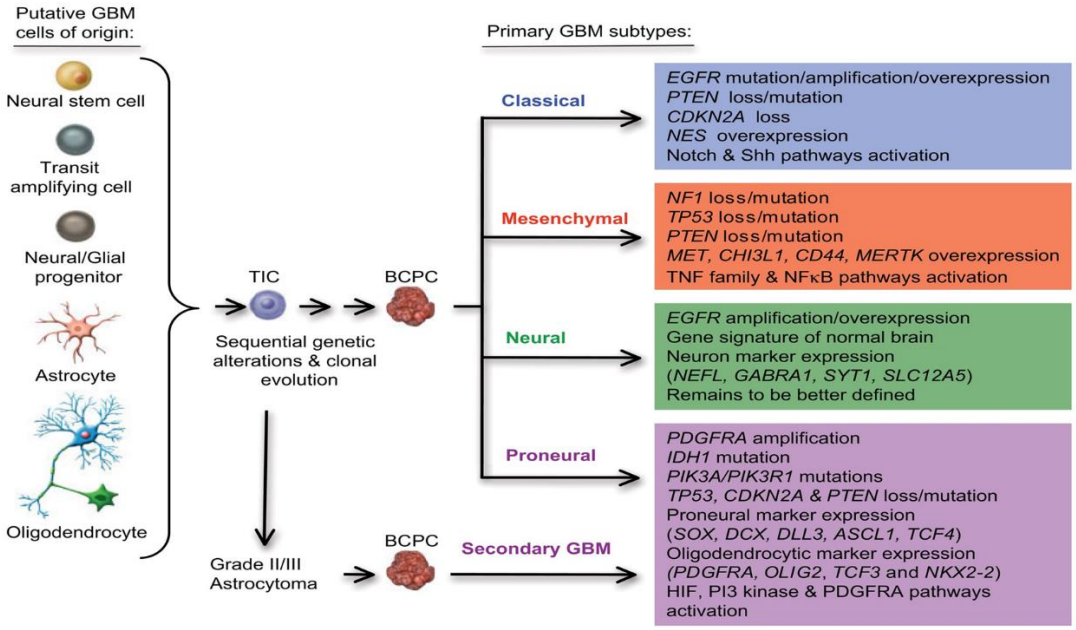


Figure 1.2. Sequential genetic changes observed in the pathogenesis of different subtypes of glioblastoma. Some cells in the normal brain undergo genetic alterations, which leads to a population of tumor initiating cells (TICs), which can then further accumulate genetic and epigenetic changes and become brain cancer propagating cells (BCPC). The latter cells are responsible for the formation of glioblastoma. GBM indicates glioblastoma multiforme; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog; TNF, tumor necrosis factor; PDGFRA, platelet-derived growth factor receptor–A; IDH, isocitrate dehydrogenase; PI3K, phosphoinositol 3–kinase; HIF, hypoxia-inducible factor. Image adapted from (Van Meir, Hadjipanayis et al. 2010)

1.2. Glioma risk factors

Many risk factors have been examined as potential contributors to glioma. Among these, the genetic risk factors, ionizing radiation, non-ionizing radiation and allergies are significant.

Genetic factors: Several Mendelian syndromes have been significantly associated with gliomagenesis. Literature pertaining to linkage studies, segregation analysis of polygenic models, genome wide association studies, genetic polymorphism (DNA repair, nonhomologous end joining, folate metabolism), germ line DNA single

nucleotide polymorphism (SNP)s have shown association with gliomagenesis (Goodenberger and Jenkins 2012; Ostrom, Bauchet et al. 2014).

Table 1.1. Monogenic Mendelian disorders associated with increased risk of glioma

Gene (chromosome location)	Disorder/Syndrome	Mode of Inheritance	Phenotypic Features	Associated Gliomas
NF1 (17q11.2)	Neurofibromatosis 1	Dominant	Neurofibromas, schwannomas, cafe´-au-lait macules	Astrocytoma, optic nerve Glioma
NF2 (22q12.2)	Neurofibromatosis 2	Dominant	Acoustic neuromas, meningiomas, neurofibromas, eye lesions	Ependymoma
TSC1,TSC2 (9q34.14,16p13.3)	Tuberous sclerosis	Dominant	Development of multisystem nonmalignant tumors	Giant cell Astrocytoma
MSH2,MLH1, MSH6,PMS2	Lynch syndrome	Dominant	Predisposition to gastrointestinal, endometrial, and other cancers	Glioblastoma, other gliomas
TP53 (17p13.1)	Li–Fraumeni syndrome	Dominant	Predisposition to numerous cancers, especially breast, brain, and soft-tissue sarcoma	Glioblastoma, other gliomas
p16/CDKN2A (9p21.3)	Melanoma-neural system tumor syndrome	Dominant	Predisposition to malignant melanoma and malignant brain Tumors	Glioma
IDH1/IDH2 (2q33.3/15q26.1)	Ollier disease/Maffucci syndrome	Acquired postzygotic mosaicism; dominant with reduced penetrance	Development of intraosseous benign cartilaginous tumors, cancer predisposition	Glioma

Abbreviations: MLH1, mutL homolog 1; MSH2/MSH6, mutS homolog 2/6; NF1/NF2, neurofibromin 1/2; PMS2, postmeiotic segregation increased 2; TSC1/TSC2, tuberous sclerosis 1/2. Table adapted from (David, Nance et al. 2012).

Ionizing radiation: An important environmental factor associated with increased risk of developing glioma is ionizing radiation, supported by many evidences like Atomic-bomb studies, nuclear test fall-out data and use of therapeutic radiations for cancer treatment. Also, occupational and environmental studies related to ionizing radiations have shown their involvement in tumorigenesis (Bondy, Scheurer et al. 2008). In Israel, individuals with Tineacapitis when treated with X-rays showed malignant brain tumors development when compared with matched population and sibling controls (Lubin, Schafer et al. 2004). Atomic bomb survivors in Japan, therapeutic radiations used on childhood tumors in US and UK and diagnostic use of radiations in CT scan have been associated with gliomagenesis (Bondy, Scheurer et al. 2008; Ostrom, Bauchet et al. 2014).

Non-ionizing radiation: Worldwide, the number of cell phone users has increased considerably. Brain is an important organ which is affected by radio frequencies when cell phones are used. With a concern for public health, cell phone usage has been extensively investigated in relation to possible risk and development of glioma and brain tumors. Several large population based studies were performed in Europe which provided conflicting evidences (Swerdlow, Feychting et al. 2011; Mrugala 2013). Cell phone usage studies in 13 countries showed increased risk for certain brain tumors but they were not statistically significant. In contrast to this, Swedish studies reported that the risk factor for glioma development is higher in the part of the brain with higher exposure to the non-ionizing electromagnetic field produced by the cell phones over 10 years exposure (Hardell, Carlberg et al. 2013). Long time (over 15 years) exposure had an increased risk on development of childhood brain tumors. This data has also been supported by U.S meta-analysis studies (Little, Rajaraman et al. 2012).

Allergies: Epidemiologic and pooled assessment studies suggest that glioma risk is reduced by allergic conditions, such as asthma, hay fever, eczema, and food allergies. (Linos, Raine et al. 2007; McCarthy, Rankin et al. 2011; McCarthy, Rankin et al. 2011).

1.4. Symptoms and diagnostics:

Glioma symptoms vary with the part of the central nervous system (location) affected and also with the size of tumor. Due to growing tumor, intracranial pressure increases and causes headaches, nausea and vomiting, seizures, and cranial nerve disorders. Depending on the location of tumor, a patient can develop symptoms such as weakness on one side of the body, memory and/or speech difficulties, and visual changes. Although gliomas do not metastasize to other organs by the bloodstream, they

can spread via the cerebrospinal fluid and cause ‘drop metastases’ to the spinal cord (Buhl, Barth et al. 1998).

Initial step in glioma diagnosis is the traditional neurological examination, which can provide clues about tumor location. Later steps involved in the diagnosis are specialized imaging techniques and laboratory tests that can detect and provide information regarding location, type and extent of spread of the tumor. Computed Tomography (CT) and MRI imaging techniques help in detecting the location, size and composition of malignant growth. CT scans have advantage in detecting calcification, skull lesions, and hyper acute hemorrhage (bleeding less than 24 hours). MRI benefits in detecting isodense lesions, edema, and infarctions. PET (Positron Emission Tomography) and SPECT (Single Photon Emission Tomography) are used for detecting the difference between radiation necrosis to tumor recurrence. However, these imaging techniques are mostly used for post-operative purposes. Final step in diagnosis is biopsy through surgical intervention wherein a small tissue sample from the tumor is excised and examined by pathologist.

1.5. Glioma therapy:

Glioma treatment is mainly influenced by location, cell type and the grade of malignancy. Standard treatment options are maximal safe resection of the tumor tissue followed by a combination of radiation and chemotherapy. In newly diagnosed GBMs, timely intervention with standard of care treatment can lead to an increased median survival time of approximately 14.6 months (Koshy, Villano et al. 2012).

Surgery: Surgery has been a critical tool in managing malignant gliomas. Maximal tumor tissue resection usually alleviates symptoms, benefits in the radiation therapy and chemotherapy with smaller tumor targets. It also provides tissues for

histopathological diagnosis which can make accurate diagnosis of tumor composition. Image guide resection with the help of MRI advances as intraoperative mappings are useful to the neurosurgeon to resect maximum tumor tissue. In recent years, role of neurosurgery has expanded to treat tumors by delivering the drugs, monoclonal antibodies, immunotherapeutic and viral gene vectors at the tumor sites precisely.

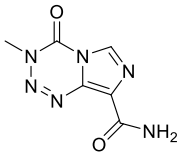
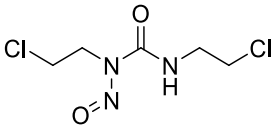
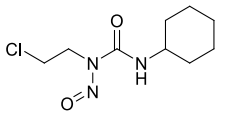
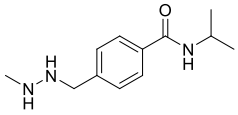
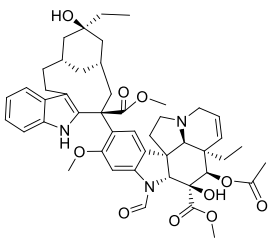
Radiotherapy: Surgical resection in GBM patients is followed by external beam radiotherapy (EBRT). Clinical trials have shown that patients who underwent surgery followed by EBRT had longer survival than surgery alone. Involved field radiotherapy is standard therapy along with adjuvant RT (dose typically 60 Gray) that helps in delivering radiation to affected regions. However, its limitation involves recurrence of glioma within 2 cm of original tumor site in 80-90% of cases when radiotherapy is applied on whole brain.

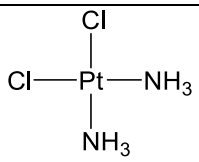
Decades of advancement in computer based 3D treatment planning, CT, MRI, PET, MRS are extremely useful in radiotherapy treatments. Intensity modulated radiation therapy (IMRT) has advantage in treating tumors which are near the radio-sensitive regions such as optic nerve. IMRT can benefit by manipulating the radiation beam into conformation shape of tumor to reduce the healthy tissue radiation exposure and damage. Disadvantage of IMRT techniques are radiations scattering into surrounding healthy region and complexity in planning, requirement of skilled physicist and hardware of linear accelerators and increased delivery time.

Stereotactic radiosurgery are used to deliver narrow collimated beam of ionizing radiation in a single dose fraction to small tumors (<4 cm). Gama knife radiosurgery

used with Cobalt 60 as the collimated beam source and fixed frame is used to stabilize the head relative to radiation source.

Chemotherapy: Chemotherapy is helpful in controlling the growth of gliomas. Many different drugs and drug combinations used in standard chemotherapy are tabulated below.

Name	Structure	Mechanism of action	Efficiency	Side effects
Temozolomide		Alkylating agent	More effective, easy to administer	Some tumor cells are able to repair damage by expressing a AGT protein.
Carmustine		Alkylating agent		Intravenous delivery causes bone marrow impairment Resulting in myelosuppression. Delivery through wafer causes seizures, swelling and infection in the brain cavity.
PCV Drug Regimen Procarbazine (Mutalane) Lomustine (CCNU) Vincristine (Oncovin)	  	Alkylating agent		Poor tolerance, rapid development of resistance

Platinum based drugs Cisplatin		Alkylating agent		
Biologic Drugs: Bevasizumab (Avastin)		Vascular endothelial growth factor	First targeted therapy approved for brain tumors	Effectiveness is based on tumor response.

Temozolomide is more effective, easy to administer and has fewer side effects. Its mode of action is depicted in **figure 1.3.**

Figure 1.3. Action mechanism of Temozolomide and chemo resistance

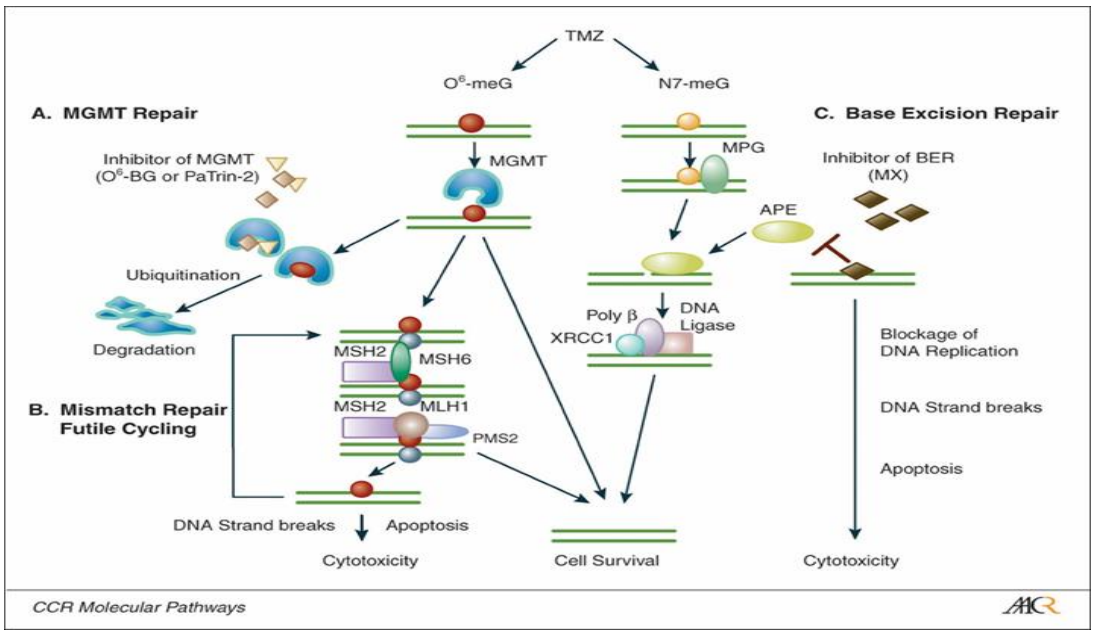


Figure 1.3. DNA repair pathways that play an important role in resistance to alkylating agents. MSH (MutS homologue), MLH (MutL homologue), and PMS2 are proteins that form the DNA MMR complex. TMZ, temozolomide; meG, methylguanine; MGMT, O6-methylguanine methyltransferase; MPG, methylpurine glycosylase; APE, AP endonuclease. Figure adapted (Liu and Gerson 2006).

1.6. Glioma animal model:

Glioma animal models help in understanding the mechanism of human glioma formation and provide a platform for evaluating the drug efficacy before going to

clinical trials. Animal models used for glioma studies are of following types. Chemical mutagen-induced models; Genetic models; Transplant or xenograft models.

Chemical mutagen induced models: is the most popular animal glioma model. In this model, brain tumors are induced by administration of chemical mutagens such as methylnitrosourea (MNU) or ethylnitrosourea (ENU) to the normal or pregnant rat via intra peritoneal route. Tumor initiation and progression in this model are similar to human glioma. Limitation of this model is that there is no precise genetic alteration in this model. Thus, it is difficult to know the function of a particular gene or pathway, and the type of cells involved in formation of tumor. Importantly, till now, there is no evidence of involvement of chemical mutagens in human glioma progression.

Genetic model: Resembles human histopathology and etiology. These are genetically engineered models having specific genetic alterations corresponding to gain or loss of function. This provides information regarding involvement of that particular gene or pathway in tumor initiation, progression, interaction between tumor and stroma, angiogenesis and malignancy. Moreover these genetic alterations can be made in specific cell type and time specific expression can be studied with available strategies such as cre-lox recombinase or **tv-a** systems. Human glioma progression is a complex and multifactorial process. However, this model explains specific alteration of one gene or pathway in the animal model which is very precise and difficult to correlate completely with human progression.

Transplant (Xenograft or Allograft) models: Where cultured human cell lines or rodent specific cell lines are transplanted into the nude rodents. These transplantations are of two types: cultured cells may be implanted subcutaneously into the flank region of animal (heterotopic tumor) or implanted into the brain (orthotopic tumor).

Heterotopic tumors require less surgical exposure, tumor growth can be observed easily and tumor size measurement is also convenient, whereas intracranial tumor (orthotopic) involves complicated surgical procedure, tumor growth is not visible outside. Also, to detect the tumor growth in live animal we need advanced imaging systems or tumor can be detected with histological observation after sacrifice. Orthotopic model is clinically more relevant model, as the tumor is located in the brain mimicking human glioma. This offers insight into the tumor-brain parenchyma interaction, which make micro environment similar to human GBM. Though cultured cells might lose most of the characteristic features in serial culture, they regain some of the properties such as invasive growth, neovascularization (angiogenesis) and cellular polymorphism. Further, blood brain barrier (BBB) vascularization is small compared to hererotopic tumors. Hence, Orthotopic tumor models clearly replicate human glioma in a sense that when a drug is administered its entry will be restricted by BBB.

1.7. Transcription factors:

In GBM patients prognosis is poor and they have a median survival of 14.6 months after all the aggressive therapies like surgery, radiotherapy and adjuvant temozolomide based chemotherapy. Although, radiotherapy is beneficial in prolonging survival time of GBM patients, presence of radio resistant cells in tumors are great obstacle for glioma therapy. Also, presence of stem cells in the tumor mass adds to their resistance to radio and chemotherapies. GBM is a heterogenic tumor with lot of molecular diversity. Participation of multiple signaling pathways renders the existing mono targeted treatment options ineffective. Novel approaches are needed to overcome this. In the present scenario understanding the transcriptional machinery which includes RNA polymerase II, transcription factors (TFs) and transcriptional regulatory

components like promoter elements, enhancers, silencers and locus control regions will help to develop novel treatment strategies. Basal level of transcription is controlled by RNA polymerase II whereas spatial and temporal expression of several transcription factors play crucial role by inducing or inhibiting their target gene expressions. Most of the transcription network systems play key role in embryonic development and in tumor progression by altering the target gene expression in response to the changes in the surrounding environment/ micro environment. Understanding the role of these transcription factors may help in formulating new targets for treating GBM.

1.8. Cell surface receptor proteins:

Like most other malignant cancers, glioma can also recognize growth promoting signals from surrounding micro environment and most of these signaling factors are related to growth, angiogenesis and migration. These factors are released by tumor cell itself and are secreted into tumor micro environment where these micro environmental factors bind to the concerned cell surface receptors which are present on tumor cells and act as autocrine for its propagation. Most of the studies on glioma cell lines and primary cultures suggested that tumor cells over express cell surface receptors which are mainly involved in cell survival, invasion, angiogenesis and migration. Glioma hall mark cell surface receptors have been reported which are related to different signaling pathways such as epidermal growth factor (EGF) and its receptor (EGFR) are crucial in tumor progression and regulate proliferation, angiogenesis, invasion, loss of cell differentiation and decreased cell death. In nearly 40 % of glioma patients EGFR specially EGFR vIII amplifications are observed (Wikstrand, Reist et al. 1998). Vascular endothelial growth factor (VEGF) and its receptor VEGFR are also overexpressed in glioma. VEGFR activation induces growth signaling pathways such

as PI3K, Akt and MAPK pathways which leads to angiogenesis, increased vascular permeability, and lymphatic vessels growth (Alitalo and Carmeliet 2002). Platelet derived growth factor (PDGF) and its receptor (PDGFR) are up regulated in human glioma and these facilitate tumor interstitial pressure, tumor growth, and angiogenesis (Ostman 2004). Recent research has focused on targeting these cell surface receptors with different monoclonal antibodies and small-molecule inhibitors (Brown and Wilson 2004).

Figure 1.4. Cell surface receptors in malignant glioma

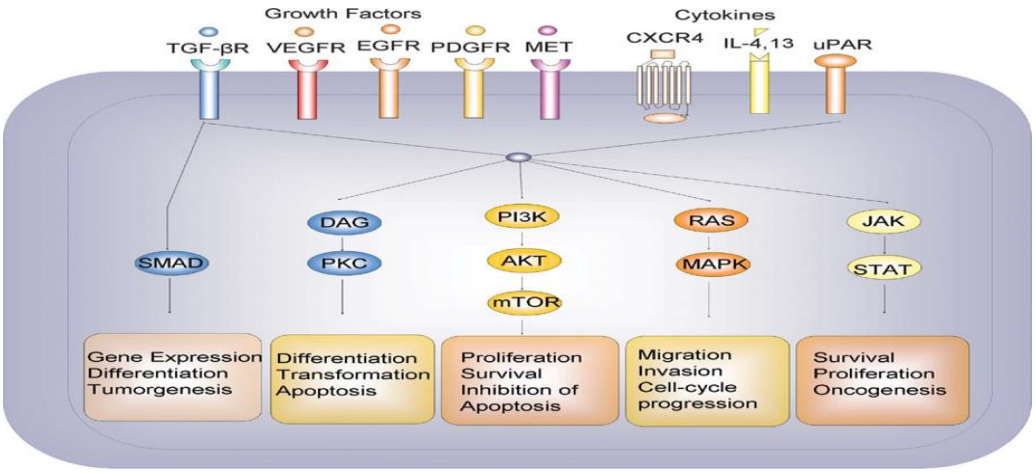


Figure 1.4. Cell surface receptor plays divergent role in glioma progression by inducing different signaling pathways. Image adapted from (Li and Hall 2011).

1.9. Angiogenesis:

Angiogenesis is the formation of the new blood vessels from existing one by remodeling or rerouting. Angiogenesis process is activated in growing gliomas when the pro-angiogenic factors preponderate the anti-angiogenic factors. These factors are secreted from cellular source (glioma cells, endothelial cells and microglia) and environmental sources (extracellular matrix (ECM) or hypoxia) (Brown and Wilson 2004; Wang, Anderson et al. 2005). In brain tumors tissue hypoxia is a strong activator of angiogenesis process, the tissue hypoxia condition along with genetic alterations dictate angiogenesis (Kaur, Tan et al. 2004) by modulating the pro and anti angiogenic

forces. Many growth factors such as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factors (FGFs), Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), and Transforming Growth Factors (TGF) had been reported in glioma angiogenesis (Dunn, Heese et al. 2000). Angiogenesis process have three distinct steps: A) Blood vessel breakdown B) basement membrane and extracellular matrix (ECM) degradation C) migration of endothelial cells into the tumor mass, forming new blood vessels.

VEGF pathway is the one well studied in glioma. VEGF is a crucial factor which plays a vital role in GBM angiogenesis by interacting with PTEN/PI3-kinase/Akt, MAPK/ERK, nitric oxide, DLL4 (Notch dltalike ligand 4), PDGF- ß, EGF, TNF-α, and bFGF (basic fibroblast growth factor) pathways. VEGF also interacts with angiopoietins, such as Ang-1 and Ang-2 through their tyrosine kinase receptors, Tie1 and Tie2. When VEGF is co-expressed with Ang-2, it promotes blood vessel development but in causes vessel regression in its absence (Gerstner and Batchelor 2012).

Gliomas are highly vascularized tumors. Among gliomas, GBM has highest degree of vascular proliferation and endothelial hyperplasia, patients with high vascularization have short survival time after surgery as compared to patients where less tumor vascularization is observed (Leon, Folkerth et al. 1996).

1.10. Migration

For glioma progression angiogenesis is needed to supply nutrients and oxygen to the tumor. Many reports suggest that with help of vascularization high grade gliomas migrate to different parts of brain. There are two different mechanisms followed by high grade gliomas for migration. First being diffuse infiltrating single cell migration

into brain parenchyma. Second one is the perivascular migration along the microvasculature. These infiltrating cells, mainly responsible for recurrence of glioma, are the cells which show radiotherapy and chemotherapy resistance (Demuth and Berens 2004; Bansal, Liang et al. 2006; Anderson, McFarland et al. 2008).

1.11. Plumbagin:

Plumbagin (5-hydroxy-2-methyl-1, 4-naphtho- quinone) is a naturally occurring pigment produced by the plumbago species such as *Plumbaginaceae, Ancestrocladaceae*, *Droseraceae,* and *Dioncophyllaceae*families. Plumbagin exhibits potent biological activities like anti-bacterial, anti-fungal and anti-inflammatory properties (Krishnaswamy and Purushothaman 1980; Checker, Sharma et al. 2010). It also has shown anti-cancer and anti-proliferative properties *in vitro* in cell cultures and *in vivo* animal models (Parimala and Sachdanandam 1993; Naresh, Udupa et al. 1996; Hazra, Sarkar et al. 2002).

Figure 1.5. Plumbagin mode of action on cancer cells

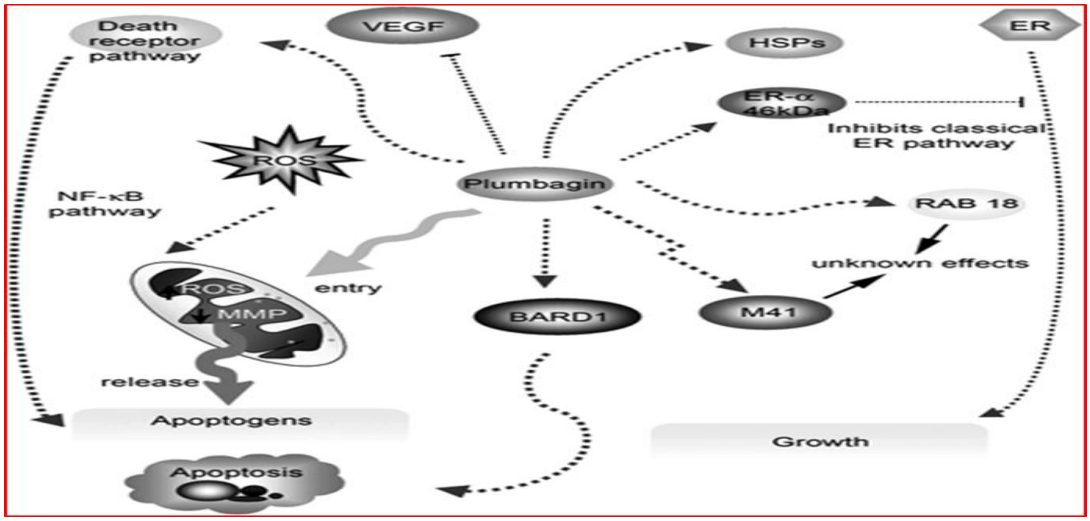


Figure 1.5. Proposed model for the action of Plumbagin on cancer cells. Single-headed arrow indicates activation and closed heads indicate inhibitory pathways. Figure adapted from (CSIR 1989).

1.12. Rationale of the present study:

Due to high proliferation, invasiveness and resistance to radiation, glioma remains incurable with very poor survival rate. Thus, there is an urgent need to reevaluate present treatments and at the same time develop new promising therapies based on better understanding of molecular basis of glioblastoma. New discoveries are being made in molecular pathways which are activated in gliomas. Cancer is a result of dysregulation of genes which either promote or inhibit proliferation by modulating several factors. These genetic alterations in tumor tissue are important in detecting the tumor stage, predicting prognosis and also for treatment.

Studying the differential genetic alteration between normal and tumor tissue and among different tumor grades, helps in the better understanding of signaling pathways alterations and their functional role in tumor progression. Broad understanding of tumor biology revealed few crucial steps in tumor progression such as uncontrolled proliferation, angiogenesis, and migration. Dysregulation of cell cycle leads to uncontrolled proliferation, which leads to tumor mass, after attaining certain tumor mass they undergo stress and hypoxic conditions due to lack of enough blood supply, to overcome this condition tumor mass undergoes angiogenesis which allows the tumor mass to grow further, finally these tumor mass spread in to other parts of the body by a process called as migration. All these steps are controlled by different factors such as transcription factors, cell surface receptors, angiogenic and migration related genes at different stages. Transcription factors influences all the stages in tumor progression and they are spatially and temporally expressed genes which play crucial role in embryo development by inducing different developmental pathways. These also regulate tumor progression by inducing pathways regulating survival, angiogenesis, invasion and

migration. Cell surface receptors play main role in tumor progression by responding to factors in the surrounding micro environment which induces many survival pathways regulating tumorigenesis. Angiogenesis factors mainly contributes to overcome hypoxic and deficient nutrients conditions by inducing angiogenesis process. Migration related factors influence at the later stages of tumor progression to facilitate the tumor cells to spread to other parts of body and these factors play major role in tumor recurrence. Angiogenesis and migration are hall mark of high grade glioma.

These observations coupled with our past experience on glioma research prompted us to focus on to study at molecular level. Further, to study differences between human astrocytoma tissue and normal healthy tissue and also compare differences between Grade II and Grade IV (GBM) using microarray analysis. From the microarray data, genes functional as transcription factors, important cell surface receptors and the genes involved in angiogenesis and migration were selected. These target genes were further validated with real time RT-PCR and from these genes, Six1 transcription factors was shortlisted and further studied. To identify novel therapeutic compounds with potential anti-glioma activity new drugs were screened *in vitro* and *in vivo.*

Objectives of the study.

- Analysis of differential gene expression in control, grade II and grade IV (GBM) human astrocytoma. Validation of selected genes.
- Involvement of transcription factors SIX1 and ZNF 24 in human astrocytoma progression.
- *In vitro* and *in vivo* drug targeting glioma.

CHAPTER 2

Analysis of differential gene expression in control, grade II and grade IV human astrocytoma. Validation of selected genes.

2.1. INTRODUCTION

According to WHO, 2007 grading system, diffusely infiltrating astrocytic tumors are diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and GBM (grade IV) (Louis, Ohgaki et al. 2007). Among them GBM is the most frequent malignant primary brain tumor. It consist about 12-20% of all intracranial tumors and occupies for 50-60% of all astrocytic tumors, with poor prognosis having the median overall survival of 14 months. Less than 10% of patients are survive up to 5 years (Vougiouklakis, Mitselou et al. 2006; Tran and Rosenthal 2010). The main reasons for GBM aggressiveness are high proliferation rate, diffuse infiltration in brain parenchyma, angiogenesis, migration, and genomic instability.

GBM is extremely heterogeneous in nature and added to this complexity is the fact that multiple pathways related to cell growth, proliferation, angiogenesis, invasion and resistance to drugs are simultaneously altered in glioma. Like other cancer cells, astrocytoma also secrete growth factors in the microenvironment, these growth factors bind to the concerned cell surface receptors, binding of ligand to the receptor leads to activation of downstream pathways such as EGFR, VEGFR, and PDGFR which play key role in tumorigenesis, proliferation, angiogenesis, invasion, migration and radio-chemo resistance. These cell surface receptors have been shown to be over expressed in glioma and are targets for chemotherapy. They are also useful in imaging techniques for tumor resection (Ohgaki, Dessen et al. 2004; Lo and Hung 2006; Schaller, Modo et al. 2007; Lima, Kahn et al. 2012). Identifying new cell surface receptors may help in improving the treatment of GBM patients who have poor prognosis.

Gliomas are highly vascularized tumors and highly vascularized GBM patients have less survival time than less vascularized GBM patients (Good, Polverini et al.

1990; Dameron, Volpert et al. 1994; Leon, Folkerth et al. 1996; Tandle, Blazer et al. 2004; Cea, Sala et al. 2012). Solid tumors such as brain tumors after reaching a critical size 1-2 mm diameter, undergo hypoxic conditions due to lack of adequate oxygen and nutrient supply. To overcome such circumstances tumor cells secrete pro-angiogenic factors such as VEGF, acidic and basic fibroblast growth factors, placental growth factors, angiopoietin 2 and interleukins into tumor micro environment. The outweigh of pro-angiogenic factors to antiangiogenic factors (angiostatin, endostatin, thrombospondin 1, and endothelial monocyte- activating polypeptide 2) leads to angiogenesis. In response to angiogenic signals, endothelial cells increase the production of matrix metalloproteinase (MMPs), such as MMP-2 which degrades the surrounding extracellular matrix and increases cell motility for invasion, and helps in cell division (Lu and Jiang 2001).

Angiogenesis also helps in tumor cell migration. Till now, glioma cell migration dynamics are not understood completely however, migrating cells need to modify its shape and stiffness to interact with the extracellular matrix to move forward. Migrating cell becomes polarized and membrane protrusions such as pseudopodia, lamellipodia, filopodia, and invadopodia develop. These structural alterations aid the cellular attachment to ECM. These infiltrating cells are mainly responsible for recurrence of glioma and cause radio and chemotherapy resistance (Demuth and Berens 2004; Bansal, Liang et al. 2006; Anderson, McFarland et al. 2008).

Transcription factors are more important in controlling the target gene or pathways expression spatially or temporally. Most of the transcription factors which play key role in embryonic development lose their expression in adult stage. Over expression of these transcription factors observed in tumor progression and they induce

survival pathways. In glioma many transcription factors reported. Understanding these transcription factors role may help in the treating GBM.

Despite great advancement in understanding biology of gliomas over the last few decades, till now there is no effective therapy for GBM except standard of care as mentioned previously. Thus there is need for discovery of new potential therapeutic targets. Understanding the molecular level alterations and gene expression patterns can help in distinguishing between different grades of glioma. For these type of applications, microarray is a very reliable and useful technique.

Hence, in the present study we used micro- arrays to compare the gene expression pattern for whole genome (44,000 genes) among the primary GBM (grade IV), diffused astrocytoma (grade II) and control brain tissue. From micro array analysis of 44 000 genes, 17 genes with high expression in GBM as compared to grade II and control samples were selected based on their functional importance as proteins related to cell surface, migration, angiogenesis and transcription factors and they are validated with real-time PCR.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection: Tissue samples were collected from Krishna Institute of Medical Science (KIMS), Secunderabad. Immediately after surgical resection, half of the tissue was snap frozen in liquid N_2 and later stored at -80^0C. Remaining half of the tissue was fixed in 4% paraformaldehyde and used for histopathological studies. Samples were graded according to WHO classification system (Louis, Ohgaki et al. 2007). From these samples one GBM, one diffused astrocytoma and one control sample was used for micro array analysis and 15 of the primary GBM, 5 of diffused

astrocytoma and 4 temporal lobe epilepsy (used as controls) samples were used for real time PCR validation.

2.3. Micro array

2.3.1. RNA extraction, labeling, and hybridization

Micro array experiments and data analysis was carried out at Genotypic Technology [P] Ltd (www.genotypic.co.in), Bangalore, India.

2.3.2. Total RNA isolation, quantification, labeling and hybridization

RNA was extracted using Qiagen's RNeasy mini kit with microsmash. The tissue sample was homogenized in a 2ml tube along with liquid nitrogen using microsmash (Tomy Digital Biology Co., Ltd., Japan) and 350 µl of RLT buffer was added and further processed for RNA extraction and on-column DNase treatment as per the manufacturer's instructions. The RNA was eluted in 30 µl of RNase-free water. RNA quantification and purity were analyzed with Nanodrop. Cy3 dye was used for labeling of complimentary RNA with Agilent's Quick-Amp labeling Kit. Quality control was performed with Nano drop. Hybridization was performed on slides containing Whole Genome Human 4x44k with an Agilent's *In situ* Hybridization kit.

2.3.3. Image quality control

The images were manually verified and found to be devoid of uneven hybridization, streaks, blobs and other artifacts. Hybridization across the slide was good based on number of feature that were "g is PosAndSignif" which indicates feature is positive and significantly above background.

2.3.4. Statistical analysis of microarray data

We analyzed expression data using Gene Spring GX version 11.0 and Microsoft Excel. Raw data were normalized using the Percentile shift normalization method. Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the percentile of the expression values for this array, across all spots (where n has a range from 0-100 and n=50 is the median). It subtracts this value from the expression value of each entity. Gene Spring GX, Gene ontology (http://www.geneontology.org) and Biointerpreter used for functional classification and significant pathways for differentially regulated genes. Biointerpreter is a user-friendly web-based Biological interpretation tool developed by Genotypic for any microarray data.

The significant representation in each category is computed using statistical evaluation method "chi-square test". This method tries to detect if the category is over represented or underrepresented in the sample set compared with the reference set (whole genome). Chi-square test finds the representation (over represented or underrepresented) of a Pathway / function / disease in the gene list compared to representation of the same Pathway / functions / disease in the whole genome. The level of significance predicted through a calculation of P-Value by Chi-Square test. P-Value less than 0.05 are usually considered as significant.

2.4. Real time PCR validation of differentially expressed genes

Total RNA isolated with TRIzol (sigma) reagent, Takara blue print cDNA synthesis kit was used for cDNA synthesis as per company manual instructions. Amplification of specific PCR products was detected using the SYBR Green PCR

Master Mix (Takara) according to the manufacturer's instruction in Applied Bio system 7500 fast real time detection system. Gene specific primers were synthesized from IDT. GAPDH was used as internal control (**Table 3.1.** End of the fourth chapter). The 2^{-ddCT} equation (Livak and Schmittgen 2001) was applied to calculate the relative mRNA expression.

2.5.1. Statistical analysis

The differences in gene expression levels were analyzed by the using the Sigma plot 11 software, differences were considered to be significant at $P<0.05$.

2.6. RESULTS

2.6.1. Clinical characteristics of human sample

For this study 31 different clinical grade astrocytoma samples and four epilepsy samples (control) were used. Among the astrocytoma clinical samples there was no gender specificity but predominant in male (21 out of 31) were observed and also predominant in age above 40 years (20 out of 31). Clinical sample are summarized in **Table 2.1.**

2.6.2. Histological features of astrocytoma samples

According to WHO classification astrocytoma divided into fallowing grades based on their cytological/nuclear atypia, mitoses, microvascular proliferation, and necrosis features. Histology of grade II astrocytoma with H&E showed increased cellularity with cytological atypia (**Figure 2.1.A, 2.1.B**). Anaplastic astrocytoma showed characteristic features of high cellularity with nuclear atypia and mitotic activity (**Figure 2.1.C, 2.1.D**). GBM showed all the features of anaplastic astrocytoma

and in addition to this presence of necrosis and micro-vascularization was observed (**Figure 2.1.E, 2.1.F**).

Table 2.1. Clinical characteristics of human

S.No.	GRADE	DISCRIPTION	AGE	GENDER
1	GBM	PRIMARY	46	M
2	GBM	SECONDAY	44	M
3	GBM	PRIMARY	40	M
4	GBM	PRIMARY	45	F
5	GBM	PRIMARY	13	F
6	GBM	PRIMARY	41	M
7	GBM	MALIGNENT	20	M
8	GBM	PRIMARY	65	M
9	GBM	PRIMARY	42	F
10	GBM	PRIMARY	52	M
11	GBM	PRIMARY	54	M
12	GBM	PRIMARY	59	M
13	GBM	PRIMARY	17	F
14	GBM	SECONDARY	43	M
15	GBM	PRIMARY	63	F
16	GBM	PRIMARY	69	F
18	III	ANAPLASTIC ASTROCYTOMA	40	M
19	III	ANAPLASTIC ASTROCYTOMA	29	F
20	III	ANAPLASTIC ASTROCYTOMA	52	M
21	III	ANAPLASTIC ASTROCYTOMA	25	M
22	III	ANAPLASTIC ASTROCYTOMA	54	F
23	III	ANAPLASTIC ASTROCYTOMA	36	M
24	II	DIFFUSED ASTROCYTOMA	27	F
25	II	DIFFUSED ASTROCYTOMA	57	M
26	II	DIFFUSED ASTROCYTOMA	31	M
27	II	DIFFUSED ASTROCYTOMA	24	F
28	II	DIFFUSED ASTROCYTOMA	55	M
29	II	OLIGODENDROGLIOMA	30	M
30	II	OLIGO ASTROCYTOMA	34	M
31	II	DIFFUSED ASTROCYTOMA	55	M
32	Control	TEMPORAL LOBE EPILEPSY	23	M
33	Control	TEMPORAL LOBE EPILEPSY	21	M
34	Control	TEMPORAL LOBE EPILEPSY	12	F
35	Control	TEMPORAL LOBE EPILEPSY	29	M

Figure 2.1: Histological features of astrocytoma grades

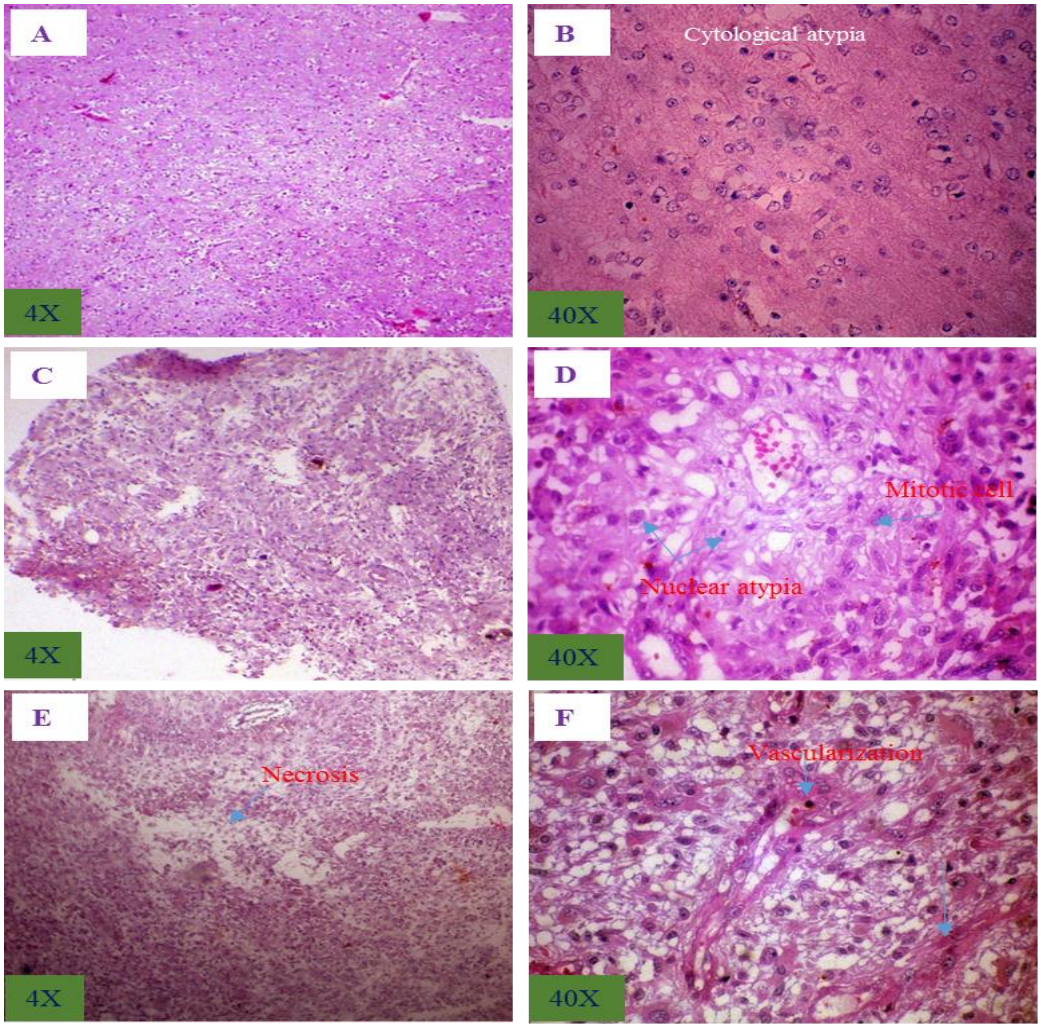


Figure 2.1: H&E staining of astrocytoma sections. Diffuse astrocytoma (grade II) showing increased cellularity and cytological atypia (A&B). Anaplastic astrocytoma (grade III) showing increased cellularity with mitotic features and nuclear atypia (C&D). GBM (grade IV) showing high cellularity with necrotic region and macro vascularization (E&F).

2.7.1. Micro array analysis

Human whole genome expression micro array profiling was (44k genes) performed on Grade IV (GBM), Grade II (Diffuse astrocytoma) and control brain sample. Here we used only one sample from each group, so expression data from these samples is pure single individual sample data, not a cumulative data. From these three

individual samples RNA and labeled complimentary RNA (cRNA) purity was checked (**Table 2.2.1, 2.2.2 and 2.2.3**) and further proceed for hybridization. These three individual sample data analyzed using Gene Spring GX version 11.0 and Microsoft Excel software, a comparison between GBM and control samples showed that 16313 genes were differentially expressed in GBM, among these 16313 genes, 7292 genes were down regulated and 9021 genes were up regulated. Differential expression analysis between Grade II and control showed 11571 genes with altered expression. Among these 5259 genes were down regulated and 6312 genes were up regulated in Grade II (**Figure 2.2**). Many Histopathological differences are present between Grade II and Grade IV. Identifying molecular level differences and similarities between the Grade II and Grade IV may help in understanding molecular aspects specific to that particular stage. Comparison between Grade II and GBM showed that nearly 7492 gene were commonly altered, of which 3679 genes were commonly down regulated and 3818 genes were commonly up regulated in both the grades **(Figure 2.2**). In cancer progression signaling pathways play a crucial role. Alterations of these pathways are hall marks of tumor stage and progression. Bio-interpreter analysis for functional and significant pathway differential expression showed many altered pathways in GBM and Grade II compared to control. Some of the important pathways and their genes which were altered significantly in Grade II are presented in (**Table 2.3.1**) and (**Table 2.3.2**). Signaling pathway alterations and number of deregulated genes in Grade IV (GBM) are shown in (**Tables 2.3.3& 2.3.4**)

From the microarray data, we selected 17 genes pertaining to functions such as cell surface receptors, transcription factors, angiogenesis and migration. Genes that are over expressed more than 2 folds in Grade IV than compare to control and Grade II

were selected. These selected genes were validated using real time PCR. For real time PCR validation total 18 clinical samples were used. We used 9 Grade IV (GBM), 5 Grade II and 4 Control (temporal epilepsy) samples for Real time PCR analysis.

Table 2.2.1: RNA concentration estimation for microarray analysis

Sl. No	Sample Name	Absorbance value 260/280	Absorbance value 260/230	RNA Concentration ng/µl	Total yield in ng	Comments
1	Control	2.2	0.2	67.0	2009.4	**Sub-OP,OC**
2	II	2.2	0.7	62.8	1885.2	**Sub-OP,OC**
3	IV	2.1	2.1	715.1	121453.0	**OP,OC**

Table 2.2.2: RNA integrity number (RIN) estimation

Sl. No.	Sample Name	QC check
1	CONTROL	Good
2	GRADE II	Admissible
3	GRADE IV	Optimal

Table 2.2.3: cRNA concentration after labeling with cy3 dye

Sample ID	Dye	pmol/ul	Conc: ng/ul	260/280	Specific Activity (pmoldye/ugcRNA)
Control	Cy3	0.74	113.54	2.35	6.52
II	Cy3	1.06	100. 78	2.45	10.52
IV	Cy3	1.58	162.50	2.26	9.72

Note: OP: Optimal Purity (OD260/280 >1.8 and <2.2; OD260/230>1.0 and <2.4); **Sub-OP**: Sub-Optimal purity (OD260/280<1.8 and OD 260/230<1.0); **OC**: optimal concentration (>50 ng/microliter and <2500 ng/microliter); **Sub-OC**: Sub-optimal concentration (<50ng/microliter); RIN (RNA integrity number) > 7 is good; RIN (RNA integrity number) 6-7 is optimal;

RIN (RNA integrity number) 5-6 is admissible;

RIN (RNA integrity number) < 5 is degraded;

Figure 2.2: Summary of micro array data

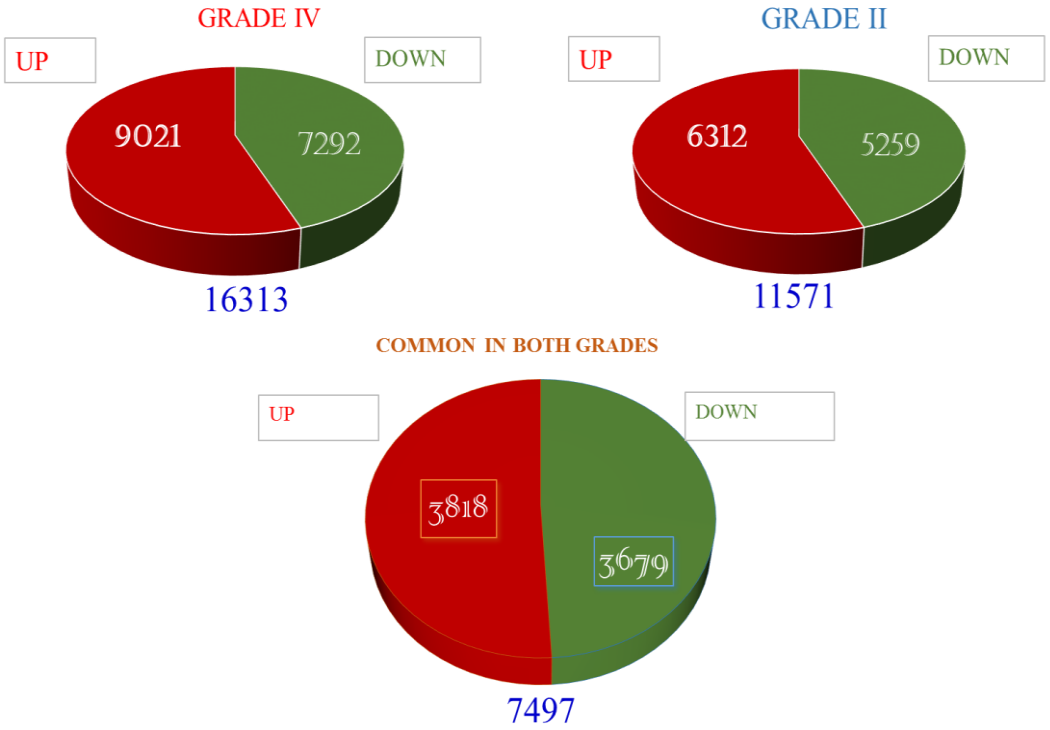


Figure 2.2: Describes the summary of complete human genome (44k genes) micro array data of 44k genes in grade IV (GBM) and grade II astrocytoma samples.

Table 2.3.1: Biointerpreter- Biological annotation for significant pathway genes up regulated in grade II astrocytoma

Name of the Pathway [total no of genes]	No of genes	P value
ABC transporters[46]		1.00E-09
Focal adhesion[201]	4	1.00E-09
Hypertrophic cardiomyopathy (HCM)[91]	2	1.00E-09
Alanine, aspartate and glutamate metabolism[31]	1	1.00E-09
Primary immunodeficiency[37]	1	1.00E-09
Non-small cell lung cancer[54]	1	1.00E-09
D-Arginine and D-ornithine metabolism[1]	1	0.0072
Neuroactive ligand-receptor interaction[312]	8	0.0098
Hematopoietic cell lineage[85]	10	0.0434
Regulation of actin cytoskeleton[216]	6	0.046
Focal adhesion[201]	5	1.00E-09
ECM-receptor interaction[86]	4	1.00E-09
Small cell lung cancer[90]	2	1.00E-09
Citrate cycle (TCA cycle)[31]	1	1.00E-09
Pathways in cancer[341]	9	0.0026
Regulation of actin cytoskeleton[216]	5	0.0105
Cytokine-cytokine receptor interaction[276]	8	0.0116
Calcium signaling pathway[189]	4	0.0127
MAPK signaling pathway[276]	9	0.0223
Endocytosis[191]	5	0.0256
Natural killer cell mediated cytotoxicity[135]	3	0.0407
Tight junction[131]	3	0.0472

Table 2.3.2: Biointerpreter- Biological annotation for significant pathway genes down regulated in grade II astrocytoma

Name of the Pathway[total no of genes]	No of genes	P value
ECM-receptor interaction[86]	17	1.00E-09
p53 signaling pathway[70]	8	1.00E-09
Glioma[66]	2	1.00E-09
Renin-angiotensin system[19]	2	1.00E-09
Type I diabetes mellitus[45]	2	1.00E-09
Allograft rejection[38]	1	1.00E-09
Alanine, aspartate and glutamate metabolism[31]	1	1.00E-09
Pathways in cancer[341]	11	0.0098
Regulation of actin cytoskeleton[216]	6	0.0222
Chemokine signaling pathway[195]	5	0.0222
Neuroactive ligand-receptor interaction[312]	12	0.0424
Epithelial cell signaling in Helicobacter pylori i[65]	3	1.00E-09
Focal adhesion[201]	17	1.00E-09
Leukocyte transendothelial migration[119]	6	1.00E-09
Valine, leucine and isoleucine degradation[45]	1	1.00E-09
ErbB signaling pathway[89]	3	1.00E-09
Vibrio cholerae infection[55]	2	1.00E-09
Proteasome[45]	1	1.00E-09
ECM-receptor interaction[86]	13	0.0062
Tight junction[131]	3	0.0444
Vascular smooth muscle contraction[129]	3	0.048

Table 2.3.3: Biointerpreter- Biological annotation for significant pathway genes up regulated in grade IV astrocytoma

Name of the Pathway [total no of genes]	No of genes	P value
ECM-receptor interaction[86]	17	1.00E-09
p53 signaling pathway[70]	8	1.00E-09
Glioma[66]	2	1.00E-09
Renin-angiotensin system[19]	2	1.00E-09
Type I diabetes mellitus[45]	2	1.00E-09
Allograft rejection[38]	1	1.00E-09
Alanine, aspartate and glutamate metabolism[31]	1	1.00E-09
Pathways in cancer[341]	11	0.0098
Regulation of actin cytoskeleton[216]	6	0.0222
Chemokine signaling pathway[195]	5	0.0222
Neuroactive ligand-receptor interaction[312]	12	0.0424
Epithelial cell signaling in Helicobacter pylori i[65]	3	1.00E-09
Focal adhesion[201]	17	1.00E-09
Leukocyte transendothelial migration[119]	6	1.00E-09
Valine, leucine and isoleucine degradation[45]	1	1.00E-09
ErbB signaling pathway[89]	3	1.00E-09
Vibrio cholerae infection[55]	2	1.00E-09
Proteasome[45]	1	1.00E-09
ECM-receptor interaction[86]	13	0.0062
Tight junction[131]	3	0.0444
Vascular smooth muscle contraction[129]	3	0.048

Table 2.3.4: Biointerpreter- Biological annotation for significant pathway genes down regulated in grade IV astrocytoma

Name of the Pathway [total no of genes]	No of genes	P value
Type I diabetes mellitus[45]	2	1.00E-09
Neuroactive ligand-receptor interaction[312]	35	0.0106
MAPK signaling pathway[276]	9	0.0125
Endocytosis[191]	6	0.0339
Adipocytokine signaling pathway[65]	2	1.00E-09
Glycerolipid metabolism[46]	2	1.00E-09
Gap junction[97]	4	1.00E-09
Epithelial cell signaling in Helicobacter pylori i[65]	2	1.00E-09
Endocytosis[191]	5	0.014
Purine metabolism[157]	4	0.0242
Chemokine signaling pathway[195]	6	0.0249
Neurotrophin signaling pathway[127]	3	0.0357

2.7.2. Real time PCR analysis of cell surface receptors genes

Here we analyzed the expression of cell surface receptor genes which were not reported earlier in glioma and those which were upregulated in microarray data. We selected STC2, LILRB3, LILRB2, OSMR, MUC20 and SRPX. Gene names, location on chromosome and fold increase in micro array has been described in **Table 2.4**. Real time PCR analysis showed that mRNA of all 6 genes were overexpressed in tumor samples compared to control.

Significant over expression of STC2, LILRB3, LILRB2, OSMR, and MUC20 was observed in Grade IV samples compared to Control. SRPX gene did not show

significant over expression in Grade IV but was overexpressed in Grade II whereas LILRB2 and LILRB3 showed significant over expression in Grade II also (**Figure 2.3**).

Table 2.4 genes related to cell surface protein

GENE SYMBOL	GENE NAME	RELATED FUNCTION	LOCATION ON CHROMOSOM	FOLD INCREASE IN GRADE IV/II COMPARE TO CONTROL
STC2	Stanniocalcin 2	cell surface receptor linked signal transduction	5q35.1	5/3
LILRB3	Lleukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	cell surface receptor linked signal transduction	19q13.4	5/4
LILRB2	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2	cell surface receptor linked signal transduction	19q13.4	4/2
OSMR	Oncostatin M receptor	cell surface	5p13.1	4/1
MUC20	Mucin 20	cell surface ssociated	3q29	3/1
SRPX	Sushi-repeat-containing protein, X-linked	cell surface	Xp21.1	3/3

Table 2.4. Six genes related to surface protein were selected from micro array. All these genes were up regulated in GBM sample more than two fold compare to control and these genes are distributed on the different chromosome locations

Figure 2.3. Real time PCR analysis of cell surface proteins related genes

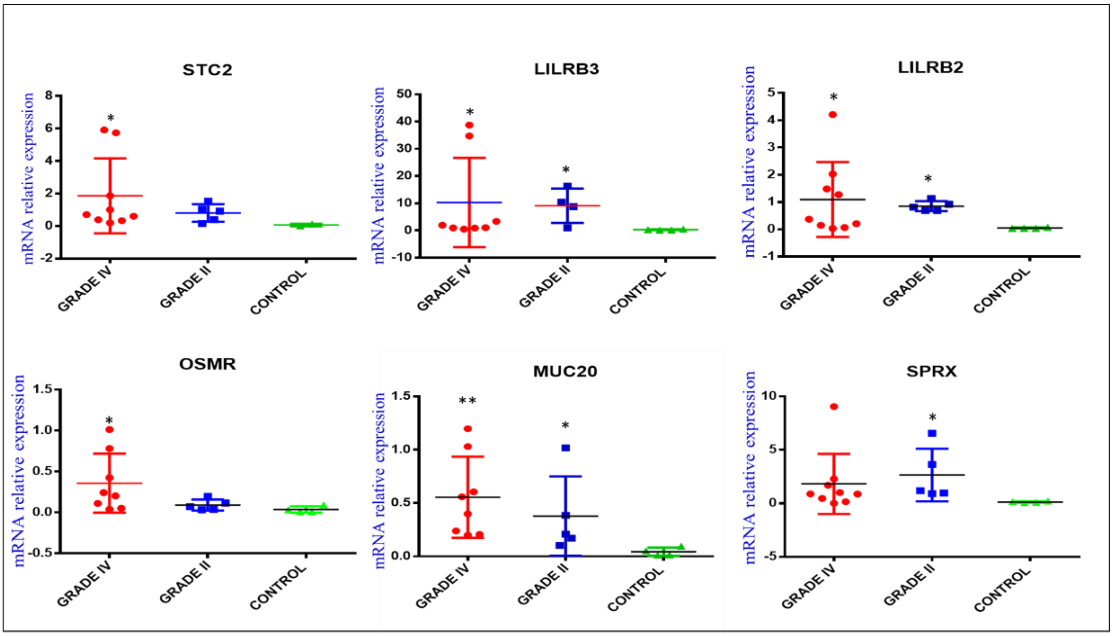


Figure 2.3. Shows real time PCR data of cell surface protein related genes. All the 6 genes showing significant level increase in astrocytoma sample compare to control. Statistical significance tested with sigma Plot 11. Kruskal-Wallis one way analysis of variance on rank, showed the median difference among the groups is statistically significant (STC2, *P = 0.029*; LILRB3, *P = 0.016; LILRB2, P = 0.046;* OSMR, *P = 0.020;* MUC20, *P = 0.007;* SPRX, *P = 0.031)*

2.7.3. Real time PCR analysis of genes regulating angiogenesis

Increased angiogenesis leads to more tumor growth and it is inversely proportional to patients' survival time. Three genes ANGPTL4, STAB1 and ELK3 were selected for our study. These genes details are shown in **Table 2.5**. Among these three genes ANGPTL4 and ELK3 are known to be involved in promotion of angiogenesis whereas STAB1 is a negative regulator of angiogenesis. Real time validation of these genes suggests that all three genes ANGPTL4, STAB1 and ELK3 were significantly over expressed in Grade IV compared to Control. Grade II also showed over expression of these genes but not at significant levels (**Figure 2.4**).

Table 2.5. Genes functionally related to Angiogenesis

GENE SYMBOL	GENE NAME	RELATED FUNCTION	LOCATION ON CHROMOSOM	FOLD INCREASE IN GRADE IV/II COMPARE TO CONTROL
ANGPTL4	Angiopoietin-like 4	Angiogenesis	19p13.3	3.5/2.3
STAB1	Stabilin 1	Negative regulation of angiogenesis	3p21.1	3/1
ELK3	ETS-domain protein (SRF accessory protein 2) (ELK3)	Angiogenesis	12q23	3/2

Table 2.5 Three genes functionally related to angiogenesis were selected from micro array. All these genes are up regulated in GBM sample more than two fold compare to control. ANGPTL4 and ELK3 are positive regulators of angiogenesis whereas STAB1 negative regulator. These genes are distributed on the different chromosome locations.

Figure 2.4. Real time PCR analysis of angiogenesis related genes

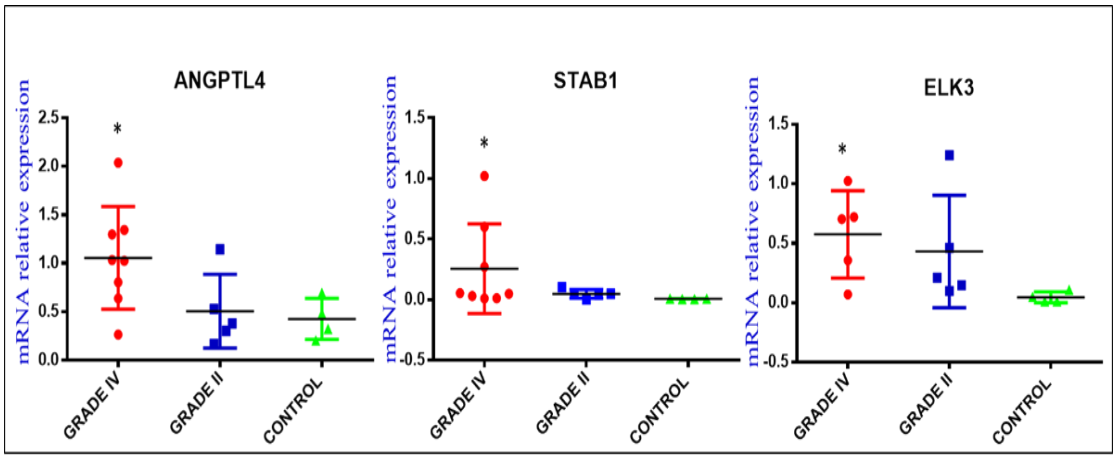


Figure 2.4 shows real time PCR analysis of angiogenesis related genes ANGPTL4, STAB1 and ELK3 expression. Kruskal-Wallis one way analysis of variance on rank, showed the median difference among the groups is statistically significant (ANGPTL4, *P = 0.047*; STAB1, *P = 0.028;* ELK3, *P = 0.026 ;)*

2.7.4. Real time PCR analysis of genes regulating migration

Complete surgical resection is not possible in high grade gliomas most of the times. One of the main reasons for this complication is migration of tumor cells from tumor site to different parts of the brain. In this migration process adhesion molecules, and extra cellular matrix proteins play a key role. Based on these observations, 6 genes (ITGB3, FN1, ITGB1, CLIC4, MMP25 and SIX1) were selected from microarray data and validated with real time PCR. Among these selected genes ITGB3, ITGB1, FN1 are adhesion molecules, MMP25 is extra cellular matrix protein, SIX1 is a transcription factor involved in migration promotion, and CLIC4 is a negative regulator of migration. For further details please refer to **table 2.6**. Real time PCR validation (**Figure 2.5**) shows that ITGB3, ITGB1, CLIC4 were significantly up regulated in Grade IV compared to Control and ITGB1 up regulated significantly in Grade II also. Whereas FN1 did not show any significant increase in Grade IV or Grade II samples when compared to Control.

Table 2.6. Six genes functionally related to migration

GENE SYMBOL	GENE NAME	RELATED FUNCTION	LOCATION ON CHROMOSOM	FOLD INCREASE IN GRADE IV/II COMPARE TO CONTROL
ITGB3	Integrin, beta 3 cell-matrix	Adhesion	17q21.32	6/4
FN1	Fibronectin 1	Adhesion	2q34	5/2
ITGB1	Integrin, beta 1 cell-matrix	Adhesion	17q21.32	3/1
CLIC4	Chloride intracellular channel 4	Negative regulation of cell migration	1p36.11	3/2
MMP25	Matrix metallopeptidase 25	Proteinaceous extracellular matrix	16p13.3	4/1
SIX1	Sine oculis homeobox homolog 1	Migration	14q23.1	2/1

Table 2.6 Six genes functionally related to migration were selected from micro array. All these genes were up regulated in GBM sample more than 2 fold compare to control. Except CLIC4, all genes are positively involve in migration. These genes distributed on the different chromosome locations.

Figure 2.5 Real time PCR analysis of migration related genes

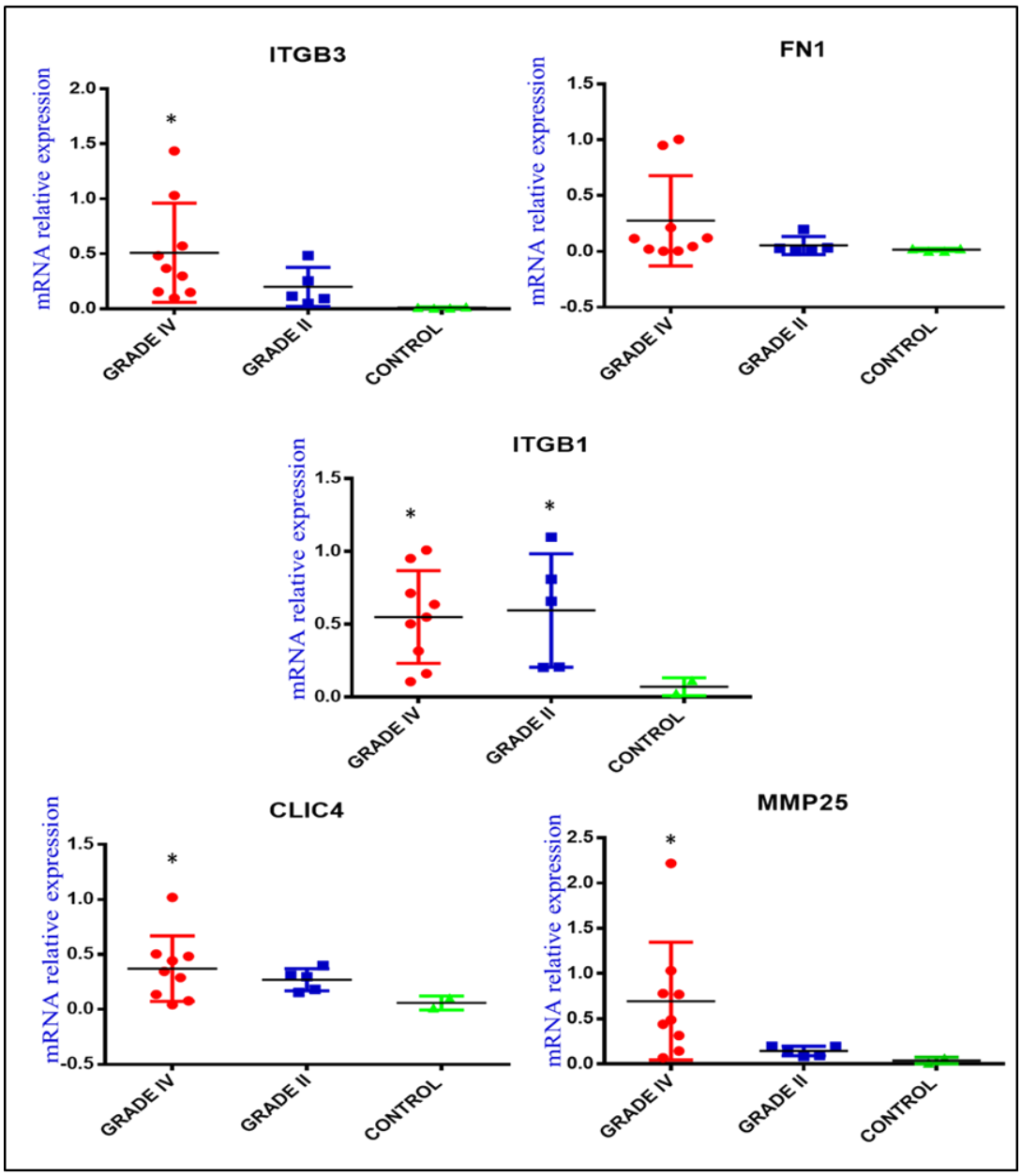


Figure 2.5 shows real time PCR analysis of migration related genes. Kruskal-Wallis one way analysis of variance on rank, except FN1 gene remaining genes showed the median difference among the groups is statistically significant (ITGB3, *P = 0.005;* ITGB1, *P = 0.015;* CLIC4, *P = 0.050;* MMP25, *P = 0.006*)

2.7.5. Real time PCR analysis of Transcription factors

Transcription factors are mainly involved in regulating pathways by enhancing the expression of other genes. Many reports have shown the involvement of transcription factors (Slug, Snail) in glioma progression by inducing developmental, cell survival

pathways and stemness of cancer cells in the tumor. In the present study we selected 4 transcription factors (ABCA13, UHRF1, SOX4, and SIX1) from the micro array data. Their scientific names, location and fold changes are presented in **Table 2.7**. Here we are presenting ABCA13, UHRF1 AND SOX4 validation data. SIX1 data forms a part of next chapter. Real time PCR validation showed that ABCA13, UHRF1and SOX4 were significantly over expressed in Grade IV samples compared to Control. ABCA13 showed significant increase in Grade II also (**Figure 2.6**).

Table 2.7 Genes functionally related to transcription activity

GENE SYMBOL	GENE NAME	RELATED FUNCTION	LOCATION ON CHROMOSOM	FOLD INCREASE IN GRADE IV/II COMPARE TO CONTROL
ABCA13	ATP-binding cassette, sub-family A (ABC1), member 13	Regulation of transcription, DNA-dependent	Xp21.1	5/4
UHRF1	Ubiquitin-like, containing PHD and RING finger domains, 1	Transcription	19p13.3	5/4
SOX4	SRY (sex determining region Y)-box 4	Regulation of transcription, DNA-dependent	6p22.3	4/1
SIX1	Sine oculis homeobox homolog 1	Regulation of transcription, DNA-dependent	14q23.1	2/1

Table 2.7 Four genes which are functionally having transcription activity were selected from micro array. All these genes were up regulated in GBM sample more than two fold compare to control. These genes distributed on the different chromosome locations.

Figure 2.6 Real time PCR analysis of transcription activity related genes

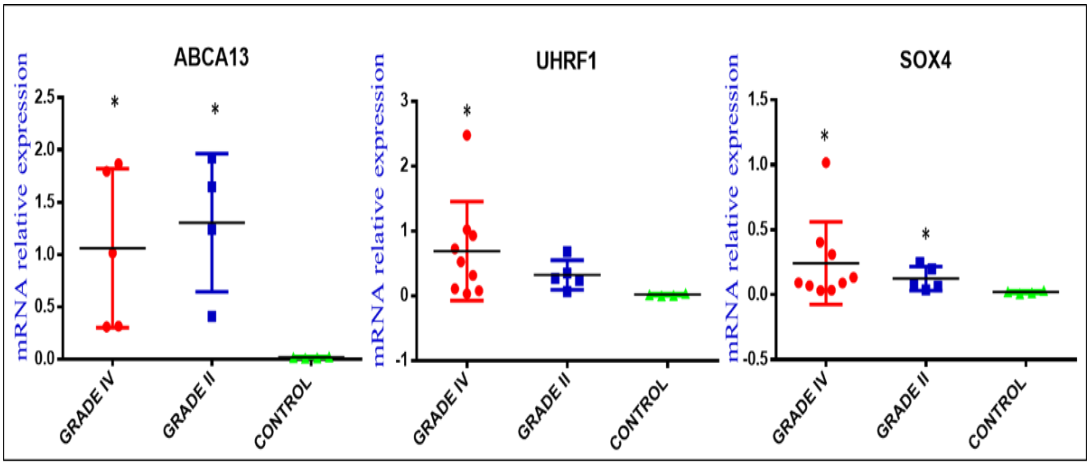


Figure 2.6 shows real time PCR analysis of Transcription activity related genes. Kruskal-Wallis one way analysis of variance on rank, showed the median difference among the groups is statistically significant (ABCA13, *P = 0.007;* UHRF1, *P = 0.013;* SOX4, *P = 0.011 ;)*

2.8. DISCUSSION

GBM is most dangerous and deadly form of astrocytoma with median survival of 14.6 months (Koshy, Villano et al. 2012). In the present study we performed micro array studies on high grade, Grade IV primary GBM, low grade, Grade II astrocytoma and Control brain sample to understand the transcript level differences among these clinical samples. Whole human genome (44k genes) micro array analysis of these clinical samples revealed that out of 44 thousand genes 16313 genes in GBM, 11572 genes in Grade II were altered compared to Control. 7497 genes were commonly altered in both the grades. We also analyzed for some important pathways related genes expression which were significantly altered in GBM and Grade II samples, with help of Bio-interpreter software.

From the microarray data we selected 18 genes which were functionally related to cell surface, transcription factors, angiogenesis and migration. Recent therapeutic strategies are targeting cell surface receptors which are over expressed in glioma. Growth factor receptors or cytokine receptor pathways are responsible for the proliferation of most cancer cells, including malignant gliomas. Autocrine and paracrine growth factor loops, that is, the presence of both growth factor ligands and their related receptors in tumor tissues, are frequently present in malignant gliomas. Over expressed cells surface receptors in gliomas include, growth receptors such as epidermal growth factor receptor (EGFR) specially EGFR variant 3 (EGFRvIII) with nearly 40% over expression (Libermann, Nusbaum et al. 1985; Kuan, Wikstrand et al. 2001).; VEGFR, PDGFR, TGF β R, FGFR, cytokine and other receptors such as IL-4, IL13, transferrin, integrin, tenascin are over expressed in glioma and they are involved in cellular functions such as proliferation, apoptotic resistance, motility, invasion, neo-

angiogenesis and metastasis. These cell surface receptors have proved to be better options in glioma treatment (Li and Hall 2011). In the current study, we analyzed the status of 6 cell surface receptors which include STC2, LILRB3, LILRB2, OSMR, MUC20 and SPRX. STC2 is known to be involved in cell surface receptor linked signal transduction in response to hormonal activity and its over expression is observed in many human cancers like gastric cancers, neuroblastomas, castration-resistant prostate cancers, breast cancer, colorectal cancer, esophageal squamous-cell cancer and renal cell carcinomas (Yeung, Law et al. 2012). Concordant to the previous reports in other cancers, we observed that STC2 expression levels were significantly increased in Grade IV astrocytoma compared to control. LILRB2 and LILRB3 belong to leukocyte Ig-like receptors (LILR, also known as ILT and LIR) family and are involved in innate immune response. They are predominantly expressed on cells of the myelomonocytic lineage (Brown, Trowsdale et al. 2004). In human non-small lung cancer LILRB2 over expression has been reported. However functional characters are still unknown. LILRB3 involvement has not been reported in any cancers till now (Sun, Liu et al. 2008). These two genes were over expressed in both the tumor grades when compared to control sample in our study. OSMR is a cell surface cytokine receptor, it over expression is observed in cervical squamous cell carcinoma (Ng, Winder et al. 2007), colon cancer (Kim, Louwagie et al. 2009) gastric cancers (Junnila, Kokkola et al. 2010). OSMR over expression has also been associated with worse clinical outcomes. It is also involved in angiogenesis, migration and invasion (Caffarel and Coleman 2014). Similar to previous reports in other cancer types, human astrocytoma samples showed overexpression of OSMR. MUC20 is a cell surface associated protein and a member of the mucin protein family. Mucins are large extracellular glycoproteins which are

produced by epithelial cells (Hollingsworth and Swanson 2004). MUC20 overexpression is associated with poor prognosis in colorectal (Xiao, Wang et al. 2013) and endometrial cancers. It promotes malignancy by inducing EGFR-STAT3 pathway (Chen, Wang et al. 2013). Similarly, we also observed an overexpression of MUC20 in tumor samples.

We observed that transcription factors ABCA13, UHRF1 and SOX4 are significantly over expressed in grade IV astrocytoma. ABCA13 belongs to family of ATP-binding cassette (ABC) transporter and it is involved in development of tumor resistance by efflux of anti-cancer drugs (Hlavata, Mohelnikova-Duchonova et al. 2012; Hlavac, Brynychova et al. 2013). UHRF1 is an oncogenic factor and its over-expression has been reported in many cancers. It has been suggested to be a biomarker for high-grade tumors. UHRF1 is associated with altered DNA methylation, inhibition of cell contact loss and tumor suppressor gene expression (Alhosin, Sharif et al. 2011). Disruption of UHRF1 with dnmt1/PCNA complex is associated with poor prognosis in GBM patients (Hervouet, Lalier et al. 2010). SOX4 is member of SOX family of transcription factors and its over expression has been reported in various cancers (Jafarnejad, Ardekani et al. 2013). In glioma, TGF beta pathway via SOX4 is involved in maintaining the stemness property in glioma initiating cells (Ikushima, Todo et al. 2009).

Angiogenesis is a hallmark of high grade glioma. Here in the present study, we analyzed 3 angiogenesis related genes, namely, ANGPTL4, STAB1 and ELK3. EGFR vIII (variant 3) over expression promote angiogenesis through ANGPTL4 up regulation in GBM cell lines *in vitro* and *in vivo* (Katanasaka, Kodera et al. 2013). In support to this our results showed up regulation of ANGPTL4 in grade IV tumor. STAB1 is

scavenger receptor expressed on macrophages and associated with clearing of SPARC (secreted protein acidic and rich in cysteine, osteonectin) which is involved in promoting the migration. Rat glioma model showed high level of STAB1 at tumor initiation period but later on STAB1 levels were decreased as the tumor progressed (David, Nance et al. 2012), in contrast to this, according to our analysis STAB1 levels were increased in grade IV compare to control and grade II. ELK3 is an ETS gene family member, Ras- activated phosphorylated ELK3 stimulates VEGF promotion and regulates angiogenesis (Zheng, Wasylyk et al. 2003), in contrast to this ELK3 suppresses angiogenesis by inhibiting the transcriptional activity of ETS-1 on MT1-MMP (Heo and Cho 2014). In our study ELK3 levels where increased in grade IV and it correlated with the angiogenesis because, according to literature, the tumor progress from low grade to high grade angiogenesis increases.

Because of their invasive and migratory nature, gliomas spread to other parts of brain and cause recurrence. Thus, complete surgical resection of these tumors is challenging. From our microarray data, we selected 5 genes (ITGB3, ITGB1, FN1, CLIC4 and MMP25), which are functionally involved in migration. We observed that ITGB3 expression levels were highly increased in grade IV which is in line with the earlier reports which showed high expression of ITGB3 in glioma and its association with negative prognosis (Schittenhelm, Schwab et al. 2013). ITGB1 overexpression was observed in tumor samples and these results match with earlier reports in GBM patients. ITGB1 is involved in migration and invasion (Fowler, Thomson et al. 2011). FN1 overexpression has been reported in a study of prognostic marker evaluation in GBM (Wei, Huang et al. 2010), but in our study FN1 did not show significant increase in GBM. CLIC4 is down regulated in many cancers compared to normal tissue but its

overexpression has been observed in tumor stromal cells, which help in tumor growth and migration (Suh, Crutchley et al. 2007). There are no earlier reports in human glioma tissues regarding the expression of this gene. In our results, CLIC4 up regulation was observed in tumor tissues. MMP25 elevated mRNA levels have been reported in GBM and other cancers. These elevated levels are known to contribute towards glioma progression (Nuttall, Pennington et al. 2003; Sohail, Sun et al. 2008). In the present study, MMP25 levels were also elevated in astrocytoma sample, supporting the earlier reports.

CONCLUSION: We analyzed differential expression of genes in Grade II and Grade IV. 17 genes related to cell surface, migration, invasion and transcription factors were selected and validated. Importantly, we report overexpression of 10 new genes in glioma samples. Further studies on these may establish some novel therapeutic targets for glioma.

CHAPTER 3

Involvement of transcription factors SIX1 and ZNF 24 in human astrocytoma progression.

3. INTRODUCTION

3.1. Six1 (Sine oculis homeobox homolog 1)

Homeobox superfamily genes which encode transcription factors play a major role in development by regulating the diverse range of target gene expression (Levine and Hoey 1988). These transcription factors are 'master regulators' of developmental processes. Transcription factors regulate many cellular process such as proliferation, differentiation, apoptosis, cell shape, cell adhesion, and migration (Pearson, Lemons et al. 2005). SIX family, sine oculis (so) is a family of proteins in the homeobox superfamily and has been identified as an essential component in eye development of drosophila (Cheyette, Green et al. 1994). In drosophila three genes (so, optix, and Dsix4) of SIX family were identified to arise through duplication of an ancestral SIX gene (Seo, Curtiss et al. 1999). Loss of so gene leads to the riddance of the compound eyes and Optix gene expression in non-retinal tissue leads to the eye formation, while Dsix4 plays crucial role in mesoderm origin of tissues, such as somatic muscles, somatic cells of the gonad, and fat tissue (Kumar 2009). Further, duplication of these three genes (so, optix, and Dsix4) in vertebrates forms six genes which are divided in to three sub families, Six1/Six2 (so subfamily), Six3/Six6 (optix subfamily), and Six4/Six5 (Dsix4 subfamily).

Six family of proteins are involved in developmental process by regulating cell growth and tissue specific mechanisms in invertebrates and vertebrates. Six family members are known to induce cell proliferation or apoptosis in progenitor cell population, and play a role in regulating migration as well as the mesenchymal phenotype. Since six family contributes in organ development in the embryonic stage,

their abnormalities lead to genetic disorders and cancers (Christensen, Patrick et al. 2008).

Figure 3.1. Six gene duplication phylogenetic tree analysis

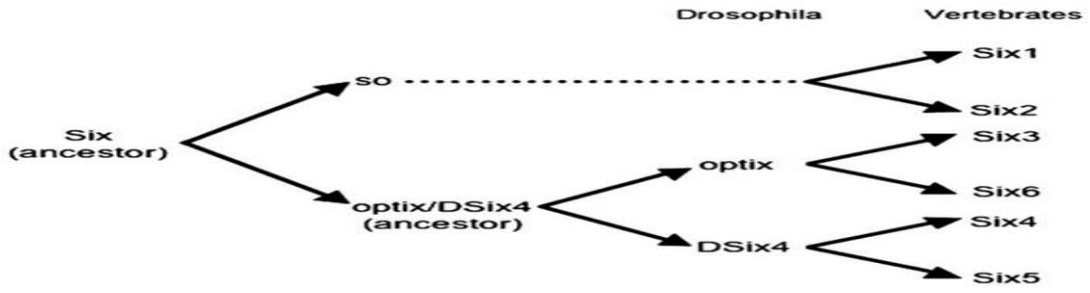


Figure 3.1.Model for SIX family duplication events. Model describing the path of evolution for the SIX genes that are found within *Drosophila* and vertebrate lineages. Figure adapted from (Kumar 2009).

3.2. Six family of homeoproteins and their structural elements

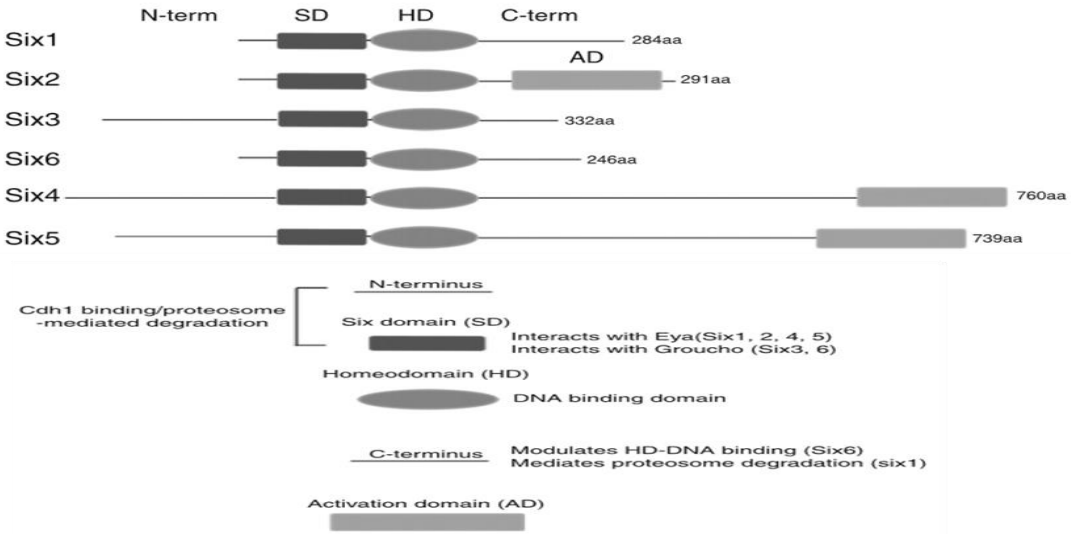


Figure 3.2. Schematic representation of six family of Homeoproteins. Structural elements of the six family members include a well-conserved six domain (SD) that is important for interactions with Eya (Six1, Six2, Six4, and Six5) and Groucho (Six3 and Six6), and a homeodomain (HD) that is responsible for DNA binding. In the case of Six1, signals that mediate Cdh1 binding and degradation by the proteosome are contained within a region that encompasses the N-terminus and the SD. The C-terminus is more divergent and also contains motifs critical for proteosome-mediated degradation of Six1, as well as an activation domain in the case of Six2, Six4, and Six5. Furthermore, the C-terminus is important for regulating Six6 HD-DNA-binding affinity. Figure adapted from (Christensen, Patrick et al. 2008).

Six1 a developmental transcription factor belongs to Six family. Six1 is widely expressed and involved in organogenesis in embryo development and has a role in muscle lineage (Laclef, Hamard et al. 2003), eye development (Relaix and Buckingham 1999), kidney development (Laclef, Souil et al. 2003), sensory structures (Konishi, Ikeda et al. 2006), auditory organ development (Zheng, Huang et al. 2003). Six1 is also expressed in rathke's pouch, through which it is involved in the pituitary, thyroid, and parathyroid glands, head muscles, facial skeleton, and cranial nerves development (Laclef, Hamard et al. 2003) (Oliver, Wehr et al. 1995). It also plays a role in normal neurogenesis (Zou, Silvius et al. 2004).

Figure 3.3. Six1 involvement in organogenesis

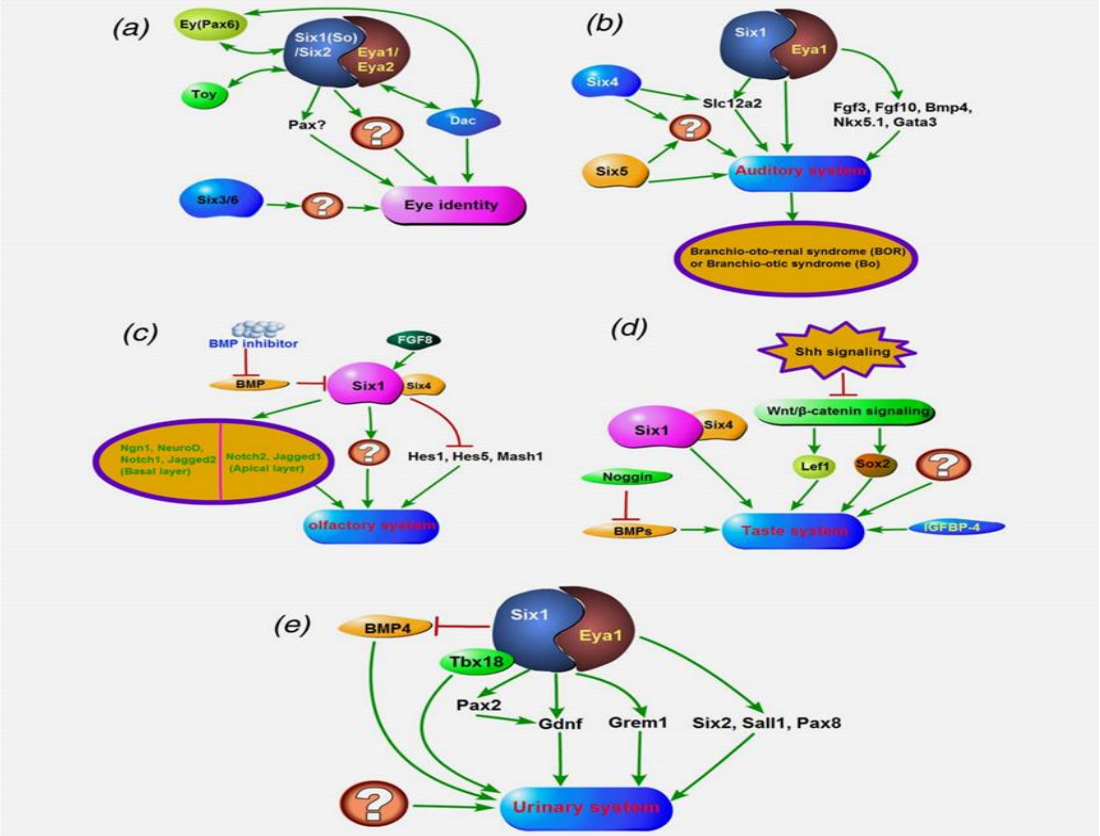


Figure 3.3. Six1 regulatory networks in the formation of sensory organs and kidney. (a) The regulation mechanism of Six1 in eye identity; (b) the regulation mechanism of Six1 in the auditory system; (c) the regulation mechanism of Six1 in the olfactory system; (d) the regulation

mechanism of Six1 in the taste bud system; (e) the regulation mechanism of Six1 in the urinary system, figure adapted from (Wu, Ren et al. 2014).

Six1 is involved in cell cycle regulation, by promoting cell cycle (Ford, Kabingu et al. 1998). This process is necessary during developmental stages to increase the cell number and then differentiate into specific cell type. Different mechanisms of Six1 cell cycle regulation have been elucidated in normal development and in cancer. In normal development six1 transcriptionally activates several cell cycle regulators which include, c-myc and gdnf (Li, Oghi et al. 2003; Yu, Davicioni et al. 2006).

Figure 3.4. Six1 role in cell cycle regulation

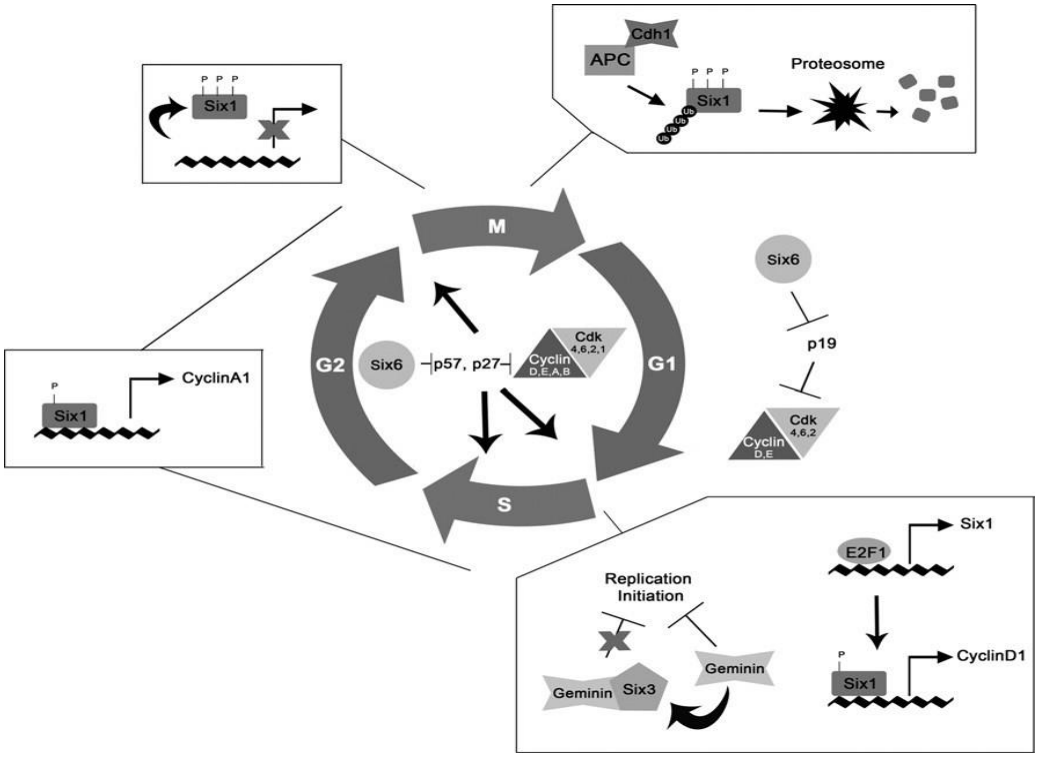


Figure 3.4. Proposed model of the role of six family members in the cell cycle. Six1 is transcriptionally activated at the G1/S boundary by E2F1, and transcript levels increase with progression through S-phase. Six1 binds to regulatory regions of its target genes during S- and G2-phases, to initiate transcription of targets such as cyclin D1 and cyclin A1. Six1 exists as a phosphoprotein throughout the cell cycle, but is hyper phosphorylated during mitosis, inhibiting its ability to bind DNA. During late mitosis, Six1 is degraded via ubiquitin-mediated proteolysis directed by APC^{Cdh1}. Six3 is able to influence the cell cycle by binding geminin, a negative regulator of replication initiation. By preventing geminin's inhibitory function, Six3 is able to initiate replication and cell cycle progression. Six6 represses transcription of the cyclin-

dependent kinase inhibitors p27, p57, and p19, and is thereby capable of influencing multiple points in the cell cycle to promote its progression. APC anaphase promoting complex. Figure adapted from (Christensen, Patrick et al. 2008).

Six1 also plays crucial role in tumorigenesis and its overexpression has been observed in a number of human cancers, where it regulates cell survival, proliferation and metastasis. Its overexpression has been reported in breast (Ford, Kabingu et al. 1998), ovarian cancer (Behbakht, Qamar et al. 2007), rhabdomyosarcoma (Yu, Khan et al. 2004), Wilms' tumors (Li, Guo et al. 2002), hepatocellular carcinoma (Ng, Man et al. 2006), cervical carcinoma (Wan, Miao et al. 2008) and colorectal cancer (Ono, Imoto et al. 2012).

Figure 3.5. Six1 role in tumorigenesis

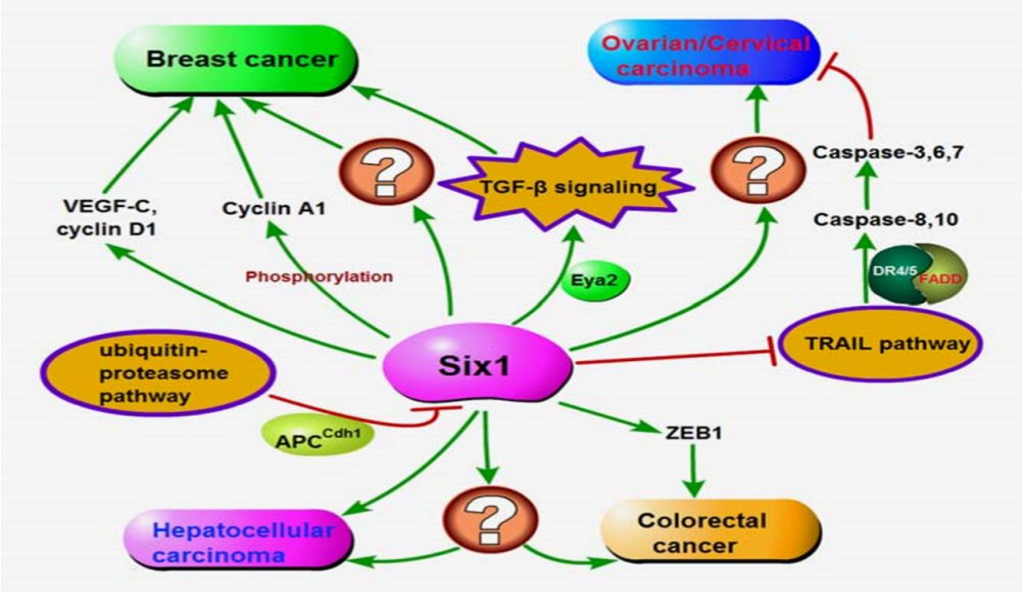


Figure 3.5. Regulatory network of Six1 in tumorigenesis. This figure shows the regulation mechanisms of Six1 in various cancers, including breast cancer, ovarian/cervical carcinoma, hepatocellular carcinoma and colorectal cancer. Figure adapted from (Wu, Ren et al. 2014).

Six1 promotes metastasis through the epithelial-mesenchymal transition in cancers by regulating ZEB1 in colorectal cancer (Ono, Imoto et al. 2012), VEGF-C in breast cancer (Wang, Jedlicka et al. 2012). In mammary cancers Six1 induces EMT and

stem cell properties through TGF-β pathway (McCoy, Iwanaga et al. 2009; Farabaugh, Micalizzi et al. 2012). Its involvement has also been reported in genetic syndromes such as Branchio-oto-renal syndrome (BOR), a human autosomal dominant disorder, with hearing impairment, auricular malformations, bronchial arch remnants, and renal anomalies (Ruf, Xu et al. 2004; Kochhar, Fischer et al. 2007).

TRAIL (Tumor Necrosis Factor- related apoptosis inducing ligand) is a member of TNF family, identified in mid-1995 by searches of TNF super family homologous in ETS data base (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996). TRAIL is expressed in human fetal and adult tissues such as thymus, spleen, prostate, placenta and small intestine (Wiley, Schooley et al. 1995). Membrane bounded TRAIL is expressed on immune cells like NK cells, B cells, dendritic and monocytes in response to stimuli of cytokines (Zamai, Ahmad et al. 1998; Fanger, Maliszewski et al. 1999; Griffith, Wiley et al. 1999; Kemp, Moore et al. 2004). TRAIL induces apoptosis in number of cancer cell lines, with very little effect on normal cells (Ashkenazi, Pai et al. 1999; Walczak, Miller et al. 1999; Allen and El-Deiry 2012). TRAIL has four receptors in humans. They are pro apoptotic receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), anti-apoptotic decoy receptors (DcR1 TRAIL-R3) and DcR2 (TRAIL-R4). TRAIL and its receptors form homo trimers in active state (Cha, Kim et al. 1999; Hymowitz, Christinger et al. 1999). Upon binding of TRAIL to death receptors, DR4 and DR5 induce cell death signaling through their intra cellular death domain (DD) (Pan, Ni et al. 1997; Walczak, Degli-Esposti et al. 1997; Wu, Burns et al. 1997), whereas decoy receptors do not induce cell death signaling because decoy receptor DcR1 lacks its intra cellular domain, DcR2 has a truncated intra cellular domain (Pan, Ni et al. 1997; Mongkolsapaya, Cowper et al. 1998; Pan, Ni et al. 1998). In addition to these receptors

TRAIL also binds to soluble osteoprotegerin, a negative regulator of osteoclastogenesis, but this binding is weaker than of other TRAIL receptors (Emery, McDonnell et al. 1998). TRAIL pathway is one of the potential targets for treatment of cancers (Micheau, Shirley et al. 2013).

3.6. TRAIL Pathway

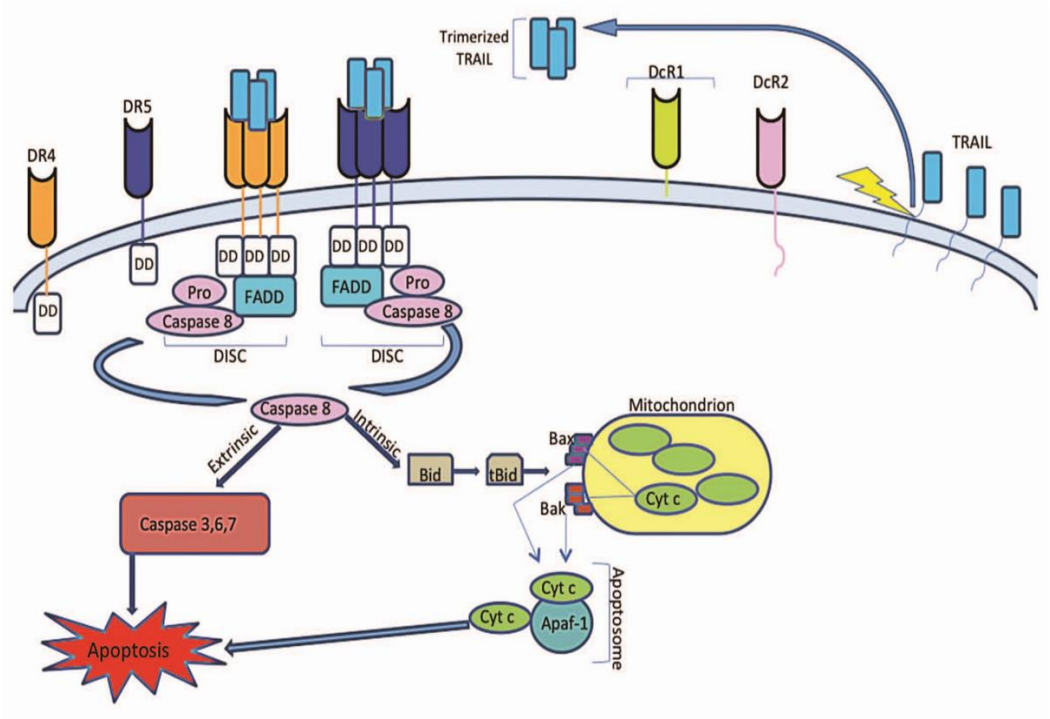


Figure 3.6. Apoptotic signaling induced by TRAIL. TRAIL initiates cell death by binding to the pro-apoptotic death receptors DR4 or DR5 that co-localizes their intracellular death domains. This clustering recruits the Fas-associated death domain (FADD) and pro-caspase-8 that results in its activation through autocatalytic cleavage. In type I cells, activate caspase-8 directly activates caspases-3, -6 and -7 to trigger the extrinsic cell death pathway. In type II cells, active caspase-8 cleaves Bid to a truncated form, tBid, which subsequently interacts with pro-apoptotic Bcl-2 family members Bax and Bak. This interaction leads to permeabilization of the mitochondrial membrane and release of cytochrome c. Cytosolic cytochrome c then combines with Apaf-1 and ATP to form the apoptosome that activates caspase-9 to trigger apoptosis through the caspase cascade. Figure adapted from (Allen and El-Deiry 2012).

However, Six1 overexpression cancer cells leads to resistance of TRAIL mediated apoptosis and tumor regression *in vivo* by TRAIL pathway inhibition had been reported (Behbakht, Qamar et al. 2007; Menke, Goncharov et al. 2011). Whereas

Six1 expression is translationally regulated by micro RNA MIR-185 in tumor cells (Imam, Buddavarapu et al. 2010).

3.2. ZNF24 (Zinc finger protein 24)

ZNF24 is also known as ZNF191/ Zfp 191, KOX17, RSG-A, NSCAN3. ZNF24 belongs to the family of SCAN box domain containing kruppel-like zinc finger transcription factors (Sander, Marsh et al. 1976) and is localized on chromosome 18q12.1 (Rousseau-Merck, Huebner et al. 1991). ZNF24 contain 4 krupel-like C2H2 zinc finger domains in C-terminus which function as DNA binding domains and bind to the TCAT repeats of the HUMTH01 microsatellite present in first intron of TH (Tyrosine hydroxylase) (Albanese, Biguet et al. 2001; Wang, Sun et al. 2008). N-terminus of ZNF24 contains SCAN domain which participates in protein–protein interactions and till now it is reported only in vertebrates (Schumacher, Wang et al. 2000; Nam, Honer et al. 2004). Gene expression data showed its involvement in hematopoiesis but its precise function is still not known (Han, Zhang et al. 1999). ZNF24 is pleiotropic factor involved in the development of brain, hematopoiesis and cancers (Li, Chen et al. 2009). Direct binding of ZNF24 to the CTNNB1 promoter of β catenin induces its expression through which it promotes cell proliferation in hepatocellular carcinoma (Liu, Jiang et al. 2012). It is already reported as negative regulator of VEGF and PDGFRB transcripts and causes their repression (Harper, Yan et al. 2007; Li, Chen et al. 2010). ZNF24 also plays a role in early embryo development (Li, Chen et al. 2006). It is necessary for neuronal cell maintenance in a cycling progenitor by inhibiting their differentiation pathways (Khalfallah, Ravassard et al. 2009) and it is also required by oligodendrocytes for central nerve system myelination

(Howng, Avila et al. 2010). However mechanism of action of ZNF24 is not yet understood completely and needs to be investigated.

Hence, the present study aims to

1. Check the status of Six1and ZNF24 proteins in human astrocytoma.
2. Expression status of TRAIL and its death receptors TRAIL-R1, TRAIL-R2 and decoy receptors DcR1, DcR2 in human astrocytoma grades and their correlation with Six1 expression
3. Investigate TRAIL pathway resistance in astrocytoma tumors and also check Six1 translational regulator MIR-185 expression in astrocytoma.

3.3. MATERIALS AND METHODS

3.3.1. Patient's samples:

32 astrocytoma samples were collected from Krishna Institute of Medical science (KIMS) hospital, Secunderabad, India. A part of the tumor sample, immediately after surgical resection was snap frozen in liquid nitrogen and stored at -80^{0}C. These samples were used for PCR analysis and western blotting. Remaining half of the samples were fixed in neutral buffered formalin and used for H&E, immunohistochemistry and immune florescence. Four normal brain specimens consisting of periventricular region obtained from patients with Temporal lobe epilepsy, which was histologically normal as confirmed by neuro-pathologist were used as the control.

3.3.2. Real time RT-PCR:

Snap frozen samples were used to isolate total RNA by Trizol method (Invitrogen, Carlsbad, CA, USA). RNA quantification was done with nano drop. cDNA

samples were prepared using blue print reverse transcript kit (TaKaRa, Japan) as per manufacturer's instructions. For cDNA preparation of micro RNA 185, we used complimentary sequence of mir-185 in place of oligo-dT while maintaining the reaction same as above. Real time RT-PCR was performed in Eppendorf multiplex 2.2 using PCR plate (Applied bio systems,) and perfect real time SYBR premix EX Taq master mix (TaKaRa, Japan). Primer sequences of the genes are listed in **Table 3.1**. (End of the fourth chapter).

3.3.3 Immunohistochemistry:

As described in earlier (Sareddy, Geeviman et al. 2012), formalin fixed, paraffin embedded 5µ sections were prepared on APES (amino-propyl tri-ethoxy-silane) coated slides. Sections were deparffinized and rehydrated by keeping at 60^0C for 3hours, followed by 3 xylene washes for 45min/wash. The slides are then treated with different graded alcohols and finally rinsed with running tap water. Antigen retrieval step was performed with Tris-EDTA buffer (PH.9) in microwave oven for three times (each time 8min). This was followed by the protocol suggested in the manual of DAKO IHC kit (cat.no.K0679). Briefly, these steps include peroxidase block for 15min, PBS wash for 5min, incubation with primary antibodies (Six1 anti body from Santa Cruz, cat no.sc-9709; ZNF24 antibody from sigma Aldrich cat. no. SAB1412359-100UG) diluted in PBS with 1:100 dilution for 1hr at room temperature or overnight at 4^0C, wash with PBS, biotinylated link for 20min, wash with PBS, treatment with streptavidin-HRP for 20min, and developed with DAB substrate and nuclear counter stained with hematoxyline. This was followed by water wash and dehydration with alcohols of different gradient. The slides were finally mounted with DPX mount (SRL, Company) and observed under Olympus microscope.

3.3.4. Immuno-florescence:

Formalin fixed, paraffin embedded 5µ sections were prepared on APES (amino-propyl tri-ethoxy-silane) coated slides. Sections were deparffinized and rehydrated by keeping at 60^0C for 3hours, followed by 3 xylene washes for 45min/wash. The slides are then treated with different graded alcohols and finally rinsed with running tap water. Antigen retrieval step was performed with Tris-EDTA buffer (PH.9) in microwave oven for three times (each time 8min).This was followed by wash with TBST buffer, blocking with 5% goat serum for 1hour, washes with TBS and TBST 4 times (TBS, TBST, TBST, and TBS). The slides were incubated with primary antibody (1:100 dilution in 1%BSA) for 1 hour at room temperature or overnight at 4^0C. After TBS washes, 1 hour incubation with FITC tagged secondary antibody was done. Further, after TBS washes, nucleus staining with DAPI was performed slides were mounted with Vectashield after TBS washing and observed in Zeiss confocal microscopy.

3.3.5. Western blot analysis:

As lab standard protocol described in (Bhaskara, Panigrahi et al. 2005), frozen tissues were homogenized in RIPA (radioimmunoprecipitaiton assay) buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.4% deoxy-cholate, 1% NP-40 containing protease inhibitors including 1 mM phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitors including 10 mM β-glycerophosphate, 10 mM NaF, 0.3mM Na3Vo4 and 0.3 mM aprotinin, using dounce homogenizer and sonicated for 2 min and then centrifuged at 14,000 g for 15 min at 4^0C. The supernatant was collected as whole cell lysates and protein concentrations were determined by Lowry method. Equal amount of proteins (30 -150ug) were resolved on 12.5% SDS-polyacrylamide gels and then transferred on to nitrocellulose membranes. After, blocking the

membranes in 5% milk solution for 1 h at room temperature, blots were probed with primary antibody overnight at 4^0C. After primary incubation blots were washed with TBS buffers 4 times, each wash for 5 min (TBS, TBST, TBST, and TBS). Then Blots were incubated with secondary antibodies conjugated with HRP/ALP for 1 h at room temperature. Later blots were washed with TBS buffer and developed with super signal west Pico chemiluminescent substrate (Thermo scientific) under versa doc instrument or with ALP method using BCIP/NBT substrates.

3.3.6. Statistical analysis:

Real time analysis for relative gene expression of all genes was done by $2^{--\Delta\Delta}$CT method as described previously (Schmittgen and Livak 2008). Statistical analysis was done by using the sigma plot 11.0. Statistical significance was analyzed with Anova and pairwise multiple comparison was performed with Student-Newman-Keuls Method with mean±S.E. Correlation Coefficient analysis was performed with Pearson Product Moment Correlation. Correlation Coefficient value (+1) was taken as very strong positive, (-1) was strong negative correlation and (0) as no correlation. For all analysis $P \leq 0.05$ was considered as statistically significant. Immunohistochemistry staining intensity was calculated by counting positive stained/unstained cells in a field. For each section, six individual fields were selected randomly.

3.4. RESULTS

3.4.1. Over expression of Six1 mRNA in human astrocytoma and correlation with tumor progression.

Preliminary studies were performed by checking the Six1 mRNA expression in astrocytoma samples with semi quantitative RT-PCR (**Figure 3.7.1.**) Then we

performed real time PCR analysis with Six1 in 21 clinical samples. Among these 7 samples were grade IV (GBM), 4 samples grade III, 5 samples were grade II and 4 temporal lobe epilepsy samples were used as control. The results showed Six1 mRNA levels were significantly over expressed in astrocytoma samples compared to control brain sample (**Figure 3.7.2. and 3.7.1.**). Six1 mRNA over expression was observed to increase with increasing tumor grade. Grade II and grade III Six1 mRNA levels showed significant increase with control ($P< 0.016$; $P< 0.029$ respectively) but there was no significant difference between the two grades. Grade IV (GBM) tumors showed significantly increased mRNA levels compared to control ($P< 0.006$) (**Figure 3.7.2.**).

Figure 3.7.1. Semi quantitative PCR of Six1 expression in human astrocytoma

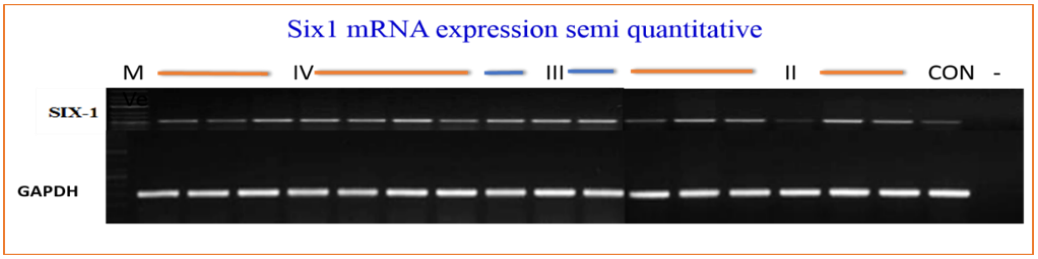


Figure 3.7.2. Real time PCR analysis of Six1 expression in human astrocytoma samples

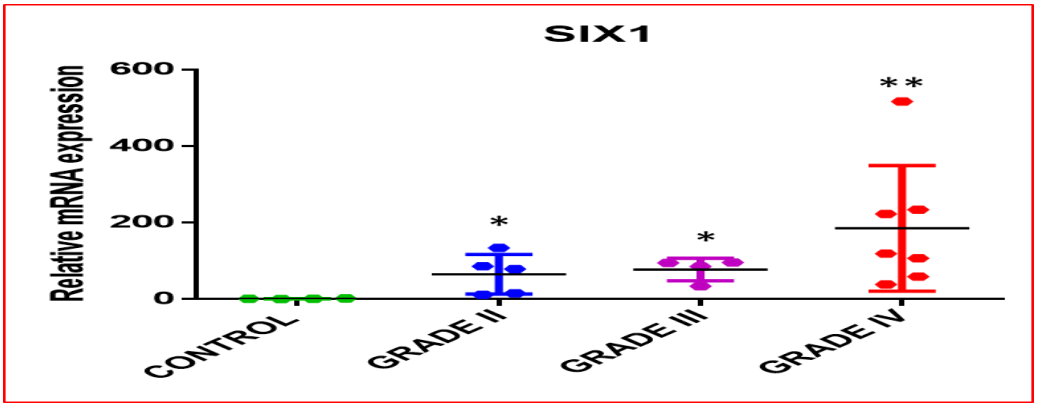


Figure 3.7.1. Representative picture of Six1 mRNA expression with semi quantitative method. 7 Grade IV, 3 Grade III, 6 Grade II and one control sample were used. GAPDH was used as internal control. **Figure 3.7.2.** Real time PCR analysis for relative Six1 expression in 7 Grade IV, 4 Grade III, 5 Grade II and 4 control samples. Statistical significance tested with one way ANOVA. (** =P<0.006). (* =$P<0.029$ & $P>0.006$ respectively).

3.4.2. TRAIL and death receptor mRNA expression in astrocytoma grades:

TRAIL pathway is important in specifically eliminating virus infected cells and cancer cells without affecting normal cells. Real time PCR analysis was performed to check the status of TRAIL, its death receptors (DR4, DR5) and decoy receptors (DcR1, DcR2) in tumor and control sample**.** Increased expression of TRAIL ligand causes elimination of cancer cell by inducing apoptosis through binding to death receptors. This apoptotic effect is nullified when TRAIL ligand binds to decoy receptors DcR1 and DcR2. In the present study, TRAIL ligand levels were increased in tumor grades compared to control samples and these elevated mRNA levels correlated positively with tumor advancement. All tumor samples (grade IV III and II) showed significant overexpression when compared to control (P<0.012, P<0.029 and P<0.032 respectively) (**Figure 3.8.1.A**). However, grade IV showed highest expression when compared to grade III and grade II but this increase among the grades was not significant. Death receptors DR4 and DR5 are important in inducing cell death and their increased levels may have a role in inducing apoptosis in cancer. We observed that death receptor DR4 mRNA levels were elevated in tumor grades but this elevated level did not correlate with tumor advancement. Grade II and grade IV showed significant increase with respect to control ($P<0.016$ and $P<0.006$). Grade IV showed higher expression than Grade II and grade III but significance was observed with grade III only ($P<0.037$). Grade III samples did not show significant increase when compared to control and its levels were lower than grade II tumor samples (**Figure 3.8.1.B**). DR5 mRNA overexpression was also observed in astrocytoma samples in comparison to control. mRNA expression levels were more in grade III than grade II and grade IV. These were observed to be statistically significant when compared to control ($P<0.029;$

P<0.016 and *P<0.006*). Grade IV showed significant increase in DR5 mRNA expression in comparison to grade II (*P<0.006*) (**Figure 3.8.1.C**).

Figure 3.8.1. Real time PCR analysis of TRAIL pathway genes in human astrocytoma

Figure 3.8.1. B

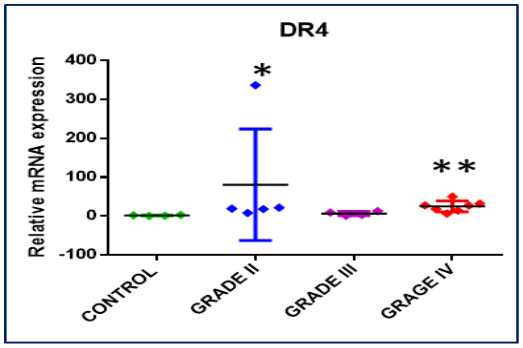


Figure 3.8.1. C

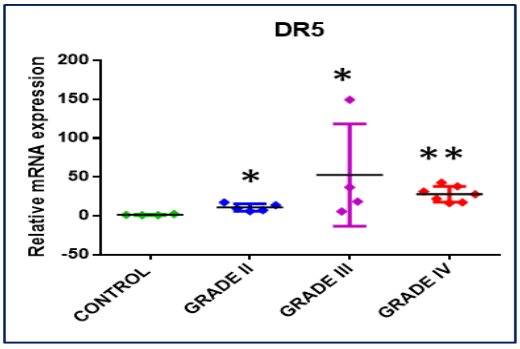


Figure 3.8.1. A

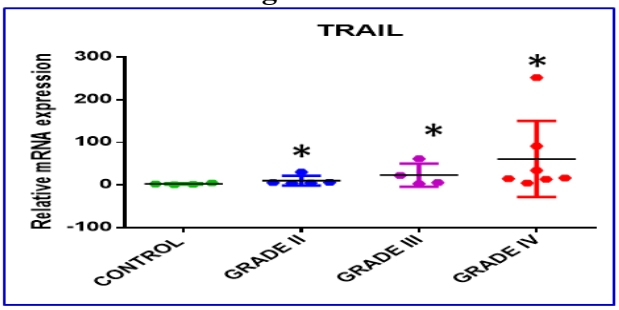


Figure 3.8.1. D

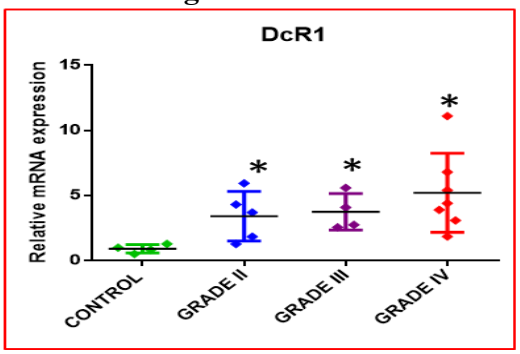


Figure 3.8.1. E

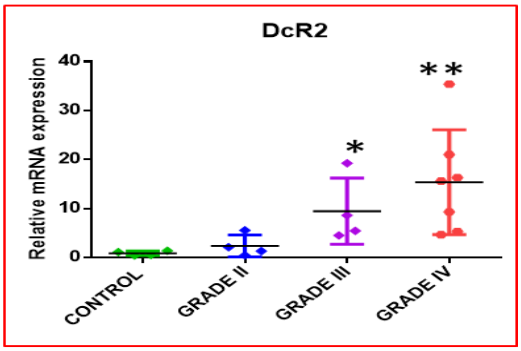


Figure 3.8.1: TRAIL and its receptors mRNA expression anlysed with real time PCR. Here 16 human tumor samples (Grade IV, 7; Grade III, 4; Grade II, 5 ;) and 4 control samples were used. **A)** TRAIL ligand expression in tumor grades and control. (**B** and **C)** TRAIL death receptors DR4 and DR5 relative mRNA expression. (**D** and **E)** Relative mRNA expression of decoy receptors DcR1 and DcR2 in astrocytoma and control samples. (*) indicates the significant level of difference in astrocytoma sample in comparison with control samples. Significance (*= $P \leq 0.05$; **= $P < 0.006$;).

Decoy receptors DcR1 and DcR2 play negative role in apoptosis by competing with death receptors. Decoy receptor DcR1 gene expression increased in astrocytoma samples compared to control. These increased mRNA levels correlated with tumor advancement. Grade IV (*P<0.022*), grade III (*P<0.008*) and grade II (*P<0.032*) showed significant overexpression when compared to control. Among the grades no significant difference was observed (**Figure 3.8.1.D**). Decoy receptor DcR2 also showed increased expression in tumor grades when compared to control and in tumor grades increased DcR2 mRNA levels correlated with tumor grade progression. Grade IV and grade III showed significantly increase of DcR2 mRNA over control (*P<0.006*, *P<0.029* respectively) (**Figure 3.8.1.E**).

3.4.3. Six1, TRAIL and Decoy receptors expressions positively correlate with tumor grade progression.

Six1 overexpression in tumors are involved in TRAIL mediated drug resistance (Behbakht, Qamar et al. 2007; Thorburn, Behbakht et al. 2008). Here we performed correlation studies on the Six1 mRNA expression with TRAIL pathway genes. Pearson correlation coefficient (C.C) showed Six1 expression showed significantly strong positive correlation with TRAIL ligand (C.C. 0.974; *P<0.0260,* **Figure 3.8.2.A**), decoy receptor DcR1 (C.C.0.945; *P<0.0450,* **Figure 3.8.2.D**) and DcR2 (C.C. 0.928; *P<0.0723*, **Figure 3.8.2.E**). Whereas death receptors DR4 (C.C. 0.124; *P<0.876,* **Figure 3.8.2.B**) and DR5 (C.C. 0.453; *P< 0.547*, **Figure 3.8.2.C)** did not show significant positive correlation **Figure 3.8.2.).**

Figure 3.8.2. Correlation analysis of TRAIL pathway genes mRNA expression with Six1 mRNA expression in human astrocytoma sample

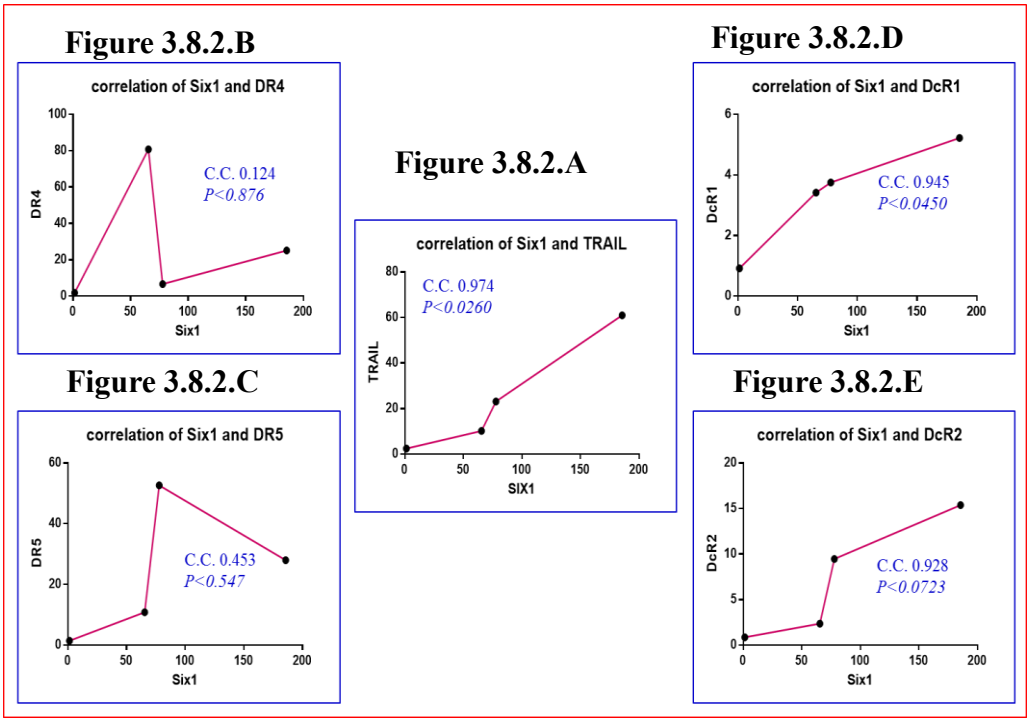


Figure 3.8.2: Correlation studies of m RNA expression of TRAIL ligand (**A**) and its death receptors DR4 and DR5 (**C, D**), and decoy receptor DcR1and DcR2 (**E, F**) with Six1 performed with Pearson correlation coefficient (C.C). Pearson correlation coefficient values range from -1 to +1. The C.C value of "-1" indicates strong negative correlation. C.C value "+1" indicates strong positive correlation and C.C value "0" indicates no correlation.

3.4.4. Six1 protein may be involved in astrocytoma progression

To check the Six1 protein expression in astrocytoma samples we performed immuno-staining with Six1 antibody (**Figure 3.9.1. and 3.9.2.).** Immuno-histochemical staining showed Six1 expression in astrocytoma samples but not in control brain sample. Six1 protein was observed to be mainly localized in nucleus.

Figure 3.9.1. Immunostaining with Six1 and GFAP of astrocytoma

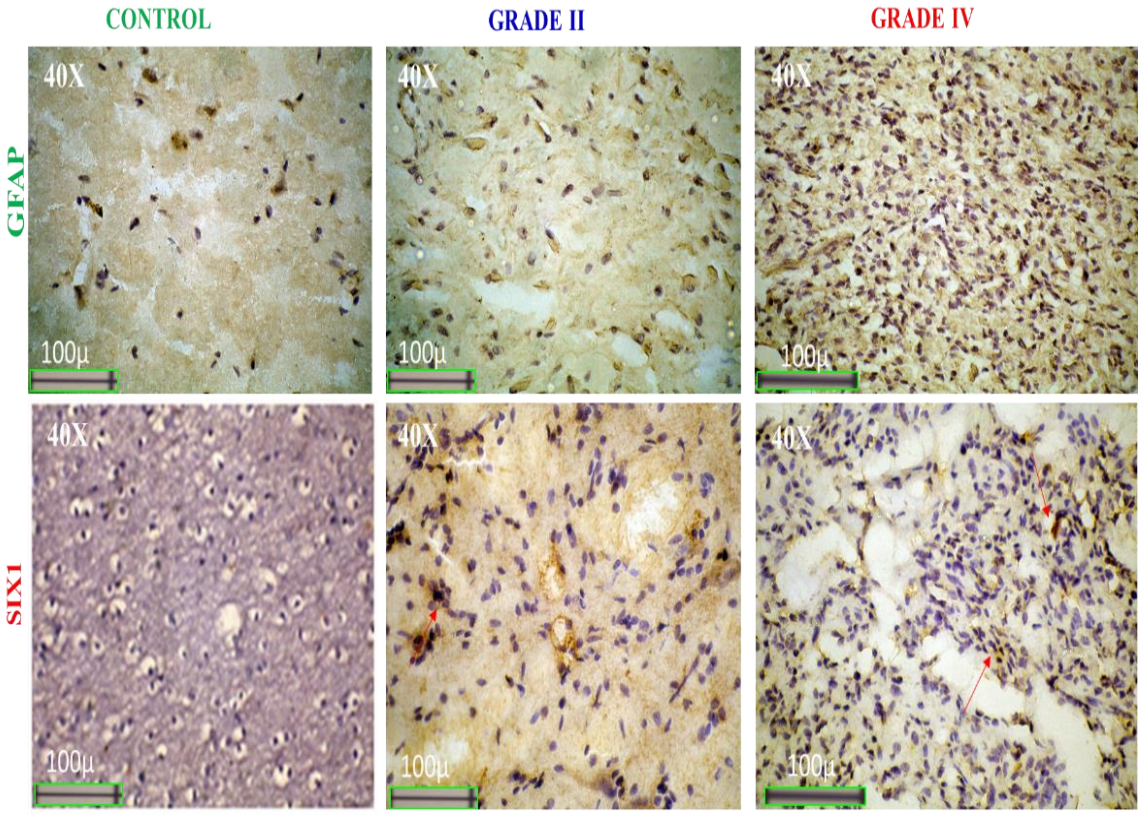


Figure 3.9.2. Very weak or negative immunostaining with Six1

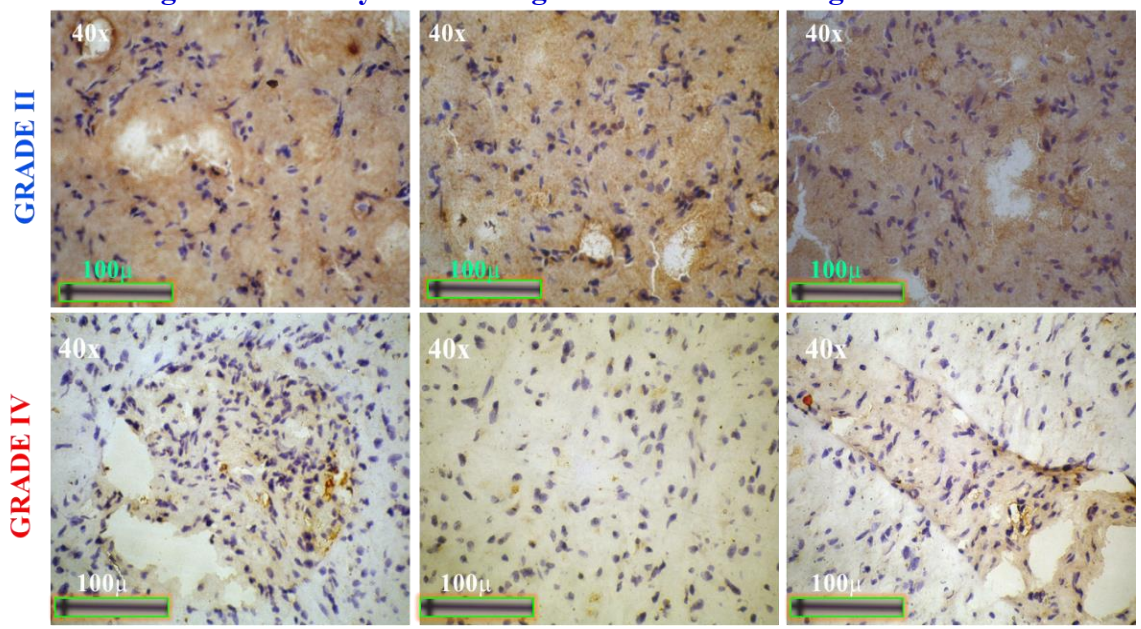


Figure 3.9. Immunohistochemistry staining of human astrocytoma samples of grade II, grade IV and control brain sample with astrocyte marker glial fibrillary acidic protein (GFAP) and Six1. **Figure 3.9.1.)** GFAP positive staining observed in control, grade II and grade IV samples. Six1 negative staining observed in control samples whereas in grade II and grade IV positive staining observed. **Figure 3.9.2.)** Shows very weak staining or no staining in grade II and grade IV astrocytoma samples. Over all Six1 expression in astrocytoma score is "1+". (Score 0 is <10%; 1+ is 10-25%; 2+ is 25-50%; 3+ is 50-75%; 4+ is >75% positive cells).

However, Six1 protein showed very less expression (<20%pasitive cells) in astrocytoma samples. Also, we did not find any significant difference between grade II and grade IV astrocytoma samples. GFAP was used as a marker for astrocytes. We also performed immuno- florescence assay (**Figure 3.9.3.)** on grade IV samples with Six1 and GFAP antibodies. Six1 was observed to localize in the nucleus whereas for GFAP cytosolic staining were observed under confocal microscopy and nucleus was with stained DAPI.

Figure 3.9.3. Six1 and GFAP immunofluorescence staining on grade IV astrocytoma sample.

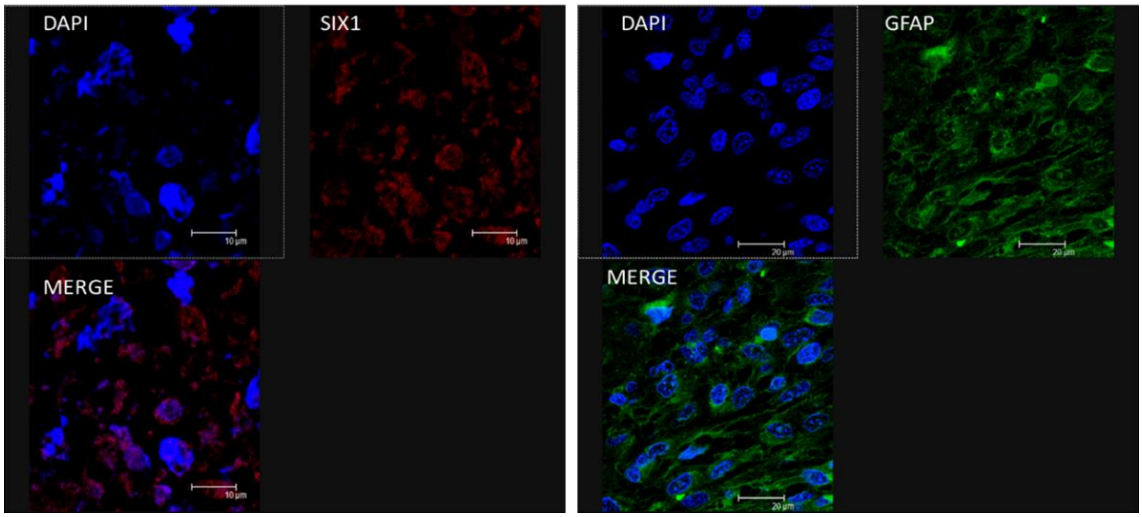


Figure 3.9.3. Immuno-fluorescence with Six1 (TRITC, red), GFAP (FITC, green) and DAPI (blue) on GBM tissue section. Six1 localized in nucleus along with DAPI. GFAP localized on cytosol.

3.4.5. Six1 translation regulator MIR-185 expression in astrocytoma

In astrocytoma samples high expression of Six1 mRNA was observed but protein expression was very less. To identify the underlying probable reason we checked Micro-RNA 185 (MIR-185) levels in the tumor samples. MIR 185 is known to regulate Six1 expression at translational level. In the present study we analyzed the

MIR-185 levels in 12 astrocytoma samples (4 grade II, 3 grade III, and 5 grade IV) and 4 control samples. MIR-185 mRNA was detected in both control and astrocytoma samples. However, among tumor grades; grade II showed high expression than grade III and grade IV. But none of them showed significant differences with respect to control. MicroRNA U6 was used as an internal control. **Figure 3.9.4.**

Figure 3.9.4. Six1 translation regulator micro RNA 185 expression

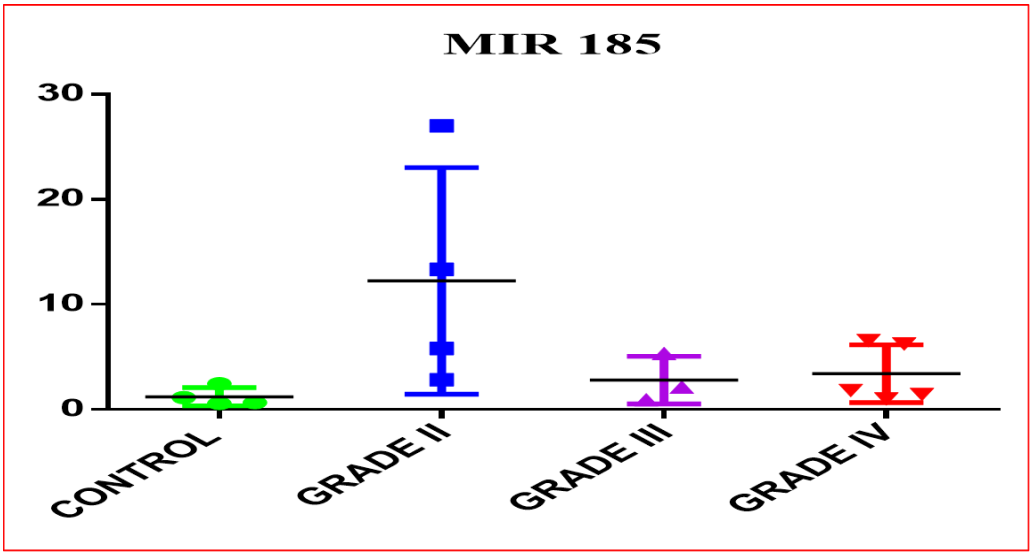


Figure 3.9.4. Six1 translational regulator MIR-185 levels were checked in 12 astrocytoma samples (grade II, 4; grade III, 3; grade IV, 5 ;) and 4 control samples. MIR-185 levels were increased in astrocytoma samples compare to control. Among the tumor samples grade II showed high expression than grade III and grade IV. But all these MIR-185 expression level changes in grades and control has no statistical significance (P<=0.05). MIR U6 used as an internal control.

3.5. ZNF24 over expression in human astrocytoma

ZNF24 had been reported as tumor promotor by inducing β-catenin expression (Liu, Jiang et al. 2012) and tumor repressor by repressing the VEGF and PDGFRβ (Harper, Yan et al. 2007; Li, Chen et al. 2010). To know the status of ZNF 24 in human astrocytoma we performed real time PCR analysis, western blot analysis and Immunohistochemistry. Real time PCR analysis of ZNF24 showed increased

expression in astrocytoma samples when compared to control and these increase in among the tumor grades is not as tumor grade progression, among the tumor grades, grade II showed high expression than grade III and grade IV, whereas grade IV showed high expression than grade III, even though we could not find any statistical significance difference among the grades (**Figure 3.10.1.**). Western blot analysis showed over expression in astrocytoma samples compare to control where no ZNF24 levels were detected. Among the tumor grades, more grade II and grade III samples showed ZNF24 expression (6 samples out of 13) compare to grade IV (2 out of 16 GBM samples) **Figure 3.10.2.** Further we check ZNF24 protein in astrocytoma sample by Immuno-staining with ZNF 24 antibody. These results showed ZNF24 overexpression in astrocytoma sample than control sample. We couldn't find much difference between the grades II and grade IV, The overall staining in astrocytoma is weak **Figure 3.20.3.**

Figure 3.10.1. Real time PCR analysis of ZNF24 mRNA expression in human astrocytoma samples

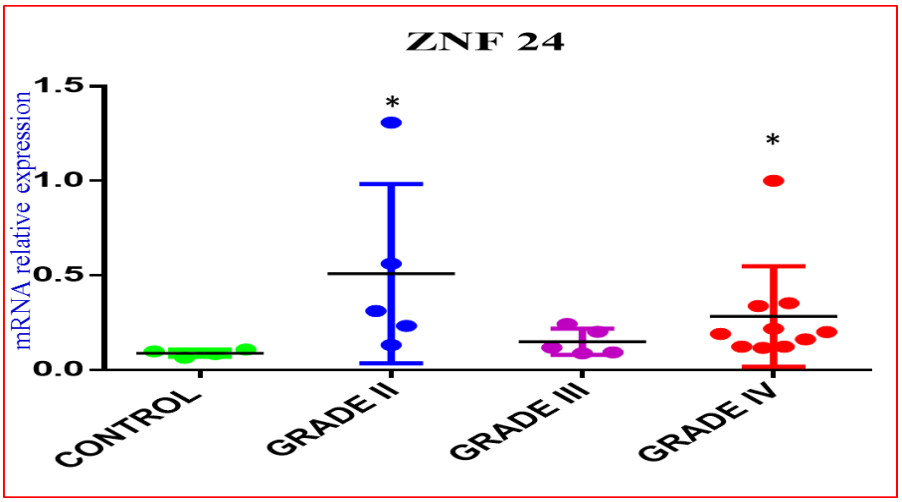


Figure 3.10.1. ZNF24 mRNA expression analysis performed with real time PCR. 20 astrocytoma samples (grade II, 5; grade III, 5; grade IV, 10) and 4 control samples were used. Grade II and grade IV samples showing significant increase with control, there is no significant difference among the tumor grades. (Significant P value <0.05).

Figure 3.10.2 Western blot analysis of ZNF24 protein in human astrocytoma samples

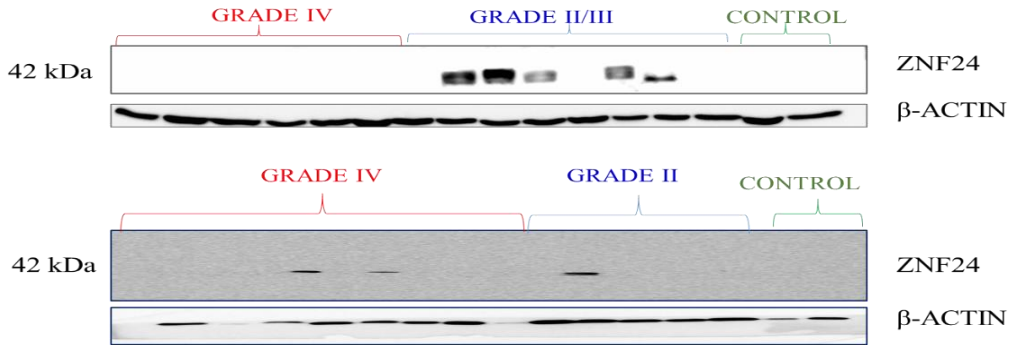


Figure 3.10.2. Western blot performed with ZNF24 antibody on 33 human brain samples (29 astrocytoma and 4 control brain samples were used). In control samples no expression was observed whereas out of 29 tumor samples (16 GBM and 13were grade III/II), 8 samples (2 GBM and 6 grade III/II) expressed ZNF24 protein.

Figure 3.10.3. Immunohistochemical staining with GFAP and ZNF24

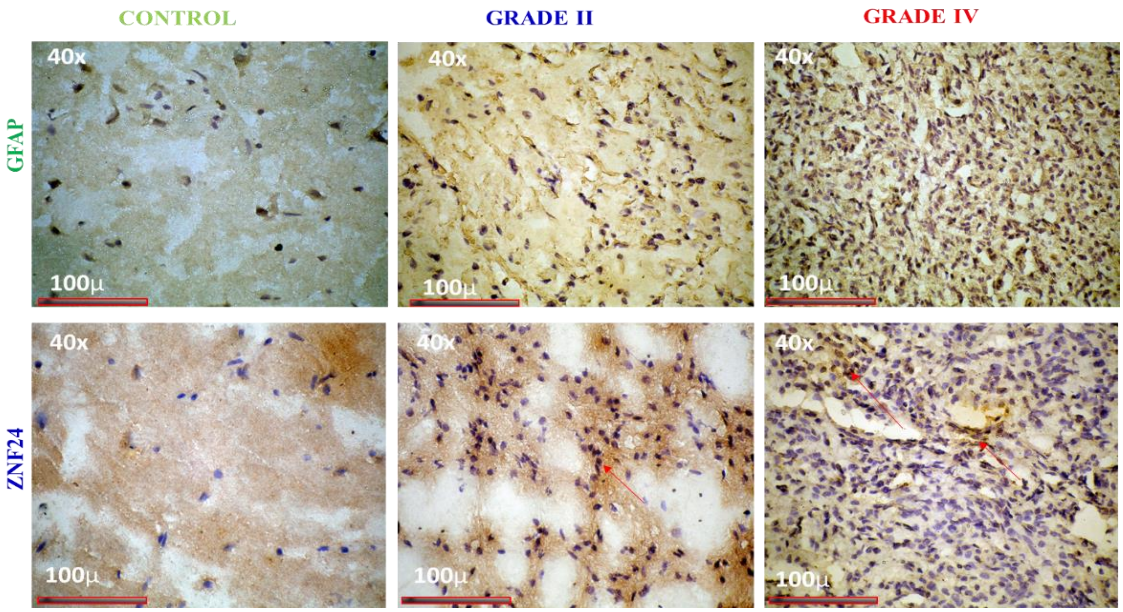


Figure 3.10.3. Immunohistochemistry staining with GFAP (glial fibrillary acidic protein, astrocytes marker) and ZNF24 on control, grade II and grade IV sections. ZNF24 nuclear positive staining observed in grade II and grade IV, control sections show negative staining. The overall staining of ZNF24 score is "1+" (where Score 0 is <10%; 1+ is 10-25%; 2+ is 25-50%; 3+ is 50-75%; 4+ is >75% positive cells).

3.6. DISCUSSION

Treatment of infiltrative astrocytomas is quite challenging, among them GBM is most invasive and lethal with a poor prognosis and less than 15 months mean survival period despite all treatment options of surgical resection, radiation and chemotherapy (Stupp, Mason et al. 2005; Furnari, Fenton et al. 2007). Till now molecular mechanisms of astrocytoma occurrence, progression and therapy resistance are not clear. Since a decade our lab had been working on human astrocytoma progression, we earlier reported, on the activation of PARP cleavage (Bhaskara, Challa et al. 2009), ERK1/2 (Bhaskara, Panigrahi et al. 2005) and Wnt/beta-catenin/tcf (Sareddy, Panigrahi et al. 2009) signaling pathways in human astrocytoma progression.

A homeodomain protein called Six1 has been reported in many cancers including breast (Ford, Kabingu et al. 1998), ovarian cancer (Behbakht, Qamar et al. 2007), rhabdomyosarcoma (Yu, Khan et al. 2004), Wilms' tumors (Li, Guo et al. 2002), hepatocellular carcinoma (Ng, Man et al. 2006), cervical carcinoma (Wan, Miao et al. 2008), colorectal cancer (Ono, Imoto et al. 2012) and pancreatic cancers (Li, Tian et al. 2013). To investigate the status of Six1 deregulation in human astrocytoma, we performed real time PCR analysis for Six1 mRNA levels. Our results showed Six1 mRNA levels were increased in astrocytoma samples compared to control sample and Six1 levels were increasing as the grade progressed from grade II to grade IV. These results support that Six1 may be involved in astrocytoma progression like in other cancers. Six1 mRNA over expression has been reported in glioma derived glial progenitor cells compared to normal glial progenitor cells (Auvergne, Sim et al. 2013). Six1 over expression is known to be involved in expansion of stem/ progenitor cells and induces the epithelial to mesenchymal transition (McCoy, Iwanaga et al. 2009).

Immunohistochemistry results showed Six1 positive staining in astrocytoma samples and negative staining in control samples. However, in tumor sections lower number of cells were stained with Six1 antibody. IHC and immunofluorescence results confirmed Six1 protein nuclear localization. These results hint towards a possible role of Six1 in astrocytoma progression. However, not all the cells expressed Six1 protein. This differential expression of Six1 protein in tumor sections may be because of higher expression in glial progenitor/stem cells as reported previously (Auvergne, Sim et al. 2013), however this needs further investigation. Six1 protein expression is translationally repressed by micro RNA-185, which binds to 3' untranslated region of Six1 mRNA (Imam, Buddavarapu et al. 2010). To identify possible reason for low expression of Six1 protein in astrocytoma samples, we analyzed the MIR-185 levels in astrocytoma and control. MIR-185 levels were detected in control and astrocytoma sample. Presence of this MIR-185 may be possible reason for low expression of Six1 protein.

TRAIL pathway is involved in the elimination of virus infected cells and cancer cell without affecting normal cells (Smyth, Takeda et al. 2003; Hayakawa, Screpanti et al. 2004; Janssen, Droin et al. 2005). This pathway is one of the promising therapeutic options in cancer. However, it has been reported that glioma cells are resistant to TRAIL therapy (Panner, Crane et al. 2009; Kuijlen, Bremer et al. 2010) . TRAIL resistance mechanism in gliomas is not understood completely. However in other cancer cells Six1 over expression is reported to be involved in the resistance of TRAIL mediated apoptosis (Behbakht, Qamar et al. 2007). Here in our study we analyzed the status of TRAIL pathway genes which include TRAIL ligand, death receptor (DR4, DR5) and decoy receptors (DcR1, DcR2) in astrocytoma and control samples. Our

results showed that mRNA levels of all the above mentioned genes were up regulated in astrocytoma samples compared to control. This is in consistence with previous reports in glioma (Frank, Kohler et al. 1999; Kuijlen, Mooij et al. 2006). In this study we performed the correlation analysis of Six1 and TRAIL pathway genes at mRNA expression. Grade wise comparison showed Six1 mRNA levels positively correlated with TRAIL ligand and decoy receptors (DcR1& DcR2). Increased decoy receptors expression leads to activation of survival pathways by competing for TRAIL ligand with death receptors (Shirley, Morizot et al. 2011). From this analysis it shows that increased expression of decoy receptors in astrocytoma may play a role in TRAIL mediated apoptosis resistant. To know the mechanism of Six1 and TRAIL mediated drug resistance further research is needed.

ZNF24 belongs to family of SCAN domine containing Kruppel-like zinc finger transcription factors (Sander, Marsh et al. 1976). ZNF24 is involved in early embryo development (Li, Chen et al. 2006). It is a pleotropic factor which is involved in development of brain, hematopoiesis and cancers (Li, Chen et al. 2009). It has been reported that ZNF24 is involved in negative regulation of VEGF and PDGFRβ by their repression (Harper, Yan et al. 2007; Li, Chen et al. 2010). Direct binding of ZNF24 to the CTNNB1 promoter of β catenin induces its expression through which it promotes cell proliferation in hepatocellular carcinoma (Liu, Jiang et al. 2012). In all these reports, ZNF24 shows contrasting functions one in promoting tumor progression and another is tumor suppression by repressing the VEGF and PDGFRβ expression. Present study is to investigate the role of ZNF24 in human astrocytoma. In human astrocytoma sample ZNF24 mRNA levels were increased in comparison to controls. Immunohistochemistry and western blot analysis showed positive protein expression in

astrocytoma samples. Here we are reporting overexpression of ZNF24 in human astrocytoma samples for the first time. This increased expression of ZNF24 in tumor samples may be involved in glioma tumor progression as reported in hepatocellular cancer (Liu, Jiang et al. 2012). Further studies are needed to identify the role of ZNF24 in astrocytoma.

CONCLUSION: From this part of study it is clear that Six1 is expressed in human astrocytoma and it may be involved in astrocytoma progression. TRAIL pathway genes were up regulated in tumor samples and increased expression of decoy receptor may be involved in tumor progression by competing with the death receptors thereby causing TRAIL resistance. In this study it is established on the role of ZNF24 over expression in human astrocytoma progression, however further studies are needed to elucidate exact mechanism.

CHAPTER 4

In vitro and *in vivo* drug targeting glioma.

4.1. INTRODUCTION

The standard treatment regimens for glioblastomas include maximal surgical resection followed by radiotherapy and chemotherapy. Despite decades of research highly invasive and heterogeneous nature makes glioblastomas difficult to treat. Surgical resection may not be possible in some cases because of its diffused infiltration, due to which tumor cannot be removed completely. Further tumors located in basal ganglia, cortex or brain stem are not accessible to surgical procedure. These patients typically show poor prognosis and in such cases chemotherapy is the better option. However in gliomas chemotherapy approaches have many limitations. The main reason is the blood brain barrier which restricts the entry of most of the anti-cancer drugs into the brain. Only some anti-cancer drugs have been reported which cross blood brain barrier and reach tumor cells, examples include temozolomide, procarbazine, lomustine (CCNU) and vincristine. These drugs are used in treating brain tumors; among these only temozolomide is the most promising agent with fewer side effects than others. But these tumors have poor prognosis and continue to grow even after treatment with temozolomide. Along with blood brain barrier, presence of active efflux pumps and hypoxic tumor environment are other factors which makes glioblastoma more resistant to chemotherapy. Hence more effective therapeutic options are urgently needed. Understanding the mechanism by which a drug induces cytotoxicity may help in developing a conceptual framework and a rational approach to anticancer drug design and therapy.

Our lab is also investigating on the therapeutic options for glioma, where we targeted glioma cell lines *in vitro* with drugs such as nonsteroidal anti-inflammatory drugs celecoxib and diclofenac drug, which were suppress the growth of glioma cells

via different pathways (Sareddy, Geeviman et al. 2012; Sareddy, Kesanakurti et al. 2013) and C6 cells proliferation inhibition by Trizolo aspirins (Anwita Mudiraj 2014) had reported from our lab. Furthermore, we screened a few more compounds naturally occurring and synthesized for their possible anti-cancer effects *in vitro* and *in vivo.*

Natural compounds are an excellent source for the discovery and development of anti-cancer drugs (Li, Backesjo et al. 2009; Choi, Lim et al. 2011), with a promising future in the treatment options. Several heteroannelated quinone and triazole derivatives either of natural or synthetic origin are known to act as anticancer compounds. Quinones are the second largest class of naturally occurring anti-cancer compounds (Sanchez-Cruz and Alegria 2009). Plumbagin is a naphthoquinone isolated from medicinal plant roots of *Plumbaginaceae, Ebenaceae* and *Droseraceae*. Plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone (**Figure 4.11.**) is an analog of vitamin K3. Plumbagin exhibits potent biological activities. Son et al reported that plumbagin is neuroprotective against cerebral ischemia, plumbagin can also radio-sensitizes cancer cells (Nair, Nair et al. 2008) and exhibits anti-cancer activity against many cancers such as growth of human breast cancers (Sugie, Okamoto et al. 1998), non-small lung cancer (Hsu, Cho et al. 2006), pancreatic and PCa (Gomathinayagam, Sowmyalakshmi et al. 2008; Hafeez, Zhong et al. 2012), squamous cell carcinomas (Ravindra, Selvi et al. 2009), ovarian cancer (Sinha, Pal et al. 2013) and intestinal tumors (Courboulin, Barrier et al. 2012) *in vitro* and *in vivo*. Plumbagin effectively induces cell death in cancer cells and causes cell cycle arrest. In a number of human cancer cell lines, its inhibitory effects have been shown to be mediated through different signaling pathways which are mainly involved in cell survival, proliferation, invasion, and metastasis. These signaling pathways include Nuclear factor-kappa B (NF-κB) pathway (Sandur, Ichikawa et al.

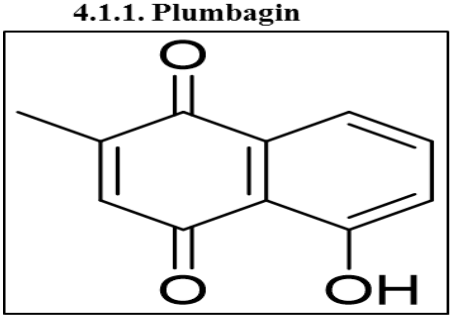
2006), PI3KAKT/mTOR pathway (Kuo, Hsu et al. 2006) and Stat3 pathway (Sandur, Pandey et al. 2010). Till now there are not many reports regarding effects of plumbagin on glioma except recent report limited to cytotoxicity on glioma cell lines (Liu, Cai et al. 2014). Therefore, in the present study we studied the cell growth inhibition activity of plumbagin on C6 rat glioma cell line *in vitro* and further studied its effects on rat glioma progression in sterotaxically induced C6 rat glioma model *in vivo.*

Triazoles, an important class of five membered nitrogen heterocycles, have received a great deal of attention for the past few decades due to their diverse biological activities. Literature is rich with reports of triazoles condensed to some Non-steroidal anti-inflammatory drug (NSAIDs) and other heterocycles and investigation of their biological activity which has emerged as part of a new class of cancer chemotherapeutic and chemo-preventive agents (Wang, Chiang et al. 2002; Serkov and Bezuglov 2009; Ramana and Reddy 2012; Anwita Mudiraj 2014). The triazoles readily associate with biological targets through hydrogen bonding and dipole interactions. Keeping in view the vital role played by these scaffold we have screened some triazoles *in vitro* to check their cytotoxicity on C6 glioma cell lines.

In the present study we have screened and investigated cytotoxicity/ anti-proliferative ability of one natural compound plumbagin and synthesized compound triazole, which has proven anticancer property and further studied the underlying mechanism of these drug (s) targeting glioma *in vitro* and *in vivo.*

Figure 4.1. Structures of Plumbagin and CMIC benzyl

4.1.1. Plumbagin



4.1.2. CMIC benzyl

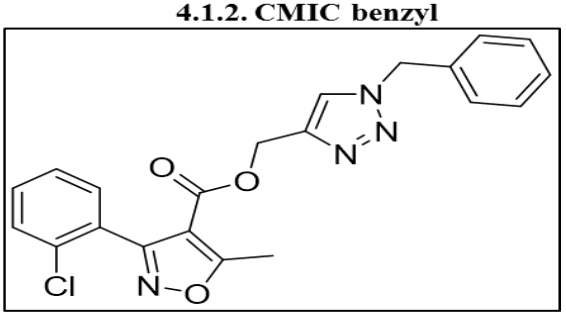


Figure 4.1.1. Plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone (C11H8O3) Molecular weight 188.1794. **Figure 4.1.2.** CMIC benzyle (1-benzyl-H-1,2,3-triazol-4-yl) methyl 3-(2,6-dichloro phenyl)-5-methyl isoxazole-4-carboxylate. Molecular weight: **409** Daltons.

4.2. MATERIALS AND METHODS

4.2.1. Materials:

Plumbagin was purchased from Sigma Aldrich Co. Saint Louis, MO 63103, USA (cat. Log # P7262). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and culture grade Dimethyl Sulphoxide (DMSO) for stock preparation was purchased from Hi-Media laboratories, Mumbai, India. Propidium Iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), (3,-4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT), and Rhodamine 123 were purchased from Sigma Chemical St Louis, MO, USA. Rat stereotaxic apparatus, Micro injector from Stoelting Company, Wood dale IL, USA.

4.2.2. Cell culture:

C6 cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM medium supplemented with 10% FBS, 1% antibiotics i.e., penicillin G and 100 mg/ml streptomycin. Cells were maintained in a

monolayer culture at 37°C with 5% CO_2 in CO_2 incubator. All experiments were carried out with exponentially growing cells.

4.2.3. MTT assay:

The cytotoxicity of drugs (Plumbagin/triazoles) were measured on C6 cell line. Briefly, cells were plated in 96-well culture plates ($1x10^4$ cells/well). After 24h incubation, cells were treated with different concentrations of drugs and control cells were treated with DMSO (0.1%) for 24h. 20µl of MTT solution, was prepared by dissolving 5mg / ml of MTT in PBS and was added to each well and incubated for 4 h. Later 100µl DMSO was added to each well and kept on rocker for 5 min. Following this, color intensity measured at 570 nm in micro plate reader TECAN infinite 200 PRO (Tecan trading AG, Switzerland).

4.2.4. Clonogenic assays:

As described earlier (Sareddy, Geeviman et al. 2012), protocol briefly, clonogenic assay was performed by plating C6 cells (500/ well) in a 6 well plate. After 12 h, adherent cells were treated with different concentrations of drugs or DMSO (0.1%) and incubated for 24 h. Then drug containing media was replaced with fresh media and cells were allowed to grow for 7 days in incubator. Later media was removed and cells were washed with PBS, fixed with 4% paraformaldehyde and stained with 0.5% methylene blue in 50% methanol. Colonies containing more than 50 cells were counted.

4.2.5. Cell Cycle Analysis:

To analyze cell cycle distribution, $5x10^5$ cells were plated in 60-mm dishes and treated with different concentrations of drugs for 24 h. Control cells were treated with vehicle alone (0.1% DMSO). After treatment, cells were collected by trypsinization,

fixed in 70% ethanol and washed with ice cold PBS, resuspended in 1 ml of PBS containing 0.5 µg/ml propidium iodide (PI), 10 µg/ml RNAase A, 0.1% sodium citrate and 0.1% Triton X-100) for 30 min at room temperature. PI stained cells were then analysed by flow cytometry, BD FACS, Caliber (Becton, Dickinson and Co, San Jose, CA, USA).

4.2.6. Estimation of reactive oxygen species (ROS):

To assess the generation of ROS, C6 cells ($1x10^6$) were seeded and treated with different concentrations of drugs for 24 h and control cells were treated with vehicle (0.1%). Cells were collected and incubated with H_2DCFDA (Life technologies, Thermo Fisher scientific Inc.) 10µM, in PBS for 30 min. Cells were washed twice with PBS and ROS levels were analyzed by flow cytometry using a FACS Aria II flow cytometer (Becton, Dickinson and Co, San Jose, CA, USA).

4.2.7. Mitochondrial membrane potential (Δψm):

Cover slips were placed in 24 well plate and 5×10^4 cells/well were seeded. After the cells adhered, they were treated with varying concentrations of drugs for 24 h. Rhodamine 123 (10µg/ml) was added to culture media and incubated for 30min in CO_2 incubator. Media was discarded and cells were washed with PBS twice and treated with DAPI for 5 min followed by PBS wash. Coverslips were mounted with mounting media on microscopic slide and florescence was observed under Zeiss laser scanning microscope.

4.2.8. *In situ* terminal-deoxytransferase mediated dUTP nick end labeling (TUNEL) assay:

An *in situ* apoptosis detection kit was used to detect DNA fragmentation in accordance with the manufacturer's procedure. Briefly, C6 cells grown on chamber slides were treated with drugs for 24 h with different concentration and control cells were treated with 0.1% DMSO. The cells were then fixed with 4% paraformaldehyde for 10 min, and incubated for 60 min with a reaction mixture containing biotin–dUTP and terminal deoxynucleotidyltransferase. Fluorescein-conjugated avidin was applied to the samples, which were then incubated in the dark for 30 min. positively stained fluorescein-labeled cells were visualized and photographed using a fluorescence microscope.

4.2.9. Western blot analysis:

As reported in earlier from our lab briefly, C6 cells were treated for 24 h with different concentrations of drugs and vehicle (0.1% DMSO). Cell lysates were prepared by suspending the cell pellets in lysis buffer (Cell Signaling Technology, Inc.). Protein concentration was estimated with Bradford reagent. 30µg protein from the samples was resolved on 12.5% and 10% SDS polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose membranes, and the membranes were blocked with 5% non-fat dry milk solution for 1 h at room temperature and incubated overnight in the primary antibodies at 4°C. Membranes were then incubated with the respective secondary antibodies for 1 h at room temperature and immune-reactivity was detected by using an ECL kit (GE Health Care, CA) in Kodak molecular imaging station (The Eastman Kodak Co.) or ALP method with BCIP/NBT as a substrates.

4.2.10. Animal model:

Male Wistar rats weighing 200 to 250g were purchased from Mahaveer agencies and Gentox Bio-services Pvt limited, Hyderabad. Animals were maintained in animal

house for acclimatization to new environment for one week just before commencement of the experiments. Rats were anesthetized with ketamine and xylazine intraperitoneal injection (ketamine 80mg/kg; xylazine 10mg/kg body weight). After anesthesia, fur on head was trimmed with trimmer and animals were fixed on stereotaxic apparatus with the help of ear bars. Midline scalp incision was made on head and skull was exposed. Bregma point was identified and then according to the rat brain atlas, the coordinates on stereotaxic apparatus guided to striatum (coordinates with regard to bregma; 0.5mm posterior; 3mm lateral; 6mm depth). 1mm hole was drilled on skull with the help of hand drill bit. C6 rat glioma cell lines around 70% confluency were trypsinized, washed once with PBS and re suspended in complete medium with a concentration of $1x10^6$ / 5µl. These one million cells were implanted in 6 mm depth into the striatum with the help of Hamilton syringe and micro injector with a speed of 1µl/1min. After stabilizing the cells in striatum syringe was removed slowly. The craniotomy was filled with dental cement. Animals were removed from the stereotaxic frame and kept back in cage and animals were maintained under aseptic conditions. All experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC), University of Hyderabad.

4.2.11. Drug administration:

After 7 days of surgery animals were randomly selected and grouped into 3 groups, which were control, intraperitoneal (i.p) and intravenous (i.v) plumbagin treated groups. Each group contained 6 animals. Animals were treated with Plumbagin 3mg/kg body weight, dissolved in 20% DMSO in saline and volume of 200µl per rat/dose was injected. Plumbagin administration started on 8^{th} day post-surgery and daily one dose up to 7 days. Animals were sacrificed on 20^{th} day by over dose anesthesia

with pentobarbital perfused intra cordially with normal saline and 4% para formaldehyde with contain monobasic Na_2HPO_4 and dibasic NaH_2PO_4. Animals were decapitated and brains were collected into 4% PFA and fixed overnight at 4^0C. Then the brains were transferred to 30% sucrose solution. After this the density settled brains were sectioned serially at 10µ thickness with cryotome (Lica) and collected on silane (3-aminopropyl triethoxysilane) coated slides and stored for further use. Tumor area was measured with Image J.softwere. H&E and immunohistochemistry protocol as described earlier.

4.3. RESULTS

4.3.1. Plumbagin showed cytotoxic effect on C6 rat glioma cells:

Cytotoxic effect of plumbagin was evaluated on rat glioma C6 cell line by MTT assay. MTT assay provides a quantitative measure of the number of cells with metabolically active mitochondria. It is based on the mitochondrial reduction of tetrazolium bromide salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]). Plumbagin had substantial detrimental effect on C6 cell line which became obvious during morphological observation. As shown in **Figure 4.2.** The number of C6 cells decreased over time when treated with plumbagin in the range of 0.5 to 5µM and showed dose dependent increase in cytotoxicity after 24h treatment. The IC_{50} value of Plumbagin was observed to be 2µM on C6 cell line. Inhibition of cellular proliferation by plumbagin was confirmed further by colony formation assay. As evident from **Figure 4.3.**, cells lost their colony forming capacity in a dose dependent manner on 24 h treatment with plumbagin.

Figure 4.2. Cell cytotoxicity assay (MTT)

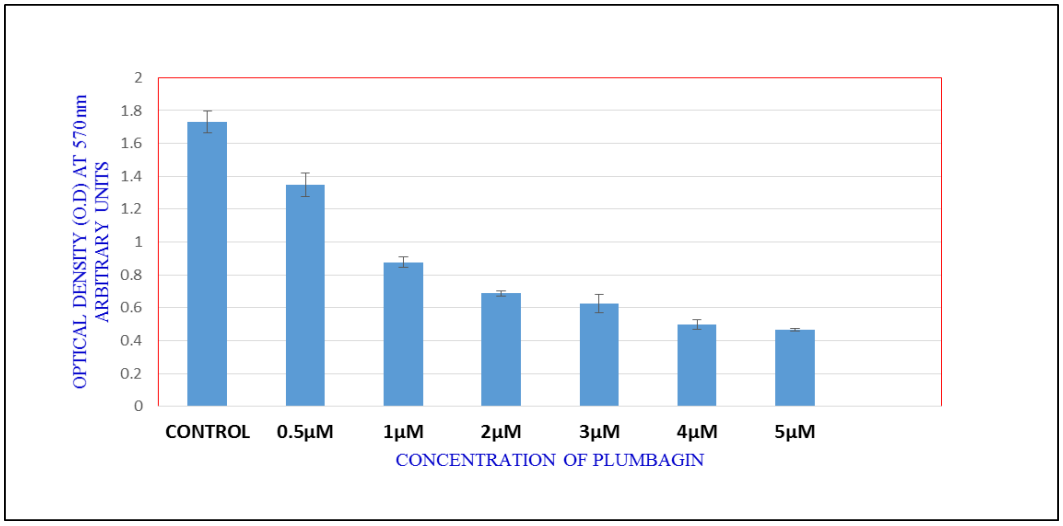


Figure 4.2. C6 cells plated on 96 well plate at 5000 cells /well. Treated for 24h with different concentrations of plumbagin and vehicle (0.1%DMSO). Then incubated with MTT, O.D observed. O.D absorbance directly proportional to cell survival or viability. Cell viability decreased by 50% at 2µM conc. This value was taken as IC_{50} Inhibitory Concentration).

Figure 4.3. Colony formation assay

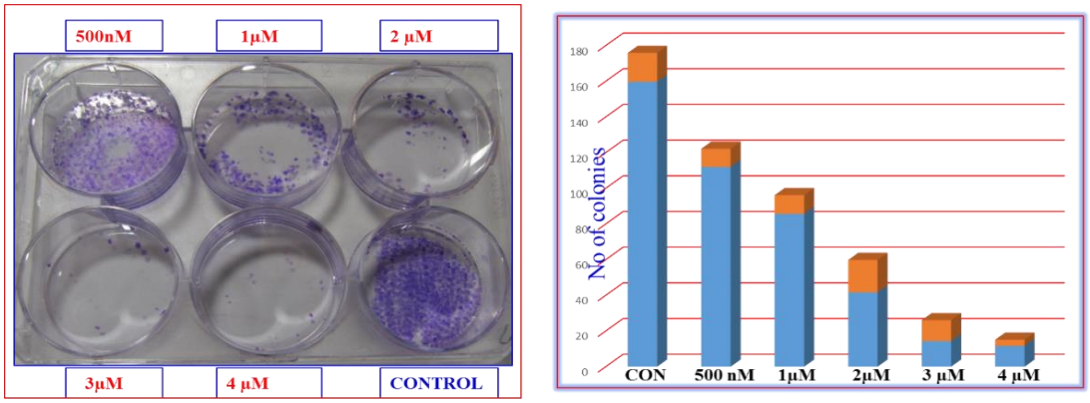


Figure 4.3. Clonogenic assay performed where cells were treated with plumbagin (500 nM, 1µM, 2µM, 3µM, 4µM) and control cells were treated with vehicle (0.1%DMSO). plumbagin concentration above 1µM showed less than 50% colonies compared to vehicle treated cells.

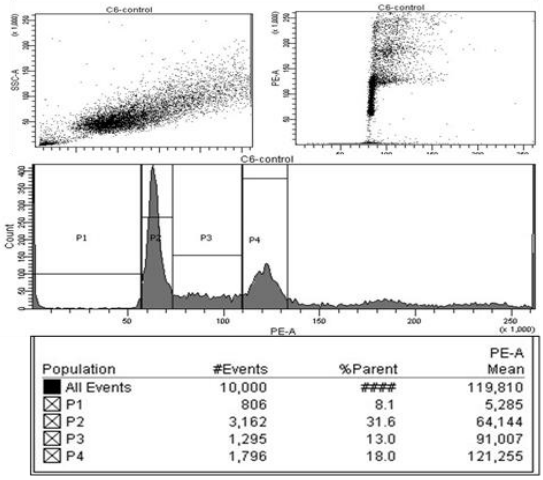
4.3.1.3. Plumbagin induces cell cycle arrest of C6 cells at S & G2 phase:

Cell cycle analysis was performed to determine whether plumbagin effect on cell toxicity or proliferation is due to the alterations in cell cycle. C6 cells were treated with different concentrations of plumbagin. Treatment with 1µM, 2µM and 3µM

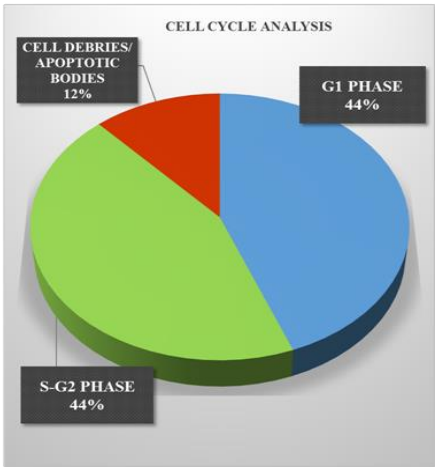
plumbagin for 24h increased accumulation of cells in S&G2 phase of the cell cycle and increased number of apoptotic bodies (**Figure 4.4.**). These results support the data from MTT and colony formation assay which were preliminarily suggesting cytotoxicity or proliferation inhibition activity of plumbagin.

Figure 4.4. Cell cycle analysis

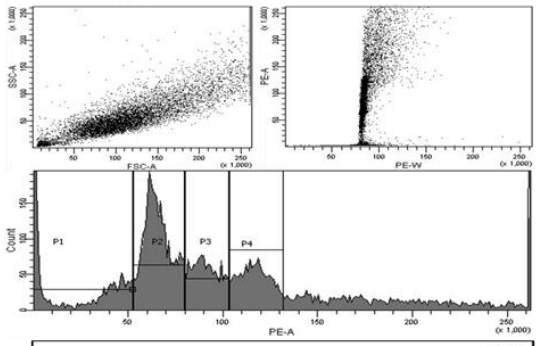
CONTROL



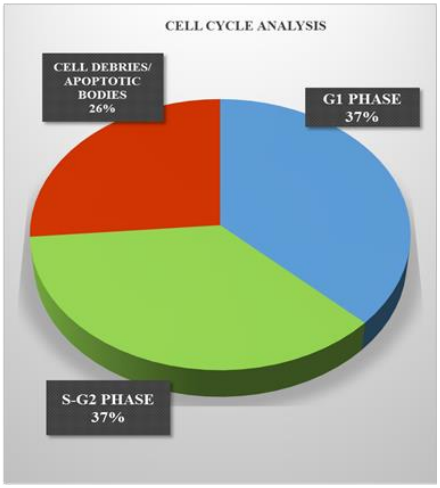
Population	#Events	%Parent	PE-A Mean
All Events	10,000	####	119,810
P1	806	8.1	5,285
P2	3,162	31.6	64,144
P3	1,295	13.0	91,007
P4	1,796	18.0	121,255



1 µM PLUMBAGIN



Population	#Events	%Parent	PE-A Mean
All Events	10,000	####	105,366
P1	1,927	19.3	16,253
P2	2,723	27.2	65,566
P3	1,308	13.1	90,909
P4	1,366	13.7	116,749



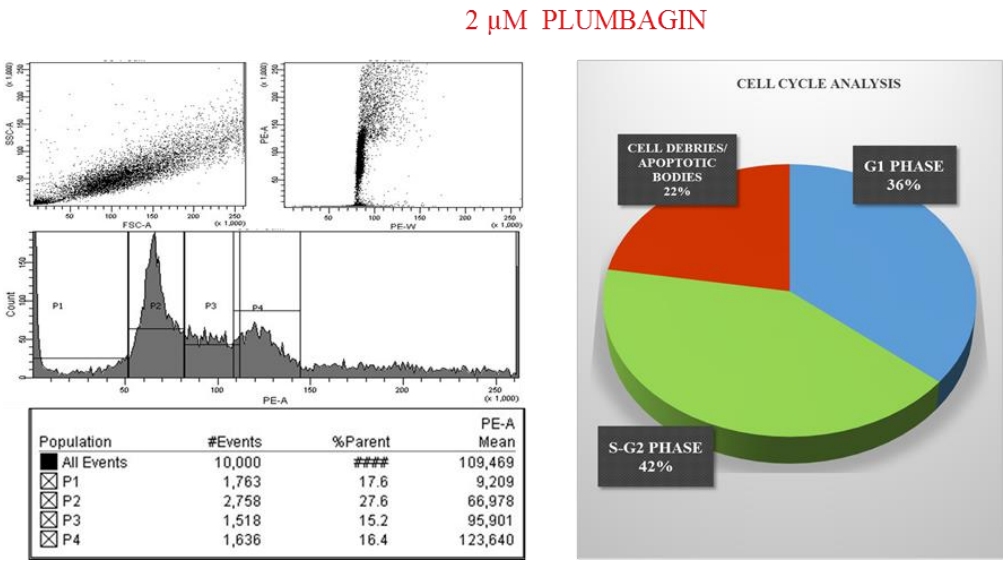


Figure 4.4. Plumbagin induces cell cycle arrest at S&G2 phase. C6 cells were treated with 0.1% DMSO (Control), 1µM and 2 µM concentration of plumbagin for 24h. FACS analysis was performed after treatment followed by PI staining. Data shows that nearly 6% of S&G2phase cells increased upon treatment with 2µM compared to vehicle treated cells. At the same time apoptotic bodies increased to 10% in 2µM plumbagin treated cells compared to vehicle treated cells.

4.3.1.4. Plumbagin affects the mitochondrial membrane potential and induces cell death through mitochondria mediated apoptosis:

To determine whether plumbagin induced cytotoxicity of C6 glioma cell lines is due to the generation of reactive oxygen species, C6 cell lines were treated with plumbagin for 24h. As expected, ROS levels were increased in plumbagin treated cells in concentration dependent manner compare to vehicle treated (**Figure 4.5.**). Increased ROS levels may lead to decreased mitochondrial membrane potential. To ascertain this, Rhodamine staining was done for the assessment of mitochondrial membrane potential. Treatment with 2µM concentration of Plumbagin for 24h showed decrease in the mitochondrial membrane potential compared to control cells as observed with Rhodamine 123 staining in confocal analysis (**Figure 4.6.**). TUNEL assay confirms apoptotic cell death at Plumbagin 2µM concentration. The data from the three experiments namely ROS estimation, Rhodamine staining and TUNEL assay supported

the mitochondrial membrane depletion and cell death indicating plumbagin induces cell death through mitochondria. These results are strongly supported by our western blot analysis. Incubation of cells for 24h with different concentration (1µM, 2µM and 3µM) showed increase in the apoptotic (BAX) and decrease in anti-apoptotic (BCL-2, BC-XL) protein levels which endorse the involvement of mitochondria (**Figure 4.8.1.**). Further, increased Caspase 3 and PARP protein cleavage support the apoptotic cell death pathway (**Figure 4.8.2.**). A decreased expression of p53 was also observed (**Figure 4.8.2.)**.

Figure 4.5. Estimation of ROS in plumbagin treated cells

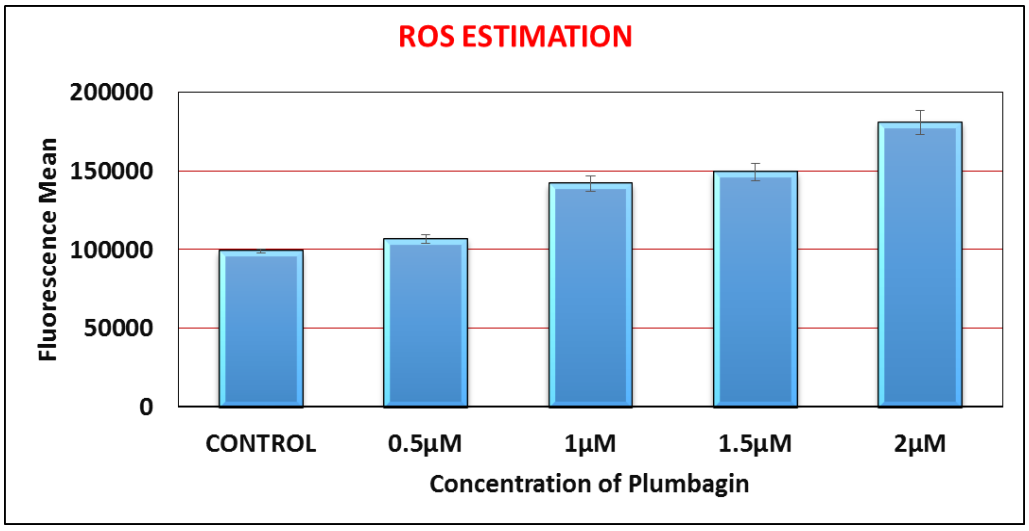


Figure 4.5. C6 cells were treated with plumbagin 0.5µM, 1µM, 1.5µM and 2µM. Control cells were treated 0.1% DMSO. DCFDA staining was used for estimation of ROS. As the concentration of the drug increases, ROS levels are also increased.

Figure 4.6. Mitochondrial membrane potential (Δψm) in plumbagin treated cells

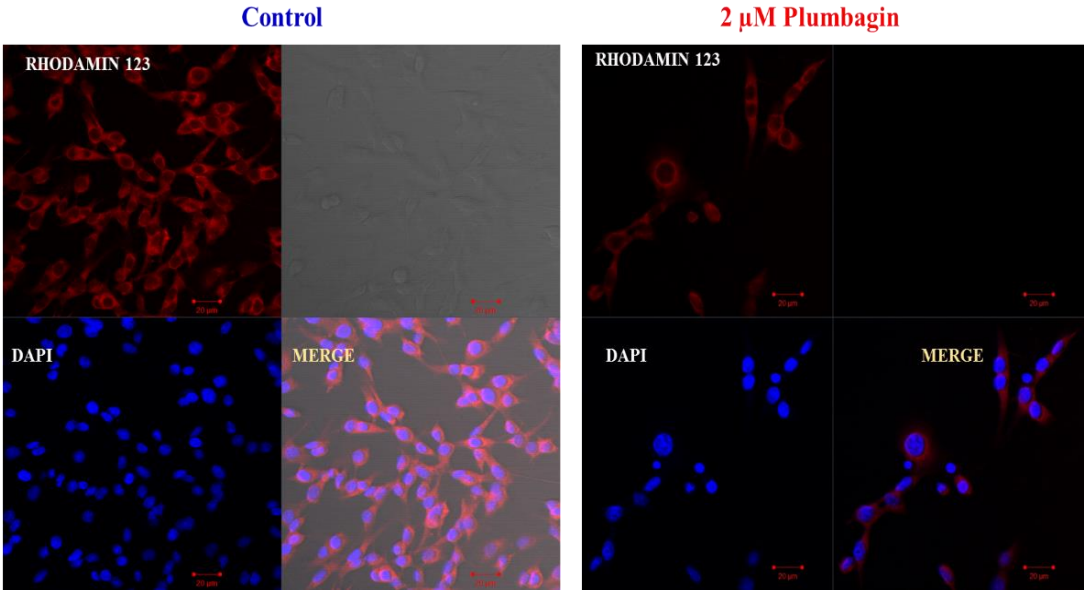


Figure 4.6. Rhodamin 123 was used as mitochondrial marker to check membrane potential. Cells were treated with plumbagin 2µM and vehicle for 24h. Staining with Rhodamin123 shows intact mitochondria in control cells whereas in plumbagin treated cells dispersed mitochondrial staining is observed indicating mitochondrial membrane potential loss.

Figure 4.7. TUNEL assay

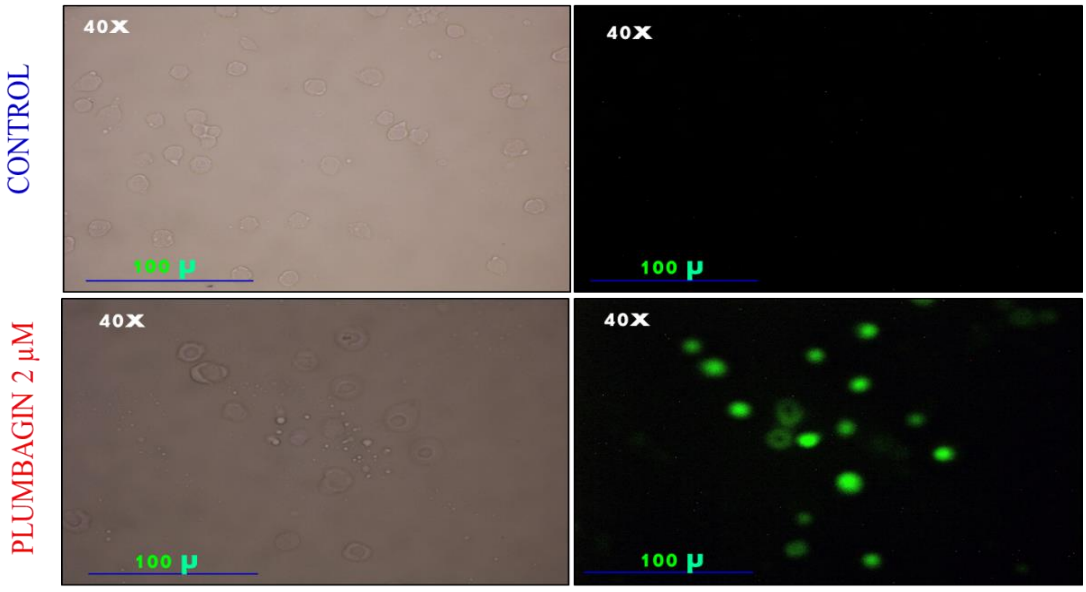


Figure 4.7. TUNEL assay was performed to detect cell death through DNA fragmentation. Cells were treated with Plumbagin (2µM) and vehicle (0.1%DMSO) for 24h. Images are taken with Olympus florescent microscope, bright field and fluorescent field at 40X. In vehicle treated cells no DNA fragmentation was observed, where plumbagin treated cells showed DNA fragmentation.

Figure 4.8. Western blot analysis of plumbagin treated cells

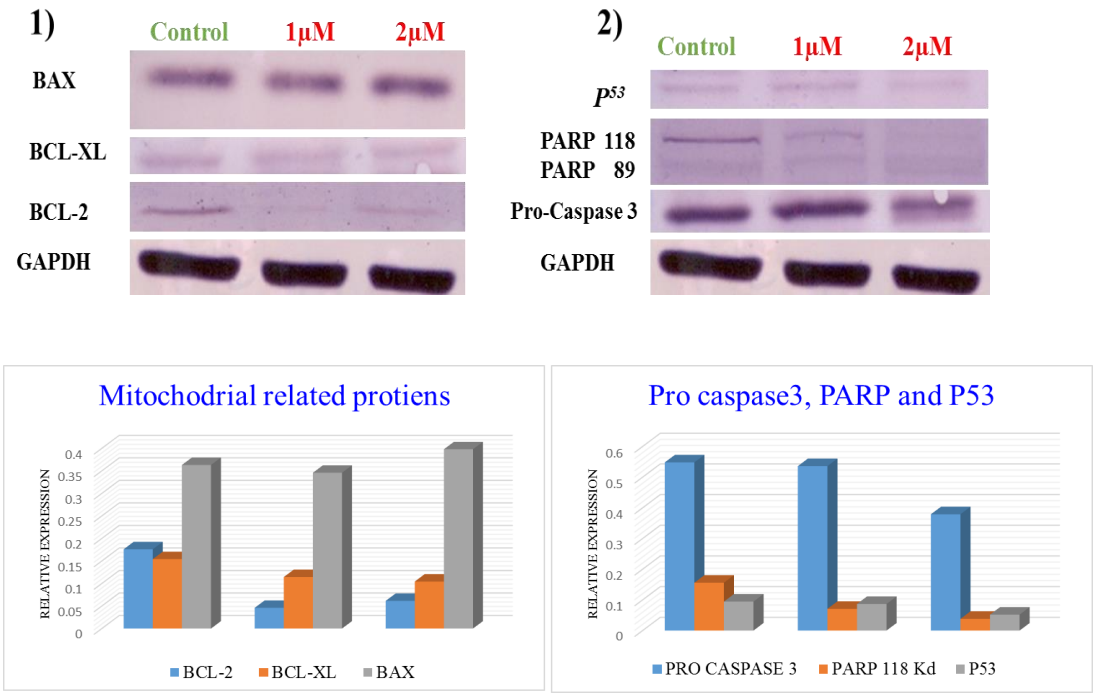


Figure 4.8. Western blot analysis of plumbagin and vehicle treated cells. **1)** Mitochondria mediated apoptotic pathway proteins and anti-apoptotic proteins BCL-2 and BCL-XL were decreased in plumbagin treated C6 cells and apoptotic protein BAX levels were increased. **2)**. Caspase 3 is an executive protein in apoptosis process, decreased Pro-caspase 3 indicates its cleavage resulting in activation. Active Caspase 3 cleaves PARP (118kd) which is observed as bands at 118 and 89kd. Decreased p53 levels are observed in plumbagin treated cells.

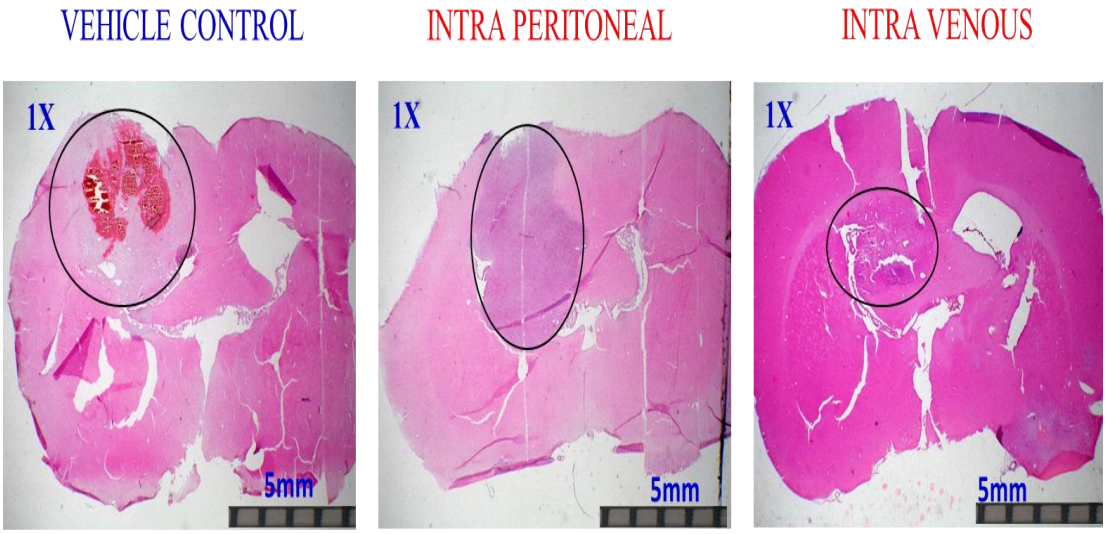
4.3.1.5. Plumbagin suppresses tumor growth in rat stereotaxic glioma model.

To validate the observed plumbagin mediated inhibition of glioma cell proliferation *in vitro*, steriotaxically implanted C6 rat glioma model were used. Brain tumor bearing rats were treated with Plumbagin and vehicle (20% DMSO in saline) for 7 days, there was no observed toxicity except in I.V treated groups where black spots were observed on caudal vein at the location of injection in both Plumbagin and vehicle treated groups. Tumor bearing rat groups treated with Plumbagin (3mg/kg body weight) administered in I.P and I.V routes showed smaller tumor size compared to vehicle (20% DMSO) treated groups, plumbagin treated I.V group showed statistically significant

smaller sized tumors than I.P treated group when compared to control group (**Figure 4.9.1. & 4.9.2.**). Tumor area was measured by image J software on H&E stained tumor bearing rat brains. I.P and I.V treated groups showed development of smaller size blood vessels compared to control groups (**Figure 4.10.**). Above results suggest that plumbagin works as a tumor growth inhibitor. To further confirm this at molecular level, IHC staining with proliferation marker Ki67, glial cell marker GFAP and angiogenic marker VEGF was performed, decreased staining of Ki67 and VEGF were observed in plumbagin treated groups. Among them I.V treated group showed significant decrease in comparison with control group (**Figure 4.11. & 4.13.**). Much difference was not observed in GFAP staining in all the plumbagin and control treated groups (**Figure 4.12.**).

Figure 4.9. H&E staining of plumbagin treated rats

1)



2)

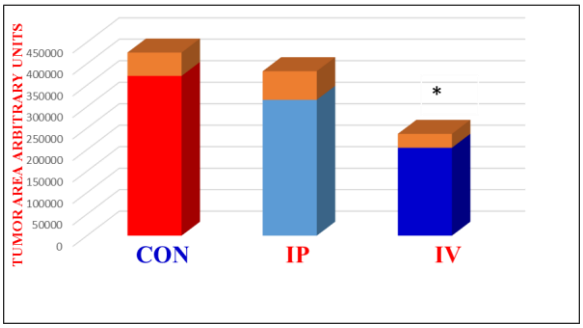


Figure 4.9.1.) Showing representative H&E stained figures of brain tumor bearing rats of 3 groups. Control, intraperitoneal and intravenous. Images are taken with Olympus microscope at 1X, tumor area indicated with circle. **Figure 4.9.2.**) Tumor area was measured with Image J software. All 3 groups; control, intraperitoneal and intravenous (each group contained 6 rats) treated with plumbagin 3mg/kg body weight and vehicle. 2) Tumor area plotted on graph where plumbagin treated animal showing smaller tumor size than vehicle treated animals. Intravenous plumbagin treated animals showed significant small tumor size compared to intraperitoneal and control group.

Figure 4.10. H&E staining of plumbagin treated tumor rat brain sections

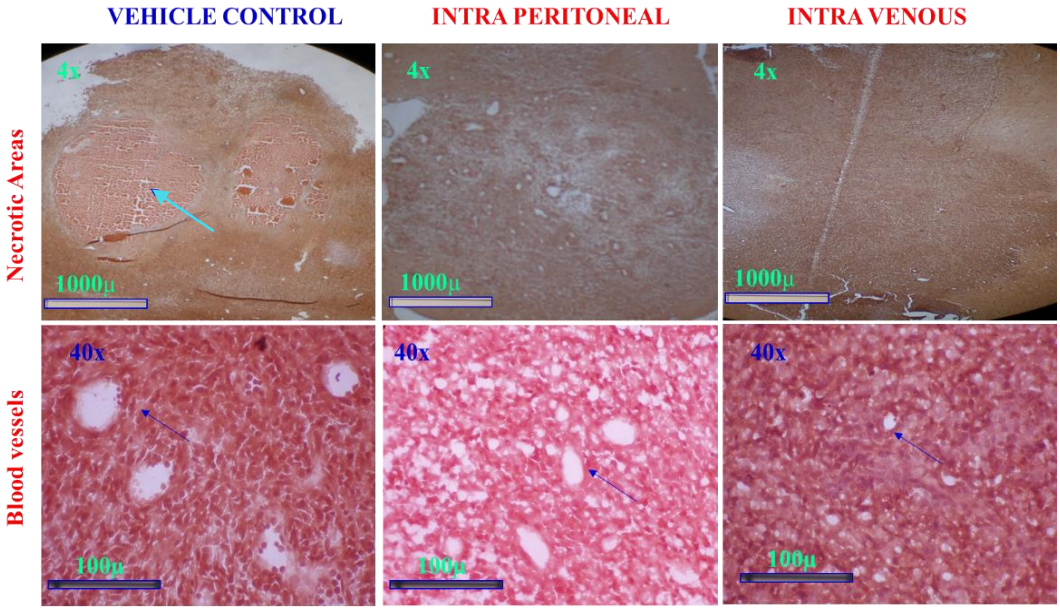


Figure 4.10. H&E sections of tumor bearing rat brains, in 4X view tumor area with necrotic regions were observed in vehicle treated animal, whereas in plumbagin treated IP and IV group didn't show any necrotic areas. Smaller blood vessels were observed in Plumbagin treated tumor rats than vehicle treated tumor rats (40X) indicating smaller size of the tumor in Plumbagin treated groups.

Figure 4.11. Immunohistochemistry with Ki67

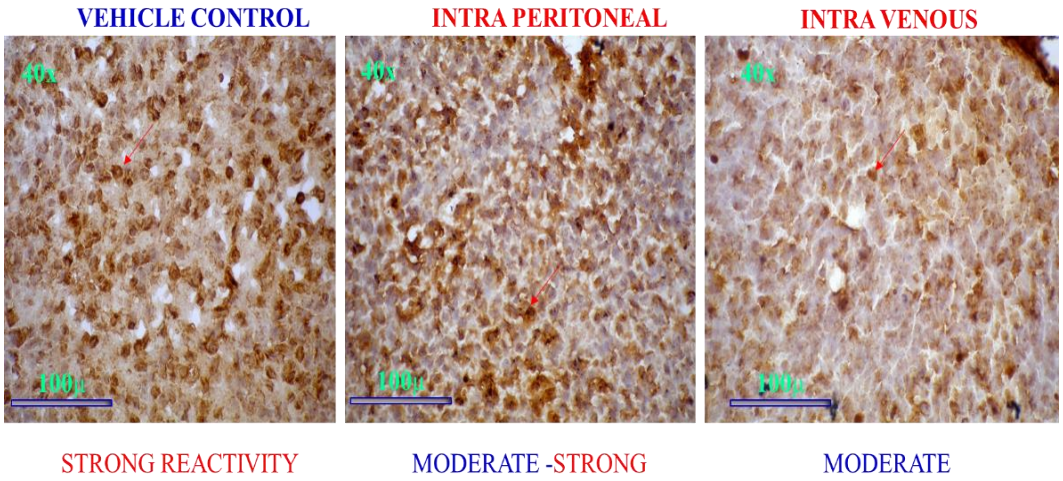


Figure 4.11. Ki67 is marker for proliferating cells. Nuclear positivity was observed. Vehicle treated group showed strong reactivity for Ki67. Intra peritoneal plumbagin treatment showed moderate to strong reactivity whereas Intravenous treatment group showed moderate reactivity. **Strong reactivity >=50% positivity; Moderate 20 to 50%; Weak reactivity < 20%;**

Figure 4.12. Immunohistochemistry with GFAP

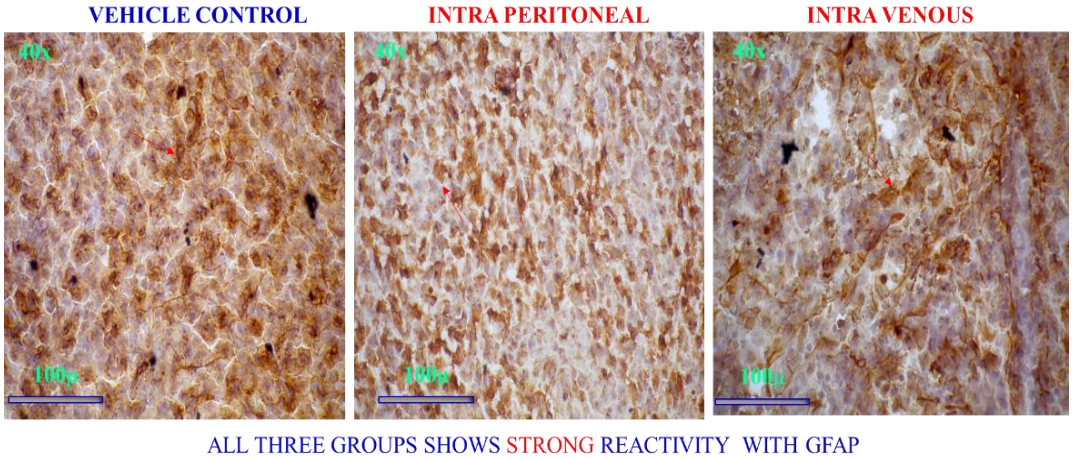
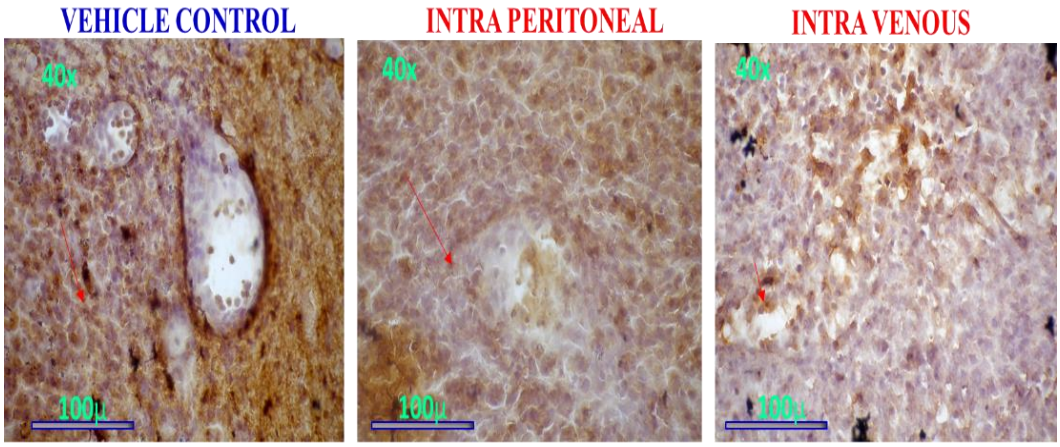


Figure 4.12. Glial fibril acidic protein (GFAP) is a marker of glial cells. Staining with GFAP on rat glioma sections showed cytoplasmic staining. All three groups stained more than 50% positively. No significant difference was observed among the 3 groups.

Figure 4.13. Immunohistochemistry with VEGF

1)



2)

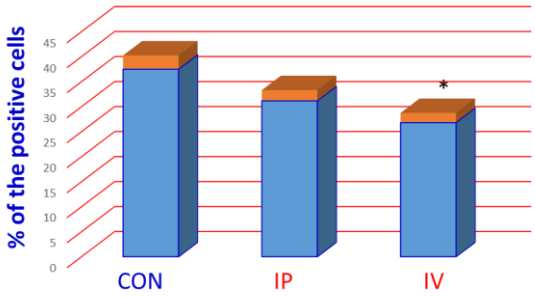


Figure 4.13. VEGF is maker for angiogenesis. **1)** All three groups (Control, IP and IV treated) stained moderately. **2)** Among these 3 groups intravenous treated group showed significantly decreased staining.

4.3.2. CMIC benzyl

4.3.2.1. Synthesized triazoles abbreviated as CMIC benzyl, DMIC benzyl and FCMIC benzyl showed cytotoxic effect on C6 rat glioma cell line

Many heteroannelated triazoles were screened for anticancer activity *in vitro* on C6 rat glioma cell line, among them 3 novel compounds namely 1-benzyl-H-1, 2, 3-triazol-4-yl) methyl 3-(2, 6-dichloro phenyl)-5-methyl isoxazole-4-carboxylate and 3-(2, 6-dichlorophenyl)-5-methyl isoxazole-4 carboxylate and 1-(2 -morpholino-2-oxoethyl-1H-1, 2, 3-triazol-4-yl) methyl 3-(2-chloro-6-fluorophenyl)-5-methyl isoxazole-4-carboxylate abbreviated as DMIC,CMIC benzyl and FCMIC respectively showed significant cytotoxicity at 100 µM -150µM concentration as confirmed by MTT assays (**Figure 4.14.**). The synthesis of these triazoles is reported. (Ramana and Reddy 2012). For furthur studies CMIC benzyl was taken up.

4.3.2.2. CMIC benzyl induces cell cycle arrest and promotes cell death through mitochondria mediated apoptosis in C6 glioma cell line:

Cells treated with CMIC benzyl were unable to form colonies compared to control. Increase in the drug concentration caused the cell to lose their colony forming ability (**Figure 4.15.**). Cell cycle analysis was performed to determine whether CMIC benzyl effect on cell toxicity is due to alterations in cell cycle. C6 cells were treated with CMIC benzyl for 24h with different concentrations. Treatment with 50µM, 100µM and 150µM concentrations showed increased accumulation of cells in G2/M phase and increased number of apoptotic bodies was also observed. These cell cycle analysis results support the MTT assay and colony formation assay there by suggesting that CMIC benzyl has cytotoxic effect on C6 cell line (**Figure 4.16.**). Treatment of C6 cell line with 150µM CMIC Benzyl for 24h showed decrease in the mitochondrial

membrane potential (Δψm) compared to DMSO treated control cells, which is also evident from Rhodamine 123 staining and confocal analysis (**Figure 4.17**.). TUNEL assay showed evidence of cell death at 150µM concentration (**Figure 4.18.**). Both Rhodamine staining and TUNEL assay support the mitochondrial membrane potential depletion and increased cell death indicating CMIC benzyl induces cell death through mitochondria. These results are strongly supported by our western blot analysis. Incubation with different concentration (50µM, 100µM and 150µM) of CMIC Benzyl showed increase in the apoptotic (BAX) decrease in anti-apoptotic (BCL-2) protein levels which endorse the involvement of mitochondria. Decrease in the Pro-Caspase 3 supports the probable involvement of apoptotic cell death pathway

Figure 4.14. Cytotoxicity assays of triazoles

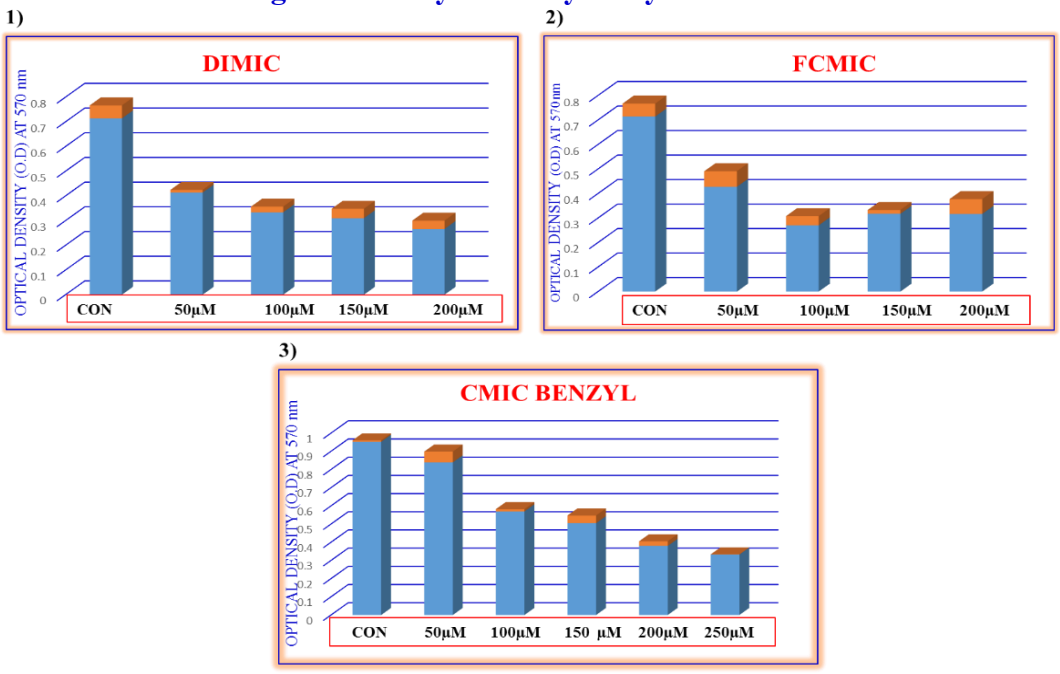


Figure 4.14. Triazoles **4.14.1.)** 3-(2, 6-dichlorophenyl)-5-methyl isoxazole-4 carboxylate (DIMIC), **Figure 4.14.2.)** (1-(2 -morpholino-2-oxoethyl-1H-1, 2, 3-triazol-4-yl) methyl 3-(2-chloro-6-fluorophenyl)-5-methyl isoxazole-4-carboxylate (FCMIC) are showing IC_{50} at 100µM concentration. **Figure 4.14.3)** (1-benzyl-H-1, 2, 3-triazol-4-yl) methyl 3-(2, 6-dichloro phenyl)-5-methyl isoxazole-4-carboxylate (CMIC benzyl) showing IC_{50} at 150µM concentration.

Figure 4.15. Clonogenic assay for CMIC benzyl treated cells

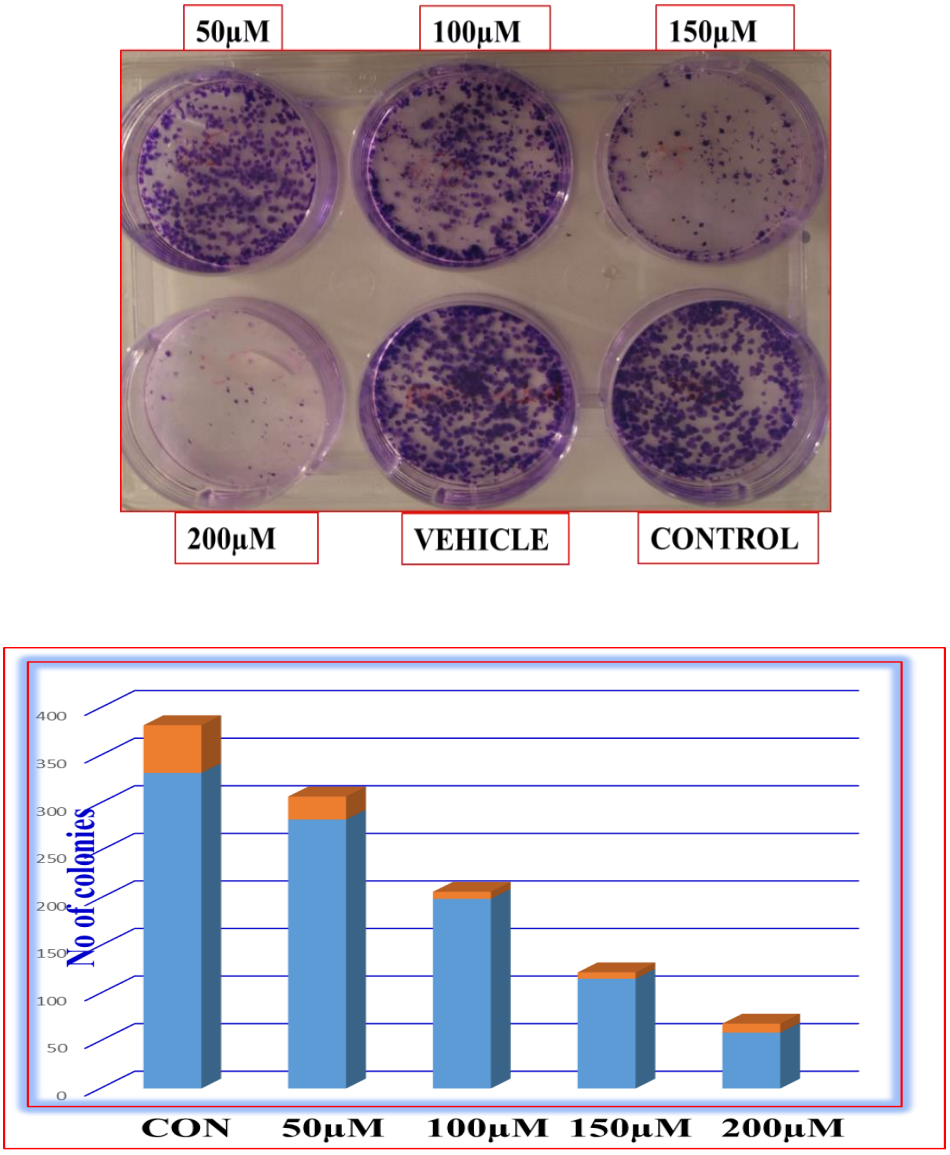
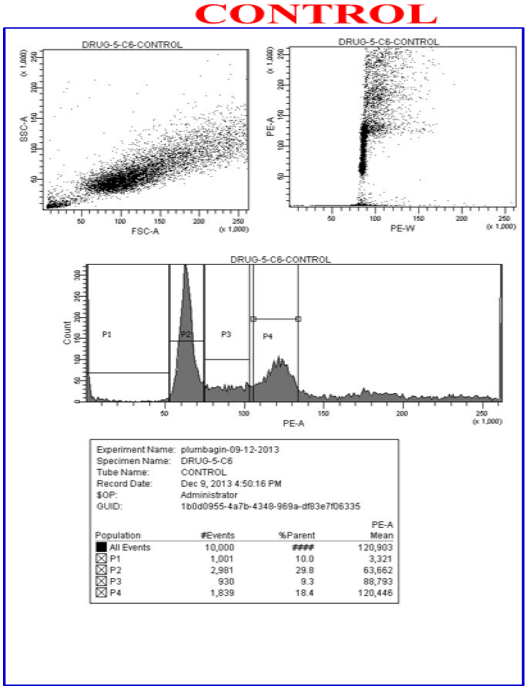


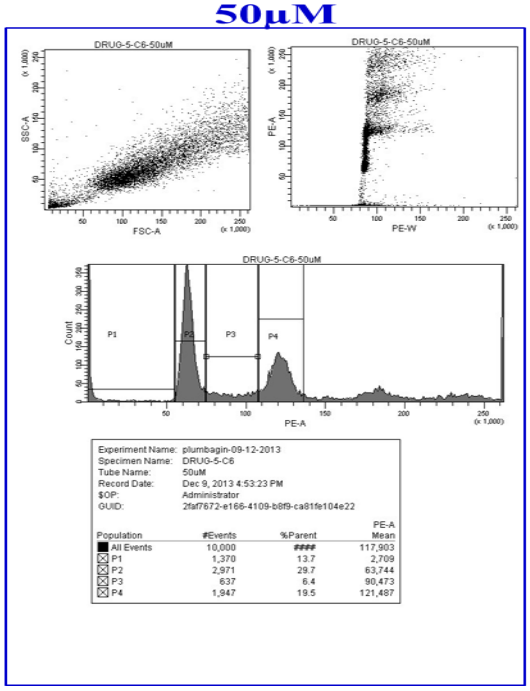
Figure 4.15. Clonogenic assay performed where cells were treated with CMIC benzyl 50µM, 100µM, 150µM, 200µM, vehicle (0.1% DMSO) and control cells. CMIC benzyl concentration at 150µM, showed less than 50% colonies compared to vehicle and control treated cells.

Figure 4.16. Cell cycle Analysis of CMIC benzyl treatment cells

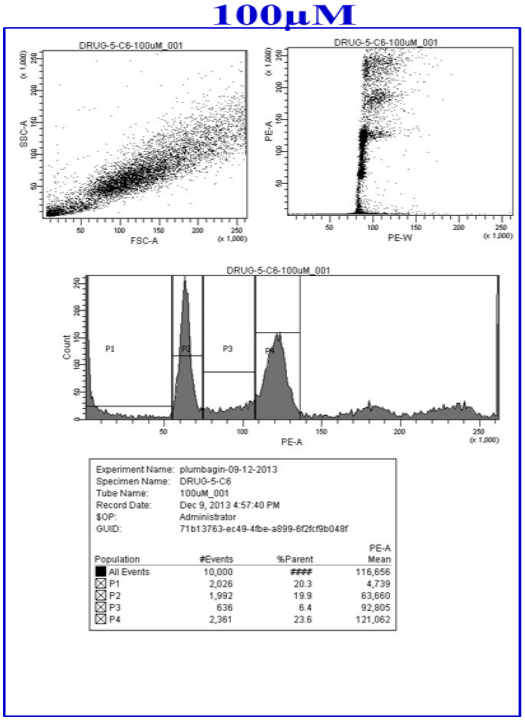
CONTROL



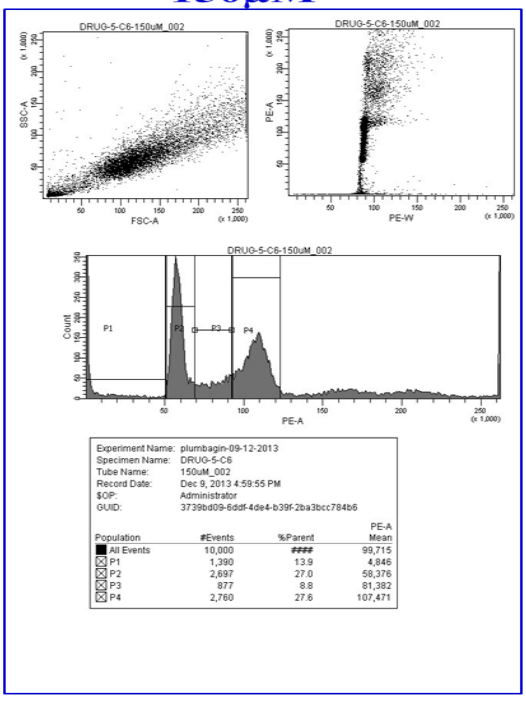
50µM



100µM



150µM



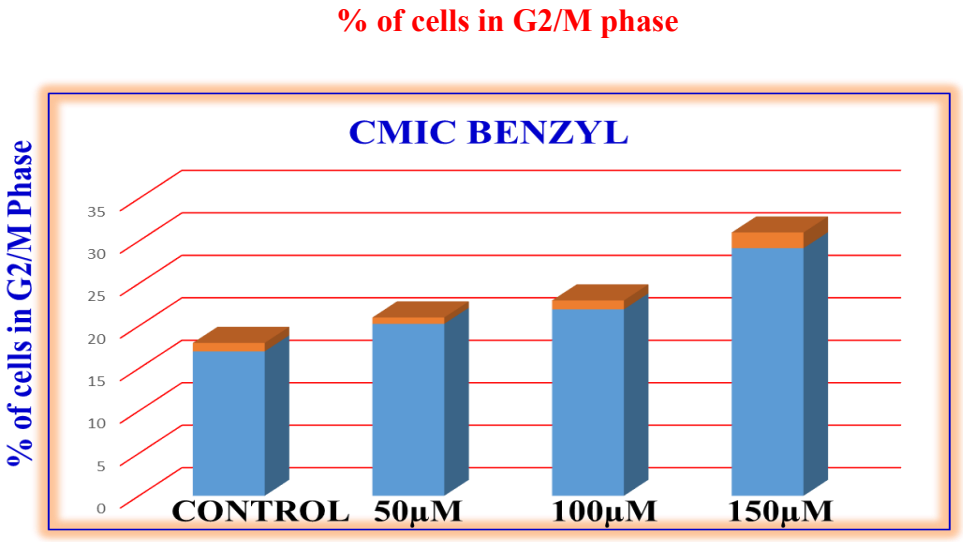


Figure 4.16. CMIC benzyl induces cell cycle arrest at G2/M phase. C6 cells were treated with 0.1% DMSO (Control), 50µM, 100µM and 150µM concentration of CMIC benzyl for 24h. FACS analysis was performed after treatment followed by PI staining. Data shows that nearly 15% of G2/M phase cells increased in 150µM compared to vehicle treated cells.

Figure 4.17. Mitochondrial membrane potential (Δψm)

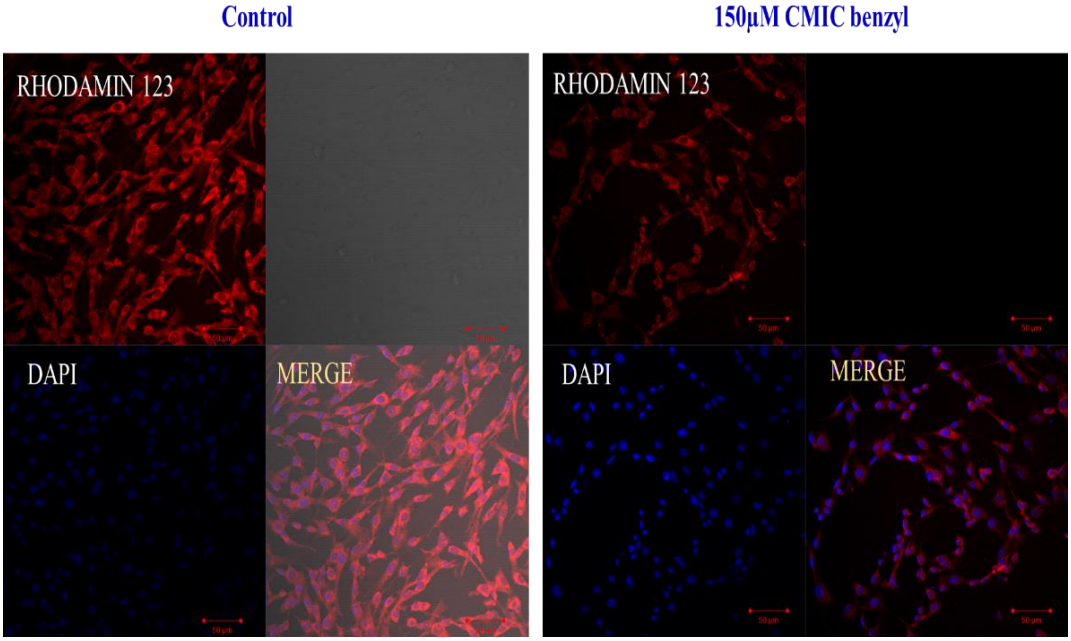


Figure 4.17. Rhodamin 123 was used as mitochondrial marker to check membrane potential **(Δψm)**. Cells were treated with CMIC benzyl 150µM and vehicle for 24hrs. Stained with Rhodamin123 show intact mitochondria in control cells whereas in CMIC benzyl treated cells disperse mitochondrial staining is observed indicating loss of mitochondrial membrane potential.

Figure 4.18. TUNEL assay of CMIC benzyl treated cells

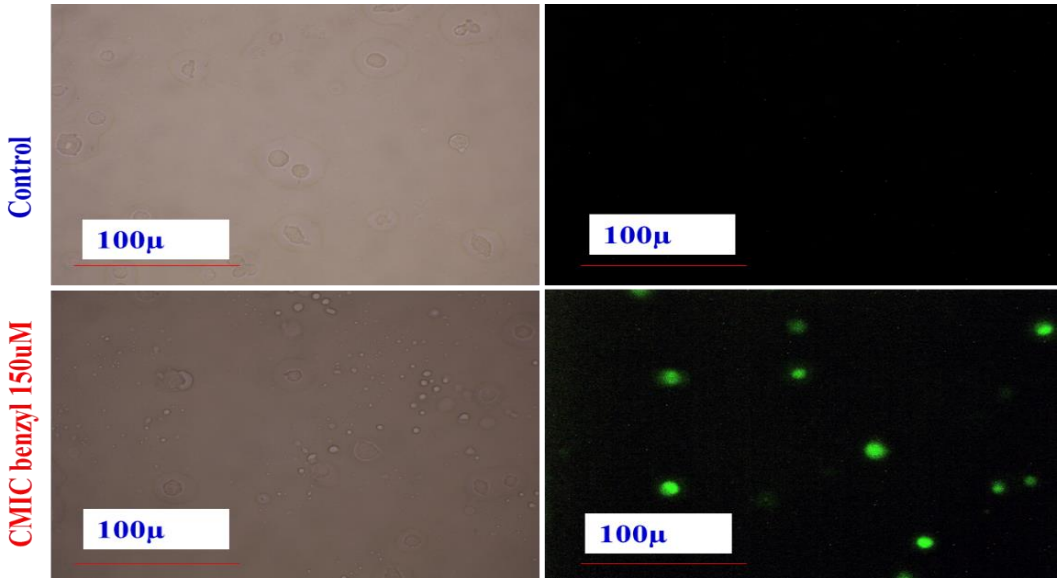
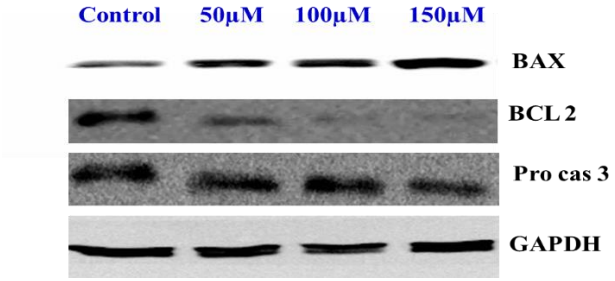


Figure 4.18. TUNEL assay was performed to detect cell death through DNA fragmentation. Cells were treated with CMIC benzyl (150µM) and vehicle (0.1%DMSO) for 24hrs. Images are taken with Olympus fluorescent microscope, bright field and florescent field at 40X. In vehicle treated cells no DNA fragmentation observed, CMIC benzyl treated cells showed DNA fragmentation.

Figure 4.19. Western blot analysis of CMIC benzyl treated cells



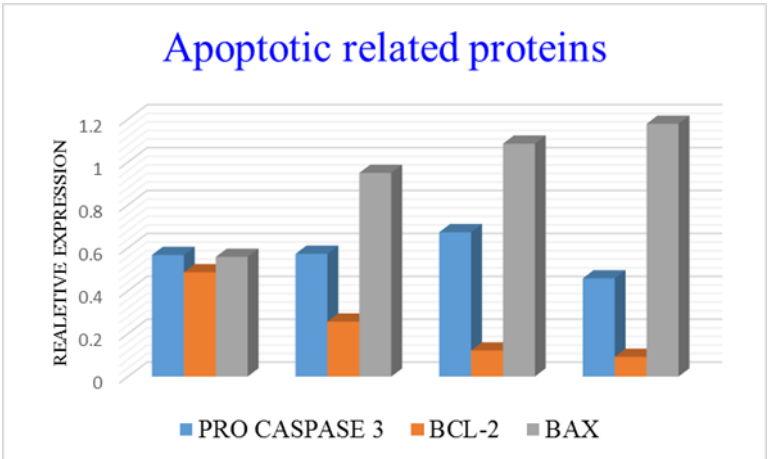


Figure 4.19. Western blot analysis of CMIC benzyl and vehicle treated cells showing mitochondria mediated apoptotic pathway proteins, anti-apoptotic BCL-2 levels were decreased in CMIC benzyl treated C6 cells. At the same time apoptotic protein BAX levels were increased. Also, a decrease in procaspase 3 levels was observed.

4.4. DISCUSSION

4.4.1. Plumbagin

Current therapy for glioblastoma patients includes surgical resection followed by radiation and chemotherapy. Despite all these aggressive therapies, the prognosis of GBM patients remains poor. Chemotherapy is one of the promising approaches for treating cancer, but most of the anti-cancer drugs do not work for brain tumors because of their low penetration into the brain and also because of heterogeneous nature of the disease. Till now temozolamide (TMZ) is the only drug qualified for use in standard of care for GBM patients. Screening of novel compounds thus will help in formulating and improving the treatment options.

Plumbagin is a natural plant product extracted from Indian medicinal plant roots of *Plumbaginaceae,* family commonly called as chitrak, plumbagin is a derivative of naphthoquinone, which can cross the blood brain barrier as reported in Parkinson disease mouse model (Choi, Son et al. 2012). In the present study plumbagin anti-cancer activity was assessed in rat glioma cell line where it showed dose dependent cytotoxicity and an IC_{50} value of 2µM for 24h treatment. Plumbagin acts as proliferation inhibitor which is evident from the results of cell cycle and colony formation analysis where cells got arrested in S&G2 phase and showed reduced colony formation capacity respectively. These results are consistent with previous reports (Wang, Chiang et al. 2008; Sinha, Pal et al. 2013).

Plumbagin induces apoptosis in C6 rat glioma cell line through mitochondrial pathway, which was confirmed by Rhodamine 123 staining (where plumbagin treated cells showed diffused mitochondria), ROS estimation and western blot analysis. Increase in ROS levels in plumbagin treated cells affects the mitochondrial proteins which is evident from the western blots which showed decreased levels of anti-apoptotic proteins BCL-2, BC-XL and up-regulation of pro-apoptotic protein BAX. The ratio of BCL-2/BAX is crucial in cells undergoing apoptosis. Our results also correlate with earlier reports (Wang, Chiang et al. 2008; Qiu, He et al. 2013; Xu, Shen et al. 2013). Mitochondrial pathway mediates apoptosis via caspase 3 cleavage which is observed by decrease in the full length pro-caspase 3 levels and cleavage of PARP which finally leads to cell death. In our results, pro-caspase 3 levels were decreased and PARP cleaved product increased which indicates cell death through apoptosis. We found p53 levels were decreased in contrast to previous cancer reports (Hsu, Cho et al. 2006; Gomathinayagam, Sowmyalakshmi et al. 2008; Tian, Yin et al. 2012). p53 expression is cell type specific and its down regulation has been reported in some cancer cell lines in response to plumbagin (Thasni, Rakesh et al. 2008; Sinha, Pal et al. 2013; Sagar, Esau et al. 2014). Although the role of decreased p53 has not been elucidated completely, it may be involved in minimizing the adverse side effects of chemo and radiotherapy (Berns 2006).

Further, we evaluated the inhibitory effect of plumbagin on glioma growth in C6 rat glioma model. Results from H&E sections of brains of tumor bearing rats suggested a clear reduction in tumor size in plumbagin treated rats. These results also correlate with our *in vitro* findings in C6 cell line. Ki67 which is a proliferative marker showed decreased staining in plumbagin treated rats compared to vehicle treated rats.

VEGF is an angiogenic marker. Its staining intensity is directly proportional to poor prognosis (Ke, Shi et al. 2000). In our experiments, VEGF Reduced VEGF staining was observed in plumbagin treated animals which suggest good prognosis.

Route of drug administration matters when we consider fast drug delivery to the site of tumor. It is already reported that plumbagin can be administered intra peritoneal (Hsu, Cho et al. 2006; Hafeez, Jamal et al. 2012; Sinha, Pal et al. 2013) but there are no reports of intravenous administration. Here we compared plumbagin administration through both the routes (I.P and I.V). Rats treated with plumbagin through I.V showed significant tumor reduction when compared to I.P treated group. This suggests that I.V route is better than I.P route for treating glioma rats with plumbagin.

4.4.2. CMIC benzyl

CMIC benzyl is a novel organic derivative. Its anti-cancer activity was assessed in rat glioma cell line were it showed dose dependent cytotoxicity and an IC_{50} value of 150µM for 24h treatment. CMIC benzyl arrest the cell cycle at G2 phase and it inhibits the colony forming capacity of C6 cells. This is in consistence with earlier report from this lab (Anwita Mudiraj 2014), CMIC benzyl showing anti-cancer activity in glioma cell line.

CMIC benzyl induces apoptosis in C6 rat glioma cell line through mitochondrial pathway, which was supported by ROS estimation, Rhodamine 123 staining and western blot analysis. CMIC benzyl treatment increases ROS levels in C6 cells which in turn affects the mitochondrial proteins which were detected by western blots showing reduced levels of anti-apoptotic proteins BCL-2 and increased levels of pro-apoptotic protein BAX. The ratio of BCL-2/BAX is crucial in cells undergoing apoptosis and this signals involvement of mitochondria in cell death mechanism. Mitochondrial pathway

mediated apoptosis occurs via Caspase 3 cleavage which is observed by decrease in the full length pro-caspase 3 levels. In our results Pro-caspase 3 levels were decreased which indicates cell death through apoptosis. Further studies are needed to elucidate the mechanism of action of CMIC benzyl induced cell death.

CONCLUSION: From this study we conclude that plumbagin is working as an anti-cancer drug by inducing cell cycle arrest and apoptosis in cell line and *in vivo* rat glioma model administration of plumbagin was more effective when compared to vehicle treated group in retarding glioma growth and progression. Thus, plumbagin treatment may have some therapeutic benefits in glioma tumors. CMIC benzyl works as a potential cytotoxic compound and induces cell death in C6 cell line. However, further studies are needed in animal model.

Table 3.1. Real time PCR primers sequences

S.NO.	GENE NAME	PRIMER SEQUENCE (5'-3')
1	ITGB3 FORWARD	TCC GGC CAG ATG ATT CGA AG
	ITGB3 REVERS	CAG CTT GGT ACC CAG GTT CT
2	CLIC4 FORWARD	AGG CTA ATG AAG CAC TGG AGA G
	CLIC4 REVERS	GCA GCA GGT TGC AAT CAG CT
3	ITGB1 FORWARD	GTT TGT GGA TCA CTG ATT GGC TG
	ITGB1 REVERS	CAA GGT GAG CAA TAG AAG GAT AAT C
4	STAB1 FORWARD	TGC CAA TCG GTG TGC AGC TGT
	STAB1 REVERS	TCT GCG GAG CAC TGG GTG TT
5	ELK3 FORWARD	CGC ACA GAC ACC AAA TGG ATT G
	ELK3 REVERS	CAT GTG GCC ATT AAG CAG TGT G
6	ANGPTL4 FORWARD	CAG CCT GCA GAC ACA ACT CAA
	ANGPTL4 REVERS	CTC ATG GTC TAG GTG CTT GTG
7	STC2 FORWARD	ATG CCC AGG GCA AGT CAT TC
	STC2 REVERS	CTT GAG GTA GCA TTC CCG CT
8	LILRB3 FORWARD	TGG TCT CAG GAC ACT CTG GA
	LILRB3 REVERS	GTT TGC TGT GAC GCT GAC GT
9	OSMR FORWARD	CAT CTG GGT GGG GAA TTA CAG
	OSMR REVERS	GAA ATT TGG CTC AGG GAA CTT GG
10	MMP25 FORWARD	TGA CAT GGA GGG TAC GTT CCT
	MMP25 REVERS	GCA AAG TCG ATG AGG ATG TCG
11	LILRB2 FORWARD	CAG ACA GGG ACC ATT CCC AA
	LILTB2 REVERS	GTA TCC GTG TAA TCC AAG ATG CT
12	MUC20 FORWARD	ACG TGA GTG CAG GTG AAA ATG GA
	MUC20 REVERS	CAG TAA GGA GAC CTG GAA GTG A
13	SRPX FORWARD	CAA ACG CTG TGG CAA ACT CAA TG
	SRPX REVERS	ATT GAC GTT CAT GGC TGC ACA G

S.NO.	GENE NAME	PRIMER SEQUENCE (5'-3')
14	ABCA13 FORWARD	GTC ACC GAG CCA GTT TAC CA
	ABCA13 REVERS	GTC TGT GGG AAT CTC CTT CAG
15	UHRF1 FORWARD	CAT GCA GTA TCC AGA AGG CTA C
	UHRF1 REVERS	CCG ACT TCC GCT TCC ACT TG
16	SOX4 FORWARD	CTG CTC AAA GAC AGC GAC AAG
	SOX4 REVERS	AGG AGC TGG AGT TGG CGT TG
17	SIX1 FORWARD	GTC CAG CTC AGA AGA GGA ATT C
	SIX1 REVERS	GGA GAG AAT AGT TTG AGC TCC TGG C
18	TRAIL FORWARD	CAT GGC TAT GAT GGA GGT CCA G
	TRAIL REVERS	CAC ACA GAG AGA CTG CAG GAG
19	DR4 FORWARD	AGA GAG AAG TCC CTG CAC CAC
	DR4 REVERS	GGC ACC CTC TGC TGC ACT T
20	DR5 FORWARD	GTG TGT CAG TGC GAA GAA GGC A
	DR5 REVERS	CAA TCA CCG ACC TTG ACC ATC C
21	DcR1 FORWARD	CTG ATG GCC GAG GCA GGG T
	DcR1 REVERS	AGT AAG CTA GGA CTG GCA GCA G
22	DcR2 FORWARD	GTA GAA CAG GGT GTC CCA GAG
	DcR2 REVERS	AGG ATG GTG GTC ACT GTC TCC
23	MIR-185 FORWARD	CAATGGAGAGAAAGGCAGTTCC
	MIR-185 REVERS	GAA GGA CCA GAG GAA AGC CA
24	U6 sn RNA FORWARD	ATTGGAACGATACAGAGAAGATT
	U6 sn RNA REVERS	GGAACGCTTCACGAATTTG
25	ZNF24 FORWARD	CTG GAC AAC CGG TTT CTC TCC
	ZNF24 REVERS	CTA CCA TCA TCA TCA CAG TGC CT
26	GAPDH FORWARD	CAT GGA GAA GGC TGG GGC TCA TTT GCA GG
	GAPDH REVERS	GTG CAG GAG GCA TTG CTG ATG ATC TTG AGG

5. SUMMARY OF WORK

Glioblastoma multiforme (GBM) is a highly aggressive subtype of glioma (brain tumor) with poor prognosis. Thus, there is a need for studying molecular, cell signaling pathways of GBM, to develop good therapeutic options and increase the survival time of glioma patients. Therefore, the present study was designed to identify new targets and novel therapeutics for the possible treatment of glioma. The important findings of the present work are summarized below:

1) **Analysis of differential gene expression in low grade (Grade II) and high grade (GBM) astrocytoma. Validation of selected genes.**

In this study control, grade II and grade IV human astrocytoma samples are used for micro array analysis. After analyzing whole human genome (44k gene) by micro array from above samples, 17 genes functioning as transcription factors, cell surface receptors, angiogenesis and migration were selected whose expression were high expression in GBM, compare to grade II and control and most of them are not reported earlier in GBM. These selected 17 genes expression were validated in GBM, grade II and control clinical samples with real time PCR. All the genes overexpression were observed in glioma samples. Importantly, overexpression of 10 new genes in glioma samples were reported. Further studies on these may establish some novel therapeutic targets for glioma.

2) **Involvement of transcription factors SIX1 and ZNF24 in astrocytoma progression**

From the micro array and real time validation data transcription factors SIX1 and ZNF24 were selected and their functional significance in glioma was explored. Six1 is a homeodomine transcription factor, having a role in embryo development and has lost expression in adults, however six1 overexpression is observed in some of the

human cancers. Six1 overexpression in cancers has been correlated with TRAIL pathway resistance and Six1 have a role inducing cancer stem cell property. In this study human astrocytoma clinical samples showed increased mRNA expression of six1 and TRAIL pathway genes (TRAIL, its receptors TRAIL R1, R2 and decoy receptors DcR1 and DcR2) and this TRAIL Decoy receptors expression correlates with six1 mRNA expression. However, six1 protein level was very low in these samples. ZNF 24 zinc finger protein, very less characterized protein. In this study we investigated into the ZNF24 mRNA levels and protein levels in clinical sample. Out of 34 clinical samples, overexpression was observed in only 8 samples.

3) *In vitro* and *in vivo* drug targeting glioma.

To identify novel therapeutic options, many synthetic and natural compounds were screened for anti-glioma activity *in vitro* followed by *in vivo* studies in case of promising compounds such as Plumbagin (natural compound) and CMIC benzyl (synthetic compound) *in vitro*. Plumbagin showed potential anti-cancer activity in glioma cell line by arresting the cell cycle and inducing the cell death *in vitro*. Plumbagin anti-cancer activity evaluated *in vivo* rat glioma model showed significant tumor growth inhibition. Thus, plumbagin treatment may have some therapeutic benefits in glioma tumors.

CMIC benzyl showed significant activity *in vitro* by inducing cell cycle arrest G2 phase and inducing apoptosis. Further *in vivo* validation of CMIC benzyl needed.

REFERENCES

Albanese, V., N. F. Biguet, et al. (2001). "Quantitative effects on gene silencing by allelic variation at a tetranucleotide microsatellite." Hum Mol Genet **10**(17): 1785-1792.

Alhosin, M., T. Sharif, et al. (2011). "Down-regulation of UHRF1, associated with re-expression of tumor suppressor genes, is a common feature of natural compounds exhibiting anti-cancer properties." J Exp Clin Cancer Res **30**: 41.

Alitalo, K. and P. Carmeliet (2002). "Molecular mechanisms of lymphangiogenesis in health and disease." Cancer Cell **1**(3): 219-227.

Allen, J. E. and W. S. El-Deiry (2012). "Regulation of the human TRAIL gene." Cancer Biol Ther **13**(12): 1143-1151.

Anderson, J. C., B. C. McFarland, et al. (2008). "New molecular targets in angiogenic vessels of glioblastoma tumours." Expert Rev Mol Med **10**: e23.

Anwita Mudiraj, Khamushavali. G., Phanithi Prakash Babu, A. Ram Reddy, (2014). "Triazolo aspirins inhibit proliferation of C6 glioma cell lines in vitro." Journal of Pharmacy Research: 741-750.

Ashkenazi, A., R. C. Pai, et al. (1999). "Safety and antitumor activity of recombinant soluble Apo2 ligand." J Clin Invest **104**(2): 155-162.

Auvergne, R. M., F. J. Sim, et al. (2013). "Transcriptional differences between normal and glioma-derived glial progenitor cells identify a core set of dysregulated genes." Cell Rep **3**(6): 2127-2141.

Bansal, K., M. L. Liang, et al. (2006). "Molecular biology of human gliomas." Technol Cancer Res Treat **5**(3): 185-194.

Behbakht, K., L. Qamar, et al. (2007). "Six1 overexpression in ovarian carcinoma causes resistance to TRAIL-mediated apoptosis and is associated with poor survival." Cancer Res **67**(7): 3036-3042.

Berns, A. (2006). "Cancer biology: can less be more for p53?" Nature **443**(7108): 153-154.

Bhaskara, V. K., S. Challa, et al. (2009). "Differential PARP cleavage: an indication for existence of multiple forms of cell death in human gliomas." Neurol India **57**(3): 264-268.

Bhaskara, V. K., M. Panigrahi, et al. (2005). "Comparative status of activated ERK1/2 and PARP cleavage in human gliomas." Neuropathology **25**(1): 48-53.

Bondy, M. L., M. E. Scheurer, et al. (2008). "Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium." Cancer **113**(7 Suppl): 1953-1968.

Brown, D., J. Trowsdale, et al. (2004). "The LILR family: modulators of innate and adaptive immune pathways in health and disease." Tissue Antigens **64**(3): 215-225.

Brown, J. M. and W. R. Wilson (2004). "Exploiting tumour hypoxia in cancer treatment." Nat Rev Cancer **4**(6): 437-447.

Buhl, R., H. Barth, et al. (1998). "Spinal drop metastases in recurrent glioblastoma multiforme." Acta Neurochir (Wien) **140**(10): 1001-1005.

Caffarel, M. M. and N. Coleman (2014). "Oncostatin M receptor is a novel therapeutic target in cervical squamous cell carcinoma." J Pathol **232**(4): 386-390.

CBTRUS (2011). CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2004-2007 United States, Central Brain Tumor Registry of the United States

Cea, V., C. Sala, et al. (2012). "Antiangiogenic therapy for glioma." J Signal Transduct **2012**: 483040.

Cha, S. S., M. S. Kim, et al. (1999). "2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity." Immunity **11**(2): 253-261.

Checker, R., D. Sharma, et al. (2010). "Plumbagin inhibits proliferative and inflammatory responses of T cells independent of ROS generation but by modulating intracellular thiols." J Cell Biochem **110**(5): 1082-1093.

Chen, C. H., S. W. Wang, et al. (2013). "MUC20 overexpression predicts poor prognosis and enhances EGF-induced malignant phenotypes via activation of the EGFR-STAT3 pathway in endometrial cancer." Gynecol Oncol **128**(3): 560-567.

Cheyette, B. N., P. J. Green, et al. (1994). "The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system." Neuron **12**(5): 977-996.

Choi, S., M. H. Lim, et al. (2011). "Cordycepin-induced apoptosis and autophagy in breast cancer cells are independent of the estrogen receptor." Toxicol Appl Pharmacol **257**(2): 165-173.

Choi, S. Y., T. G. Son, et al. (2012). "Naphthazarin has a protective effect on the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine-induced Parkinson's disease model." J Neurosci Res **90**(9): 1842-1849.

Christensen, K. L., A. N. Patrick, et al. (2008). "The six family of homeobox genes in development and cancer." Adv Cancer Res **101**: 93-126.

Courboulin, A., M. Barrier, et al. (2012). "Plumbagin reverses proliferation and resistance to apoptosis in experimental PAH." Eur Respir J **40**(3): 618-629.

CSIR (1989). The Wealth of India. . New Delhi, New Delhi: CSIR.

Dameron, K. M., O. V. Volpert, et al. (1994). "Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1." Science **265**(5178): 1582-1584.

David, C., J. P. Nance, et al. (2012). "Stabilin-1 expression in tumor associated macrophages." Brain Res **1481**: 71-78.

Demuth, T. and M. E. Berens (2004). "Molecular mechanisms of glioma cell migration and invasion." J Neurooncol **70**(2): 217-228.

Dunn, I. F., O. Heese, et al. (2000). "Growth factors in glioma angiogenesis: FGFs, PDGF, EGF, and TGFs." J Neurooncol **50**(1-2): 121-137.

Emery, J. G., P. McDonnell, et al. (1998). "Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL." J Biol Chem **273**(23): 14363-14367.

Fanger, N. A., C. R. Maliszewski, et al. (1999). "Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)." J Exp Med **190**(8): 1155-1164.

Farabaugh, S. M., D. S. Micalizzi, et al. (2012). "Eya2 is required to mediate the pro-metastatic functions of Six1 via the induction of TGF-beta signaling, epithelial-mesenchymal transition, and cancer stem cell properties." Oncogene **31**(5): 552-562.

Ford, H. L., E. N. Kabingu, et al. (1998). "Abrogation of the G2 cell cycle checkpoint associated with overexpression of HSIX1: a possible mechanism of breast carcinogenesis." Proc Natl Acad Sci U S A **95**(21): 12608-12613.

Fowler, A., D. Thomson, et al. (2011). "miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion." Eur J Cancer **47**(6): 953-963.

Frank, S., U. Kohler, et al. (1999). "Expression of TRAIL and its receptors in human brain tumors." Biochem Biophys Res Commun **257**(2): 454-459.

Furnari, F. B., T. Fenton, et al. (2007). "Malignant astrocytic glioma: genetics, biology, and paths to treatment." Genes Dev **21**(21): 2683-2710.

Gerstner, E. R. and T. T. Batchelor (2012). "Antiangiogenic therapy for glioblastoma." Cancer J **18**(1): 45-50.

Gomathinayagam, R., S. Sowmyalakshmi, et al. (2008). "Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells." Anticancer Res **28**(2A): 785-792.

Good, D. J., P. J. Polverini, et al. (1990). "A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin." Proc Natl Acad Sci U S A **87**(17): 6624-6628.

Goodenberger, M. L. and R. B. Jenkins (2012). "Genetics of adult glioma." Cancer Genet **205**(12): 613-621.

Griffith, T. S., S. R. Wiley, et al. (1999). "Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL." J Exp Med **189**(8): 1343-1354.

Hafeez, B. B., M. S. Jamal, et al. (2012). "Plumbagin, a plant derived natural agent inhibits the growth of pancreatic cancer cells in in vitro and in vivo via targeting EGFR, Stat3 and NF-kappaB signaling pathways." Int J Cancer **131**(9): 2175-2186.

Hafeez, B. B., W. Zhong, et al. (2012). "Plumbagin inhibits prostate cancer development in TRAMP mice via targeting PKCepsilon, Stat3 and neuroendocrine markers." Carcinogenesis **33**(12): 2586-2592.

Han, Z. G., Q. H. Zhang, et al. (1999). "Molecular cloning of six novel Kruppel-like zinc finger genes from hematopoietic cells and identification of a novel transregulatory domain KRNB." J Biol Chem **274**(50): 35741-35748.

Hardell, L., M. Carlberg, et al. (2013). "Use of mobile phones and cordless phones is associated with increased risk for glioma and acoustic neuroma." Pathophysiology **20**(2): 85-110.

Harper, J., L. Yan, et al. (2007). "Repression of vascular endothelial growth factor expression by the zinc finger transcription factor ZNF24." Cancer Res **67**(18): 8736-8741.

Hayakawa, Y., V. Screpanti, et al. (2004). "NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy." J Immunol **172**(1): 123-129.

Hazra, B., R. Sarkar, et al. (2002). "Synthesis of plumbagin derivatives and their inhibitory activities against Ehrlich ascites carcinoma in vivo and Leishmania donovani Promastigotes in vitro." Phytother Res **16**(2): 133-137.

Heo, S. H. and J. Y. Cho (2014). "ELK3 suppresses angiogenesis by inhibiting the transcriptional activity of ETS-1 on MT1-MMP." Int J Biol Sci **10**(4): 438-447.

Hervouet, E., L. Lalier, et al. (2010). "Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells." PLoS One **5**(6): e11333.

Hlavac, V., V. Brynychova, et al. (2013). "The expression profile of ATP-binding cassette transporter genes in breast carcinoma." Pharmacogenomics **14**(5): 515-529.

Hlavata, I., B. Mohelnikova-Duchonova, et al. (2012). "The role of ABC transporters in progression and clinical outcome of colorectal cancer." Mutagenesis **27**(2): 187-196.

Hollingsworth, M. A. and B. J. Swanson (2004). "Mucins in cancer: protection and control of the cell surface." Nat Rev Cancer **4**(1): 45-60.

Howng, S. Y., R. L. Avila, et al. (2010). "ZFP191 is required by oligodendrocytes for CNS myelination." Genes Dev **24**(3): 301-311.

Hsu, Y. L., C. Y. Cho, et al. (2006). "Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo." J Pharmacol Exp Ther **318**(2): 484-494.

Hymowitz, S. G., H. W. Christinger, et al. (1999). "Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5." Mol Cell **4**(4): 563-571.

Ikushima, H., T. Todo, et al. (2009). "Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors." Cell Stem Cell **5**(5): 504-514.

Imam, J. S., K. Buddavarapu, et al. (2010). "MicroRNA-185 suppresses tumor growth and progression by targeting the Six1 oncogene in human cancers." Oncogene **29**(35): 4971-4979.

Jafarnejad, S. M., G. S. Ardekani, et al. (2013). "Pleiotropic function of SRY-related HMG box transcription factor 4 in regulation of tumorigenesis." Cell Mol Life Sci **70**(15): 2677-2696.

Janssen, E. M., N. M. Droin, et al. (2005). "CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death." Nature **434**(7029): 88-93.

Junnila, S., A. Kokkola, et al. (2010). "Genome-wide gene copy number and expression analysis of primary gastric tumors and gastric cancer cell lines." BMC Cancer **10**: 73.

Katanasaka, Y., Y. Kodera, et al. (2013). "Epidermal growth factor receptor variant type III markedly accelerates angiogenesis and tumor growth via inducing c-myc mediated angiopoietin-like 4 expression in malignant glioma." Mol Cancer **12**: 31.

Kaur, B., C. Tan, et al. (2004). "Genetic and hypoxic regulation of angiogenesis in gliomas." J Neurooncol **70**(2): 229-243.

Ke, L. D., Y. X. Shi, et al. (2000). "The relevance of cell proliferation, vascular endothelial growth factor, and basic fibroblast growth factor production to angiogenesis and tumorigenicity in human glioma cell lines." Clin Cancer Res **6**(6): 2562-2572.

Kemp, T. J., J. M. Moore, et al. (2004). "Human B cells express functional TRAIL/Apo-2 ligand after CpG-containing oligodeoxynucleotide stimulation." J Immunol **173**(2): 892-899.

Khalfallah, O., P. Ravassard, et al. (2009). "Zinc finger protein 191 (ZNF191/Zfp191) is necessary to maintain neural cells as cycling progenitors." Stem Cells **27**(7): 1643-1653.

Kim, M. S., J. Louwagie, et al. (2009). "Promoter DNA methylation of oncostatin m receptor-beta as a novel diagnostic and therapeutic marker in colon cancer." PLoS One **4**(8): e6555.

Kochhar, A., S. M. Fischer, et al. (2007). "Branchio-oto-renal syndrome." Am J Med Genet A **143A**(14): 1671-1678.

Konishi, Y., K. Ikeda, et al. (2006). "Six1 and Six4 promote survival of sensory neurons during early trigeminal gangliogenesis." Brain Res **1116**(1): 93-102.

Koshy, M., J. L. Villano, et al. (2012). "Improved survival time trends for glioblastoma using the SEER 17 population-based registries." J Neurooncol **107**(1): 207-212.

Krishnaswamy, M. and K. K. Purushothaman (1980). "Plumbagin: A study of its anticancer, antibacterial & antifungal properties." Indian J Exp Biol **18**(8): 876-877.

Kuan, C. T., C. J. Wikstrand, et al. (2001). "EGF mutant receptor vIII as a molecular target in cancer therapy." Endocr Relat Cancer **8**(2): 83-96.

Kuijlen, J. M., E. Bremer, et al. (2010). "Review: on TRAIL for malignant glioma therapy?" Neuropathol Appl Neurobiol **36**(3): 168-182.

Kuijlen, J. M., J. J. Mooij, et al. (2006). "TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme." J Neurooncol **78**(2): 161-171.

Kumar, J. P. (2009). "The sine oculis homeobox (SIX) family of transcription factors as regulators of development and disease." Cell Mol Life Sci **66**(4): 565-583.

Kuo, P. L., Y. L. Hsu, et al. (2006). "Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells." Mol Cancer Ther **5**(12): 3209-3221.

Laclef, C., G. Hamard, et al. (2003). "Altered myogenesis in Six1-deficient mice." Development **130**(10): 2239-2252.

Laclef, C., E. Souil, et al. (2003). "Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice." Mech Dev **120**(6): 669-679.

Leon, S. P., R. D. Folkerth, et al. (1996). "Microvessel density is a prognostic indicator for patients with astroglial brain tumors." Cancer **77**(2): 362-372.

Levine, M. and T. Hoey (1988). "Homeobox proteins as sequence-specific transcription factors." Cell **55**(4): 537-540.

Li, C. M., M. Guo, et al. (2002). "Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition." Am J Pathol **160**(6): 2181-2190.

Li, J., X. Chen, et al. (2009). "A transcript profiling approach reveals the zinc finger transcription factor ZNF191 is a pleiotropic factor." BMC Genomics **10**: 241.

Li, J., X. Chen, et al. (2010). "The transcriptional repression of platelet-derived growth factor receptor-beta by the zinc finger transcription factor ZNF24." Biochem Biophys Res Commun **397**(2): 318-322.

Li, J., X. Chen, et al. (2006). "The zinc finger transcription factor 191 is required for early embryonic development and cell proliferation." Exp Cell Res **312**(20): 3990-3998.

Li, X., K. A. Oghi, et al. (2003). "Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis." Nature **426**(6964): 247-254.

Li, Y., C. M. Backesjo, et al. (2009). "Resveratrol inhibits proliferation and promotes apoptosis of osteosarcoma cells." Eur J Pharmacol **609**(1-3): 13-18.

Li, Y. M. and W. A. Hall (2011). "Cell surface receptors in malignant glioma." Neurosurgery **69**(4): 980-994; discussion 994.

Li, Z., T. Tian, et al. (2013). "Six1 promotes proliferation of pancreatic cancer cells via upregulation of cyclin D1 expression." PLoS One **8**(3): e59203.

Liang, M. L., J. Ma, et al. (2008). "Tyrosine kinase expression in pediatric high grade astrocytoma." J Neurooncol **87**(3): 247-253.

Libermann, T. A., H. R. Nusbaum, et al. (1985). "Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin." Nature **313**(5998): 144-147.

Lima, F. R., S. A. Kahn, et al. (2012). "Glioblastoma: therapeutic challenges, what lies ahead." Biochim Biophys Acta **1826**(2): 338-349.

Linos, E., T. Raine, et al. (2007). "Atopy and risk of brain tumors: a meta-analysis." J Natl Cancer Inst **99**(20): 1544-1550.

Little, M. P., P. Rajaraman, et al. (2012). "Mobile phone use and glioma risk: comparison of epidemiological study results with incidence trends in the United States." BMJ **344**: e1147.

Liu, G., S. Jiang, et al. (2012). "Zinc finger transcription factor 191, directly binding to beta-catenin promoter, promotes cell proliferation of hepatocellular carcinoma." Hepatology **55**(6): 1830-1839.

Liu, L. and S. L. Gerson (2006). "Targeted modulation of MGMT: clinical implications." Clin Cancer Res **12**(2): 328-331.

Liu, X., W. Cai, et al. (2014). "Plumbagin induces growth inhibition of human glioma cells by downregulating the expression and activity of FOXM1." J Neurooncol.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lo, H. W. and M. C. Hung (2006). "Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival." Br J Cancer **94**(2): 184-188.

Lokker, N. A., C. M. Sullivan, et al. (2002). "Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors." Cancer Res **62**(13): 3729-3735.

Louis, D. N. (2006). "Molecular pathology of malignant gliomas." Annu Rev Pathol **1**: 97-117.

Louis, D. N., H. Ohgaki, et al. (2007). "The 2007 WHO classification of tumours of the central nervous system." Acta Neuropathol **114**(2): 97-109.

Lu, J. and C. Jiang (2001). "Antiangiogenic activity of selenium in cancer chemoprevention: metabolite-specific effects." Nutr Cancer **40**(1): 64-73.

Lubin, J. H., D. W. Schafer, et al. (2004). "A reanalysis of thyroid neoplasms in the Israeli tinea capitis study accounting for dose uncertainties." Radiat Res **161**(3): 359-368.

Mason, W. P. and J. G. Cairncross (2008). "Invited article: the expanding impact of molecular biology on the diagnosis and treatment of gliomas." Neurology **71**(5): 365-373.

McCarthy, B. J., K. Rankin, et al. (2011). "Assessment of Type of Allergy and Antihistamine Use in the Development of Glioma." Cancer Epidemiology Biomarkers & Prevention **20**(2): 370-378.

McCarthy, B. J., K. M. Rankin, et al. (2011). "Risk factors for oligodendroglial tumors: a pooled international study." Neuro Oncol **13**(2): 242-250.

McCoy, E. L., R. Iwanaga, et al. (2009). "Six1 expands the mouse mammary epithelial stem/progenitor cell pool and induces mammary tumors that undergo epithelial-mesenchymal transition." J Clin Invest **119**(9): 2663-2677.

Menke, C., T. Goncharov, et al. (2011). "TRAIL receptor signaling regulation of chemosensitivity in vivo but not in vitro." PLoS One **6**(1): e14527.

Micheau, O., S. Shirley, et al. (2013). "Death receptors as targets in cancer." Br J Pharmacol **169**(8): 1723-1744.

Mongkolsapaya, J., A. E. Cowper, et al. (1998). "Lymphocyte inhibitor of TRAIL (TNF-related apoptosis-inducing ligand): a new receptor protecting lymphocytes from the death ligand TRAIL." J Immunol **160**(1): 3-6.

Mrugala, M. M. (2013). "Advances and challenges in the treatment of glioblastoma: a clinician's perspective." Discov Med **15**(83): 221-230.

Nair, S., R. R. Nair, et al. (2008). "Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway." Mol Carcinog **47**(1): 22-33.

Nam, K., C. Honer, et al. (2004). "Structural components of SCAN-domain dimerizations." Proteins **56**(4): 685-692.

Naresh, R. A., N. Udupa, et al. (1996). "Niosomal plumbagin with reduced toxicity and improved anticancer activity in BALB/C mice." J Pharm Pharmacol **48**(11): 1128-1132.

Network-CGA (2008). "Comprehensive genomic characterization defines human glioblastoma genes and core pathways." Nature **455**(7216): 1061-1068.

Ng, G., D. Winder, et al. (2007). "Gain and overexpression of the oncostatin M receptor occur frequently in cervical squamous cell carcinoma and are associated with adverse clinical outcome." J Pathol **212**(3): 325-334.

Ng, K. T., K. Man, et al. (2006). "Clinicopathological significance of homeoprotein Six1 in hepatocellular carcinoma." Br J Cancer **95**(8): 1050-1055.

Nuttall, R. K., C. J. Pennington, et al. (2003). "Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells." Mol Cancer Res **1**(5): 333-345.

Ohgaki, H., P. Dessen, et al. (2004). "Genetic pathways to glioblastoma: a population-based study." Cancer Res **64**(19): 6892-6899.

Oliver, G., R. Wehr, et al. (1995). "Homeobox genes and connective tissue patterning." Development **121**(3): 693-705.

Ono, H., I. Imoto, et al. (2012). "SIX1 promotes epithelial-mesenchymal transition in colorectal cancer through ZEB1 activation." Oncogene **31**(47): 4923-4934.

Ostman, A. (2004). "PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma." Cytokine Growth Factor Rev **15**(4): 275-286.

Ostrom, Q. T., L. Bauchet, et al. (2014). "The epidemiology of glioma in adults: a "state of the science" review." Neuro Oncol.

Pan, G., J. Ni, et al. (1997). "An antagonist decoy receptor and a death domain-containing receptor for TRAIL." Science **277**(5327): 815-818.

Pan, G., J. Ni, et al. (1998). "TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling." FEBS Lett **424**(1-2): 41-45.

Panner, A., C. A. Crane, et al. (2009). "A novel PTEN-dependent link to ubiquitination controls FLIPS stability and TRAIL sensitivity in glioblastoma multiforme." Cancer Res **69**(20): 7911-7916.

Parimala, R. and P. Sachdanandam (1993). "Effect of Plumbagin on some glucose metabolising enzymes studied in rats in experimental hepatoma." Mol Cell Biochem **125**(1): 59-63.

Parsons, D. W., S. Jones, et al. (2008). "An integrated genomic analysis of human glioblastoma multiforme." Science **321**(5897): 1807-1812.

Pearson, J. C., D. Lemons, et al. (2005). "Modulating Hox gene functions during animal body patterning." Nat Rev Genet **6**(12): 893-904.

Pitti, R. M., S. A. Marsters, et al. (1996). "Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family." J Biol Chem **271**(22): 12687-12690.

Qiu, J. X., Y. Q. He, et al. (2013). "Plumbagin induces the apoptosis of human tongue carcinoma cells through the mitochondria-mediated pathway." Med Sci Monit Basic Res **19**: 228-236.

Ramana, P. V. and A. R. Reddy (2012). "Synthesis of 1,2,3-Triazole Substituted Isoxazoles via Copper (I) Catalyzed Cycloaddition." Journal of Heterocyclic Chemistry **49**(3): 621-627.

Rao, R. D., J. H. Uhm, et al. (2003). "Genetic and signaling pathway alterations in glioblastoma: relevance to novel targeted therapies." Front Biosci **8**: e270-280.

Ravindra, K. C., B. R. Selvi, et al. (2009). "Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin." J Biol Chem **284**(36): 24453-24464.

Reifenberger, G., L. Liu, et al. (1993). "Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations." Cancer Res **53**(12): 2736-2739.

Relaix, F. and M. Buckingham (1999). "From insect eye to vertebrate muscle: redeployment of a regulatory network." Genes Dev **13**(24): 3171-3178.

Rousseau-Merck, M. F., K. Huebner, et al. (1991). "Chromosomal localization of two human zinc finger protein genes, ZNF24 (KOX17) and ZNF29 (KOX26), to 18q12 and 17p13-p12, respectively." Genomics **9**(1): 154-161.

Ruf, R. G., P. X. Xu, et al. (2004). "SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes." Proc Natl Acad Sci U S A **101**(21): 8090-8095.

Sagar, S., L. Esau, et al. (2014). "Cytotoxicity and apoptosis induced by a plumbagin derivative in estrogen positive MCF-7 breast cancer cells." Anticancer Agents Med Chem **14**(1): 170-180.

Sanchez-Cruz, P. and A. E. Alegria (2009). "Quinone-enhanced reduction of nitric oxide by xanthine/xanthine oxidase." Chem Res Toxicol **22**(5): 818-823.

Sander, G., R. C. Marsh, et al. (1976). "Activity of the 30-S CsCl core in elongation-factor-dependent GTP hydrolysis." Eur J Biochem **61**(1): 317-323.

Sandur, S. K., H. Ichikawa, et al. (2006). "Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents." J Biol Chem **281**(25): 17023-17033.

Sandur, S. K., M. K. Pandey, et al. (2010). "5-hydroxy-2-methyl-1,4-naphthoquinone, a vitamin K3 analogue, suppresses STAT3 activation pathway through induction of protein tyrosine phosphatase, SHP-1: potential role in chemosensitization." Mol Cancer Res **8**(1): 107-118.

Sareddy, G. R., K. Geeviman, et al. (2012). "Increased beta-catenin/Tcf signaling in pilocytic astrocytomas: a comparative study to distinguish pilocytic astrocytomas from low-grade diffuse astrocytomas." Neurochem Res **37**(1): 96-104.

Sareddy, G. R., K. Geeviman, et al. (2012). "The nonsteroidal anti-inflammatory drug celecoxib suppresses the growth and induces apoptosis of human glioblastoma cells via the NF-kappaB pathway." J Neurooncol **106**(1): 99-109.

Sareddy, G. R., D. Kesanakurti, et al. (2013). "Nonsteroidal anti-inflammatory drugs diclofenac and celecoxib attenuates Wnt/beta-catenin/Tcf signaling pathway in human glioblastoma cells." Neurochem Res **38**(11): 2313-2322.

Sareddy, G. R., M. Panigrahi, et al. (2009). "Activation of Wnt/beta-catenin/Tcf signaling pathway in human astrocytomas." Neurochem Int **55**(5): 307-317.

Sathornsumetee, S. and J. N. Rich (2008). "Designer therapies for glioblastoma multiforme." Ann N Y Acad Sci **1142**: 108-132.

Schaller, B. J., M. Modo, et al. (2007). "Molecular imaging of brain tumors: a bridge between clinical and molecular medicine?" Mol Imaging Biol **9**(2): 60-71.

Schittenhelm, J., E. I. Schwab, et al. (2013). "Longitudinal expression analysis of alphav integrins in human gliomas reveals upregulation of integrin alphavbeta3 as a negative prognostic factor." J Neuropathol Exp Neurol **72**(3): 194-210.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Schumacher, C., H. Wang, et al. (2000). "The SCAN domain mediates selective oligomerization." J Biol Chem **275**(22): 17173-17179.

Seo, H. C., J. Curtiss, et al. (1999). "Six class homeobox genes in drosophila belong to three distinct families and are involved in head development." Mech Dev **83**(1-2): 127-139.

Serkov, I. V. and V. V. Bezuglov (2009). "Synthesis of new nitroxyalkylamides as potential prototypes of hybrid nonsteroidal anti-inflammatory drugs containing NO-donating fragment." Doklady Chemistry **425**: 88-90.

Shih, A. H. and E. C. Holland (2006). "Platelet-derived growth factor (PDGF) and glial tumorigenesis." Cancer Lett **232**(2): 139-147.

Shirley, S., A. Morizot, et al. (2011). "Regulating TRAIL receptor-induced cell death at the membrane : a deadly discussion." Recent Pat Anticancer Drug Discov **6**(3): 311-323.

Sinha, S., K. Pal, et al. (2013). "Plumbagin inhibits tumorigenesis and angiogenesis of ovarian cancer cells in vivo." Int J Cancer **132**(5): 1201-1212.

Smyth, M. J., K. Takeda, et al. (2003). "Nature's TRAIL--on a path to cancer immunotherapy." Immunity **18**(1): 1-6.

Sohail, A., Q. Sun, et al. (2008). "MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer." Cancer Metastasis Rev **27**(2): 289-302.

Soni, D., J. A. King, et al. (2005). "Genetics of glioblastoma multiforme: mitogenic signaling and cell cycle pathways converge." J Clin Neurosci **12**(1): 1-5.

Stupp, R., W. P. Mason, et al. (2005). "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma." N Engl J Med **352**(10): 987-996.

Sugie, S., K. Okamoto, et al. (1998). "Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats." Cancer Lett **127**(1-2): 177-183.

Suh, K. S., J. M. Crutchley, et al. (2007). "Reciprocal modifications of CLIC4 in tumor epithelium and stroma mark malignant progression of multiple human cancers." Clin Cancer Res **13**(1): 121-131.

Sun, Y., J. Liu, et al. (2008). "Expression of Ig-like transcript 4 inhibitory receptor in human non-small cell lung cancer." Chest **134**(4): 783-788.

Swerdlow, A. J., M. Feychting, et al. (2011). "Mobile phones, brain tumors, and the interphone study: where are we now?" Environ Health Perspect **119**(11): 1534-1538.

Tandle, A., D. G. Blazer, 3rd, et al. (2004). "Antiangiogenic gene therapy of cancer: recent developments." J Transl Med **2**(1): 22.

Thasni, K. A., S. Rakesh, et al. (2008). "Estrogen-dependent cell signaling and apoptosis in BRCA1-blocked BG1 ovarian cancer cells in response to plumbagin and other chemotherapeutic agents." Ann Oncol **19**(4): 696-705.

Thorburn, A., K. Behbakht, et al. (2008). "TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them." Drug Resist Updat **11**(1-2): 17-24.

Tian, L., D. Yin, et al. (2012). "Plumbagin induces apoptosis via the p53 pathway and generation of reactive oxygen species in human osteosarcoma cells." Mol Med Rep **5**(1): 126-132.

Tran, B. and M. A. Rosenthal (2010). "Survival comparison between glioblastoma multiforme and other incurable cancers." J Clin Neurosci **17**(4): 417-421.

Van Meir, E. G., C. G. Hadjipanayis, et al. (2010). "Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma." CA Cancer J Clin **60**(3): 166-193.

Verhaak, R. G., K. A. Hoadley, et al. (2010). "Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1." Cancer Cell **17**(1): 98-110.

Vougiouklakis, T., A. Mitselou, et al. (2006). "Sudden death due to primary intracranial neoplasms. A forensic autopsy study." Anticancer Res **26**(3B): 2463-2466.

Walczak, H., M. A. Degli-Esposti, et al. (1997). "TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL." EMBO J **16**(17): 5386-5397.

Walczak, H., R. E. Miller, et al. (1999). "Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo." Nat Med **5**(2): 157-163.

Wan, F., X. Miao, et al. (2008). "Gene expression changes during HPV-mediated carcinogenesis: a comparison between an in vitro cell model and cervical cancer." Int J Cancer **123**(1): 32-40.

Wang, C. A., P. Jedlicka, et al. (2012). "SIX1 induces lymphangiogenesis and metastasis via upregulation of VEGF-C in mouse models of breast cancer." J Clin Invest **122**(5): 1895-1906.

Wang, C. C., Y. M. Chiang, et al. (2008). "Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells." Cancer Lett **259**(1): 82-98.

Wang, D., J. C. Anderson, et al. (2005). "The role of the extracellular matrix in angiogenesis in malignant glioma tumors." Brain Pathol **15**(4): 318-326.

Wang, H., R. Sun, et al. (2008). "Characterization of the target DNA sequence for the DNA-binding domain of zinc finger protein 191." Acta Biochim Biophys Sin (Shanghai) **40**(8): 704-710.

Wang, L. F., H. N. Chiang, et al. (2002). "Kinetics and hydrolysis mechanism of polymeric prodrugs containing ibuprofen, ketoprofen, and naproxen as pendent agents." J Biomater Sci Polym Ed **13**(3): 287-299.

Wei, K. C., C. Y. Huang, et al. (2010). "Evaluation of the prognostic value of CD44 in glioblastoma multiforme." Anticancer Res **30**(1): 253-259.

Wikstrand, C. J., C. J. Reist, et al. (1998). "The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target." J Neurovirol **4**(2): 148-158.

Wiley, S. R., K. Schooley, et al. (1995). "Identification and characterization of a new member of the TNF family that induces apoptosis." Immunity **3**(6): 673-682.

Wu, G. S., T. F. Burns, et al. (1997). "KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene." Nat Genet **17**(2): 141-143.

Wu, W., Z. Ren, et al. (2014). "Six1: A critical transcription factor in tumorigenesis." Int J Cancer.

Xiao, X., L. Wang, et al. (2013). "Role of MUC20 overexpression as a predictor of recurrence and poor outcome in colorectal cancer." J Transl Med **11**: 151.

Xu, T. P., H. Shen, et al. (2013). "Plumbagin from Plumbago Zeylanica L induces apoptosis in human non-small cell lung cancer cell lines through NF- kappaB inactivation." Asian Pac J Cancer Prev **14**(4): 2325-2331.

Yan, H., D. W. Parsons, et al. (2009). "IDH1 and IDH2 mutations in gliomas." N Engl J Med **360**(8): 765-773.

Yeung, B. H., A. Y. Law, et al. (2012). "Evolution and roles of stanniocalcin." Mol Cell Endocrinol **349**(2): 272-280.

Yu, Y., E. Davicioni, et al. (2006). "The homeoprotein six1 transcriptionally activates multiple protumorigenic genes but requires ezrin to promote metastasis." Cancer Res **66**(4): 1982-1989.

Yu, Y., J. Khan, et al. (2004). "Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators." Nat Med **10**(2): 175-181.

Zamai, L., M. Ahmad, et al. (1998). "Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells." J Exp Med **188**(12): 2375-2380.

Zheng, H., C. Wasylyk, et al. (2003). "The transcription factor Net regulates the angiogenic switch." Genes Dev **17**(18): 2283-2297.

Zheng, W., L. Huang, et al. (2003). "The role of Six1 in mammalian auditory system development." Development **130**(17): 3989-4000.

Zou, D., D. Silvius, et al. (2004). "Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes." Development **131**(22): 5561-5572.