Activation of Wnt/β-catenin/Tcf signaling pathway in gliomas: A potential target for nonsteroidal anti-inflammatory drugs celecoxib and diclofenac

A thesis submitted to the University of Hyderabad
for the award of a Ph.D. degree in Department of Animal Sciences

By

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DECLARATION

I **Gangadhara Reddy Sareddy** hereby declare that this thesis entitled **"Activation of Wnt/β-catenin/Tcf signaling pathway in gliomas: A potential target for nonsteroidal anti-inflammatory drugs celecoxib and diclofenac"** 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.

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This is to certify that this thesis entitled **"Activation of Wnt/β-catenin/Tcf signaling pathway in gliomas: A potential target for nonsteroidal anti-inflammatory drugs celecoxib and diclofenac"** is a record of bonafide work done by **Gangadhara Reddy Sareddy** a research scholar for Ph.D. programme in Department of Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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Prof. P. Prakash Babu
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//Countersigned//

Head
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Dedicated to my beloved Grandparents

LATE. SMT. Y. LAKSHAMMA

LATE. SRI. Y. OBI REDDY

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S. Gangadhara Reddy

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ABBREVIATIONS

AA: Anaplastic astrocytoma

ALP: Alkaline peroxidase

APC: Adenomatous polyposis coli

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

Bcl-2: B-cell lymphoma protein-2

CDK4: Cyclin-dependent kinase 4

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CK1α: Casein kinase 1α

CNS: Central nervous system

Cox-2: Cyclo-oxygenase-2

DA: Diffuse astrocytoma

Dvl: Dishevelled

EDTA: Ethylene diamine tetra acetate

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ENU: n-Ethyl n-nitrosourea

ERK[1]/2: Extracellular signal regulated kinase ½

FGF-2: Fibroblast growth factor-2

FZD: Frizzled

GBM : Glioblastoma multiforme

GFAP: Glial fibrillary acidic protein

GSK3β: Glycogen synthase kinase 3β

HNGC-1: Human neural glial cell line-1

HNGC-2: Human neural glial cell line-2

IκB: Inhibitor of κB

JNK: c-Jun NH_2 -terminal kinase

LRP 5, 6: LDL receptor-related protein 5, 6

LEF1: Lymphoid enhancing factor 1

LOH: Loss of heterozygosity

MAPK: Mitogen activated protein kinase

MDM2: Mouse double minute-2 protein

MEF: Mouse embryo fibroblasts

MNU: n-Methyl n-nitrosourea

NBT: Nitro-blue tetrazolium chloride

NF-κB: Nuclear factor κB

NPC: Neuronal precursor cells

P180: Postnatal day-180

P135: Postnatal day-135

P90: Postnatal day-90

PARP: Poly (ADP-ribosyl) polymerase

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

PDGFR: Platelet derived growth factor receptor

PGE2: Prostaglandins E2

PI3K: Phospho-Inositol-3-Kinase

PKB: Protein kinase B

PMSF: Phenyl methyl sulphonyl fluoride

PTEN: Phosphatase tensin homologue

Rb1: Retinoblastoma 1 protein

RIPA: Radioimmunoprecipitation assay

RTK: Receptor tyrosine kinase

SAPK: Stress activated protein kinase

SVZ: Sub-ventricular zone

TBS: Tris-buffered saline

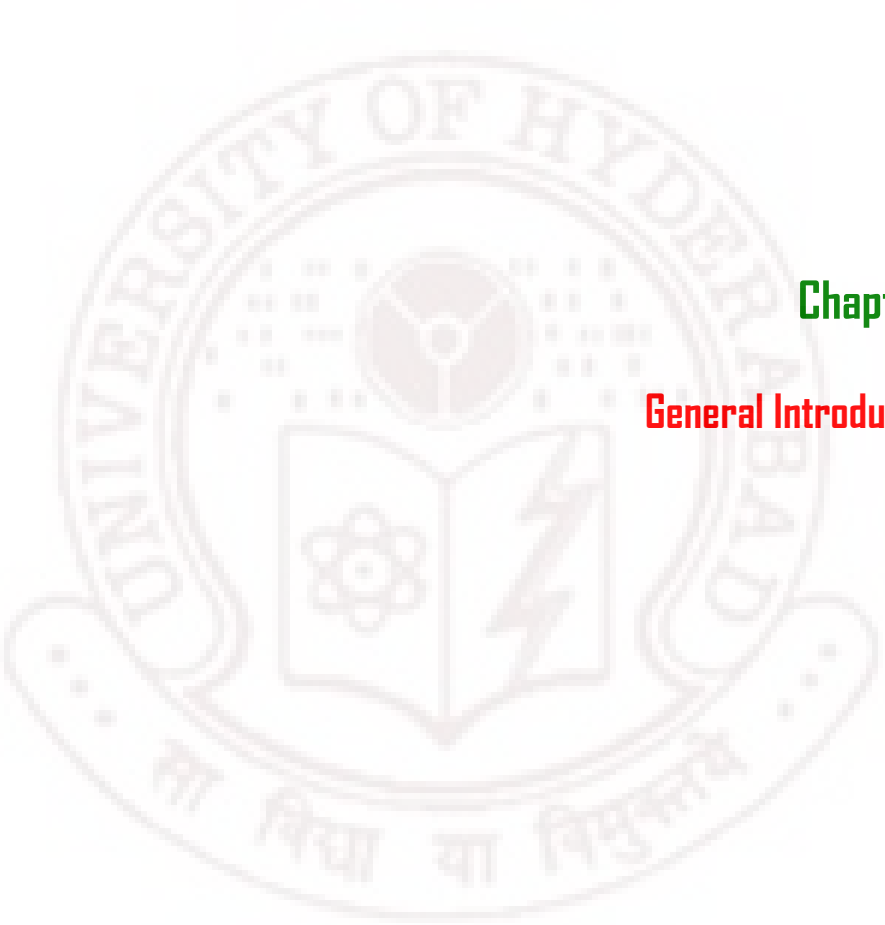
TBST: Tris-buffered saline Tween 20

TCF: T cell facotr

TEP1: TEnsin-like phosphatase

TNFα: Tumor necrosis factor α

WHO: World health organization



Chapter-1

General Introduction

1.1. Introduction

Gliomas are the most common primary brain tumors and accounts for about 1.7% of all cancers and 77% of all brain tumors. The term “glioma” was coined by Rudolf Virchow in 1860 and encompasses all tumors that are originated from glial cells. Gliomas have an annual incidence of 5-10 cases per 100,000 in western population and are a leading cause of death among children and adults diagnosed with a neoplasia of the brain. Gliomas are the heterogeneous group of tumors that are broadly classified into oligodendrogliomas, ependymomas, astrocytomas and mixed oligoastrocytomas based on the specific type of cell to which they closely resemble.

1.2. World Health Organization Classification

World Health Organization (WHO) classified the astrocytomas into four clinical grades on the basis of analysis of the most malignant tumor region, number of mitoses, nuclear atypia, microvascular proliferation and presence of necrosis. These includes pilocytic astrocytoma (WHO grade I), diffuse astrocytoma (WHO grade II) anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (WHO grade IV). Among these glioblastoma multiforme (GBM) is most malignant, aggressive and devastating form with a worse prognosis. Patients with GBM have a mean survival of about 1 year, where as patients with grade III or anaplastic gliomas survive for 2-3 years, and those with grade II gliomas can survive for as long as 10-15 years (Kleihues and Cavenee, 1997; Kleihues et al, 2000; Louis et al, 2007; Gonzales, 2001; Lopes et al, 2001). Grade I gliomas, also known as pilocytic

astrocytomas are childhood brain tumors, benign in nature with circumscribed and well differentiated tumor margins. The tumor cells are in piloid shape and the presence of microvascular proliferation is evident. These tumors are curable by surgery and might represent a separate disease from the gliomas of other grades.

Diffuse astrocytomas (grade II) are predominantly manifested in young adults (~34 years), characterized by high degree of cellular differentiation, slow growth and diffuse infiltration of neighboring structures and absence of microvascular proliferation and necrosis and single mitoses may be or may not be present. The tendency for malignant progression to anaplastic astrocytoma and eventually to GBM is evident. Molecular genetic features of diffuse astrocytoma include point mutations in the TP53 tumor suppressor gene (50-80%), overexpression of PDGF-A and PDGFR-α is observed in astrocytic tumors of all stages (60%), but gene amplification was only detected in a small subset (<10%) of secondary GBM (Nupponen et al, 2006). Anaplastic astrocytoma (grade III) arise *de novo* or from less malignant diffuse astrocytoma. Males are frequently affected and the mean age is 41 years. Histopathologically, there is increased cellularity, distinct nuclear atypia and marked mitotic activity. Micro vascular proliferation and necrosis are not present. They show tendency for malignant progression to GBM. Anaplastic astrocytoma (III) has a higher frequency of TP53 mutations. Additional genetic changes include p16 and p19 deletion, RB alterations, and LOH on chromosome 19q (50%) (Nupponen et al, 2006; Von Deimling et al, 1993).

Glioblastomas are the heterogeneous intraparenchymal masses and the most common malignant primary brain tumor in adults. These tumors

show the histological evidences of cellular polymorphism, nuclear atypia, mitotic activity, microvascular proliferation, vascular thrombosis and necrosis. Microscopically, they consists of several cell types: the glioma cell proper, hyper proliferative endothelial cells, macrophages and trapped cells of normal brain structures that are overrun by the invading glioma. Several histological characteristics are used to grade and define gliomas. These include regions of necrosis, in which necrotic areas are surrounded by densely packed tumor cell nuclei and are referred as “pseudopalisading” necrosis. In addition, the blood vessels both with and adjacent to the tumor are hypertrophied. Furthermore, the nuclei of tumor cells are extremely variable in size and shape, a characteristic called nuclear polymorphism. Tumor cells characteristically invade the adjacent normal brain parenchyma, migrating through the white matter tracts to collect around blood vessels, neurons and at the edge of the brain parenchyma in the subpial region (Ohgaki and Kleihues, 1999). Characteristic histopathological features of astrocytomas of all grades are summarized in table 1.1.

GBM may develop *de novo* (primary GBM) or from less malignant precursor lesion (secondary GBM). However, the majority of develop *de novo* with short clinical history usually less than 3 months. They may manifest at any age, but are more common in adults (~55 years). The males are more frequently affected. The secondary GBM occur in younger age group (~39 years), show a slightly more favorable outcome and develop far less often than primary GBM (Lacroix et al, 2001; Simmons et al, 2001; Holland, 2001). The time interval for progression from diffuse low-grade astrocytoma to secondary GBM varies considerably (~4-5 years). However, with regard to

histopathological and immunohistochemical features there are no differences between primary and secondary GBM (Ohgaki and Kleihues, 1999). Comparative gene expression analysis

Table 1.1. Clinico-pathological features of astrocytomas

Tumor	Age	Mean survival (years)	Histological characteristics
Pilocytic astrocytoma (grade I)	children's	almost curable	marked heterogeneity in cellular density, pleomorphism, proliferation and microglia distribution
Diffuse astrocytoma (grade II)	~35	~10-15 years	high degree of cellular differentiation, slow growth and diffuse infiltration of neighboring structures
Anaplastic astrocytoma (grade III)	~41	~2-3 years	increased cellularity, marked mitotic activity and nuclear atypia
Glioblastoma multiforme (grade IV)	~55 (1^0 GBM) ~39 (2^0 GBM)	~1 year	cellular pleomorphism, nuclear atypia, mitotic activity, microvasular proliferation, vascular thrombosis and necrosis

showed that primary and secondary GBM have distinct expression profiles, however, half of the clinically determined primary GBM has a similar pattern to

the secondary GBM. These suggest that primary GBM may originate from a clinically undiagnosed lower grade lesion.

1.3. Genetic alterations associated with primary GBM and Secondary GBM

Primary and secondary GBM are distinct in their molecular features. Chromosomal aberrations of gliomas have been studied using karyotyping, comparative genomic hybridization (CGH), or chromosome painting (Hui et al, 2001). The chromosomal aberrations identified in primary GBM are distinct from those of secondary GBM. However, there are also chromosomal aberrations that are shared by both primary and secondary GBM. The most chromosomal aberrations associated with primary GBM are amplifications and gains of 7p, 12q13-21, and chromosome 19. The main chromosomal regions showing losses are 10q, 9p, 13q, and 22q. In addition primary GBM may harbors additional aberrations such as losses of 18q, 16p, and 19q, and gains of 20q, and 12q. Diffuse astrocytoma associated with gains of 3q, 4q, 7q, 12p, and 19p, and losses of Xp, 1p, and 19q. Anaplastic astrocytoma associated with losses of 9p, 10q, 13q and gains of 1q, 7p, 11q, and Xq (Koschny et al, 2002; Roerig et al, 2005).

The genetic hallmark of primary GBM that typically lack a TP53 mutation is MDM2 amplification/overexpression (50%). Additional genetic changes are EGFR amplification (40% of cases) and/or overexpression (60%), CDKN2-A, CDKN2-B and PTEN mutations (30%), RB alteration and p16 deletion (30-40%) (Hayashi et al, 1997). Their genetic characteristics are LOH on the entire chromosome 10 (50-80%). The sequence in which gene

alterations are acquired is not known since these neoplasms develop very rapidly, without a clinically or histopathologically identifiable precursor lesion. The TP53 mutations are less common in primary GBM (<10%). Secondary GBM frequently associate with mutations of gene TP53. These mutations in more than 90% cases are already present in the first biopsy of diffuse low grade or anaplastic astrocytoma. Most likely, the TP53 mutation is the initial gatekeeper lesion in astrocytic tumors, which then, through genetic instability undergoes malignant progression. The pathway to secondary glioblastoma is further characterized by LOH on chromosomes 19q and 10q (but not on the entire chromosome 10 as it is seen in primary GBM) (Lang wt al, 1994; Von Deimling et al, 2000). Genetic alterations that are characteristic of primary and secondary GBM are represented in figure 1.1. Recently it was pointed out that genomic alterations of LOH 1p and 19q, which are observed in the majority of oligodendroglioma, may be observed in GBM. However, in contrast to oligodendroglioma, in GBM loss of 19q is more likely to be partial than complete and loss of 1p is uncommon (approx. 10%). It was suggested that combined losses of chromosome arms 1p and 19q may indicate better prognosis and potential sensitivity to chemotherapy in GBM patients, while isolated loss of either 1p or 19q is of no prognostic significance (Smith et al, 2000).

The genetic alterations identified to date in gliomas ultimately result in the abnormal activation of signal transduction pathways, down stream of receptor tyrosine kinases or disruption of cell cycle arrest pathways (Cavenee, 1992). For example, amplification or activating mutations of EGFR, over-expression of FGF, FGFR, and PDGF and PDGFR due to either gene

amplification or other epigenetic mechanisms, all lead to constitutive activation of corresponding receptor tyrosine kinase signaling (Hayashi et al, 1997; Louis, 1997). Subsequently, a number of down stream signal transduction pathways are activated, including the PI3 kinase/AKT pathway, RAS/MAP kinase pathway, C-MYC pathway, protein kinase C pathway, and STAT pathways (Holland, 2001; Ohgaki et al, 2007).

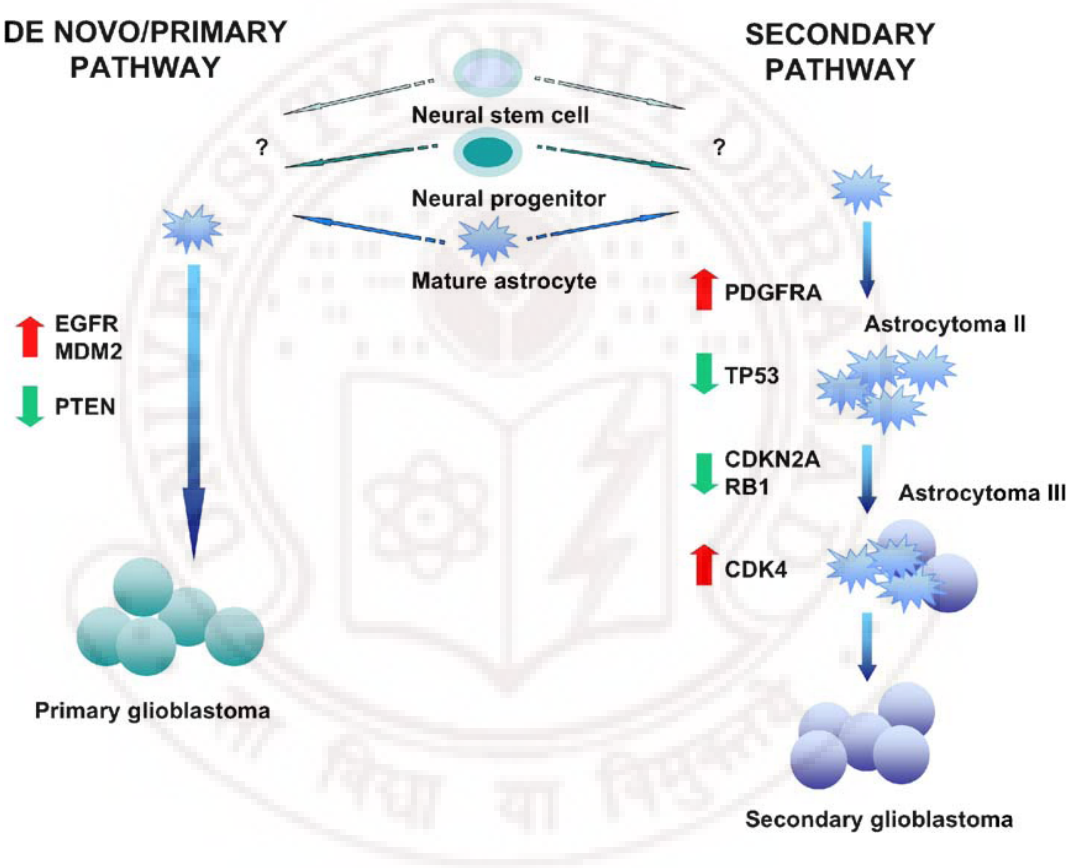


Figure 1.1. Genetic alterations associated with primary and secondary GBM

1.4. Epidemiology and clinical features

Approximately 13,000 deaths and 22,000 new cases of primary brain tumors are diagnosed annually in the United States. The Central Brain Tumor Registry of the United States (CBTRUS) annual reports indicated that brain tumor incidence is increasing and also showed that glioma accounts for 77%

of primary brain tumors (Inskip et al, 1995; CBTRUS, 2009). During the years 1998-2002, the average annual age-adjusted incidence rate of primary brain tumors among adults in the US was 9.0 per 100,000 persons. At population level most of the GBMs are primary GBM (95%), while the occurrence of secondary GBM is very low (5%) (Ohgaki et al, 2004; Ohgaki et al, 2005a). The incidence rate of low-grade and anaplastic astrocytomas is approximately two to three times higher than that of secondary GBM (Dropcho et al, 1996). This may be explained at least in part by the fact that a significant fraction of patients with low grade or anaplastic astrocytoma succumb to the disease before progression to GBM occurs (Ohgaki et al, 2005b). At the population level, the majority of patients with primary GBM (68%) had a clinical history of less than 3 months. The mean period from first symptoms to histological diagnosis was 6.3 months and the mean time to progression from anaplastic glioma to GBM was 2 years and that from low grade glioma to GBM was 5 years (Ohgaki et al, 2005a). In addition, there is striking difference in the age distribution of patients with primary GBM and secondary GBM. The prevalence of gliomas is more common in men than in women, although these gender differences are quite small in the case of oligodendroglioma. At population level, primary GBM develop more frequently in men (M/F ratio, 1:33), whereas secondary GBM are more frequent in women (M/F ratio, 0:65) (Ohgaki et al, 2004). This corroborates a previous observation that GBMs with TP53 mutations (a genetic hallmark of secondary GBM) are more common in women (Louis et al, 1993). The median survival of secondary GBM patients is 7.8 months, where as with that of primary GBM patients are 4.7 months. There is around four fold differences in the incidence of primary brain tumors

between countries with a high incidence (e.g. Australia, Canada, Denmark, Finland, New Zealand and the U S) and regions with a low incidence (e.g. Philippines and India) (Wrensch et al, 2002; Inskip et al, 1995).

1.5. Causes and risk factors for glioma development

The exact causes of gliomas are not known. But epidemiological data suggested that there are several factors that slightly increase the risk of developing gliomas including: 1) environmental and genetic risk factors, 2) genetic syndromes, familial aggregation and linkage, 3) immunologic risk factors and germline polymorphisms, 4) DNA repair gene polymorphisms, and 5) polymorphisms in cell cycle regulation.

The established environmental cause of glioma is exposure to therapeutic agents or high-dose radiation, although high-dose chemotherapy for treatment of cancers at sites other than brain has also been linked to this condition (Edick et al, 2005; Ohgaki et al, 2005). Recently it was reported that (Relling et al, 1999) among childrens treated with cranial irradiation and intensive antimetabolite therapy for acute lymphocytic leukemia, and those with germline polymorphisms leading to low or absent thiopurine methyltransferase activity were significantly more likely than those without such polymorphisms to subsequently develop gliomas.

Hereditary genetic disorders such as neurofibromatosis (type 1 and type 2) and tuberous sclerosis complex, retinoblastoma, Li-Fraumeni syndrome, and Turcot's syndrome and multiple hamartoma have been associated with increased risks for gliomas and are known to predispose to their development (Ichimura et al, 2004). Obesity during adolescence has a

three to four times greater risk of developing glioma than having normal weight. Being tall also increases the risk; each 10 centimeter increase in height increased the risk nearly 20 percent (schwartzbaum et al, 2006).

Recently it was reported that (Schwartzbaum et al, 2006) there was inverse/negative relationship between allergy histories or chicken pox, IgG antibodies to varicella-zoster virus (VZV), and high levels of IgE. In addition, single-nucleotide polymorphisms (SNPs) in genes related to GBM and genotypes that increase asthma risk confirming the inverse association between asthma and GBM (Brenner et al, 2002). Inherited variations in components of DNA repair pathways represent other risk factors in glioma development. Gliomas have been significantly associated with variants in ERCC1, ERCC2, the nearby gene GLTSCR1 (glioma tumor suppressor candidate of unknown function), PRKDC and MGMT (Wang et al, 2004). Moreover, there is positive correlation between glioma risk and cell cycle genes and metabolic enzymes (schwartzbaum et al, 2006).

1.6. Symptoms

The symptoms among glioma patients are highly variable and depend on the size of the tumor and its position in the brain. The most common symptoms are headaches and seizures (fits) affect 71.4% and 52.2% of glioma patients respectively. Headaches are usually worse in the morning but wear off as the day goes on. As the tumor grows, the intracranial pressure is increased in the skull which does not allow the brain to expand significantly. Other symptoms are secondary to effects upon nearby structures and inflammation associated with malignant invasion. Other symptoms include vomiting, deterioration in mental status (lethargy, personality changes,

combative or aggressive behavior, impaired cognition, etc.), memory loss, generalized or focal seizures (new onset), impairment of motion or sensation in a body region (often unilateral), difficulty in walking, hearing loss, visual field defects or loss of vision, giddiness, acquired inability to smell, tremor, nystagmus, urinary urgency or incontinence, difficulty in speaking or understanding what others are saying, excessive thirst or appetite, inappropriate denial of illness, injury or bodily defects, visual hallucinations are also observed.

1.7. Diagnosis

Patients with the symptoms of headache, seizures, vision problems, hearing problems, and alterations in balance, coordination and reflexes will be suspected of having brain tumor and neurosurgeon will suggest the following tests for further confirmation. Magnetic Resonance Imaging (MRI) scan uses magnetic fields to generate images of the brain and is particularly useful in diagnosing the brain tumors, because they outline the normal brain structures in detail. Sometimes a special dye is injected into the bloodstream during the procedure to help better distinguish tumors from healthy tissue (MRI angiogram). A Computed Tomography (CT) scan uses a sophisticated X-ray machine to produce detailed two dimensional images of the brain. A special dye may be injected into the bloodstream after a few CT scans to help better distinguish tumors (CT angiogram). In addition other brain scans such as angiogram, magnetic resonance spectroscopy (MRS), single-photon emission computed tomography (SPECT) or positron emission tomography (PET) scanning also helps in the diagnosing brain tumor.

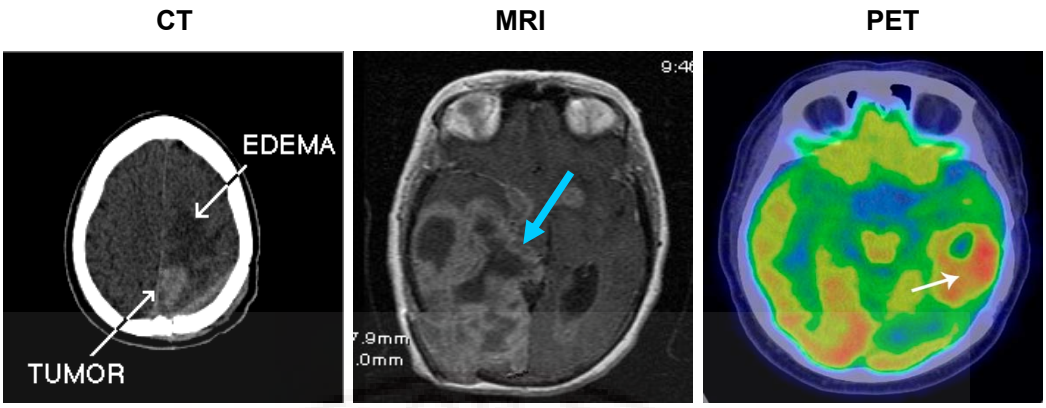


Figure 1.2. CT, MRI and PET scans of GBM

Respective CT, MRI and PET scans of GBM is shown in figure 1.2. These scans can be combined with MRIs, to understand a tumor's effects on brain activity and function. Biopsy is usually required to diagnose a brain tumor and confirm its type.

1.8. Treatment of gliomas

Treatment of the patient depends on the glioma type, size and location, as well as the patient's age and overall health. Surgery is the first line of treatment and neurosurgeons will remove as much of the tumor as possible, a process known as “debulking”. Surgical resection of tumor can cure most of the benign tumors such as pilocytic astrocytomas as well as meningiomas. But with the case of diffuse astrocytomas complete resection is not possible. Owing to the lack of clear cut boarders and diffusion or invasion in to the surrounding normal brain structures, most of the diffuse astrocytomas are not completely removed by the surgery. But tumor resection helps relieve symptoms by reducing pressure on the brain and reducing the size of the glioma to be treated by radiation and chemotherapy. Surgery is not always

possible with all gliomas, especially gliomas in the sensitive areas such as brainstem. In these situations chemotherapy and radiotherapy are the only advisable treatment options.

Often surgery is followed by the radiation therapy. Radiation therapy is an essential component of treatment for many patients with gliomas. It can be curative for some patients and prolongs survival for most. Clinically a wide variety of radiation techniques have been used in the treatment of GBM, including conventional radiotherapy, intensity-modulated radiotherapy, temporary or permanent brachytherapy, single or multi-fraction stereotactic radiosurgery, and photodynamic radiotherapy. It has been shown that the median survival time for patients undergoing repeated irradiation is between 10-12 months. Radiation therapy is associated with major disadvantages of affecting both systemic and local side effects and patients suffer from long term and short term side effects.

Chemotherapy is an important part of the care of glioma patients. For patients with glioblastomas, the most rapidly growing and aggressive glioma, the addition of chemotherapy to the radiation has been shown to significantly extend a patient's lifespan. However, chemotherapy is of limited use in the treatment procedure, due to heterogeneous biologic properties despite indistinguishable history, presence of blood-brain barrier, poorly understood cell kinetics, variations in tumor vasculature and structure, blood flow, permeability and due to variation in type and or extent of necrosis. Corticosteroids are indispensable for reducing the size of gliomas and controlling increased intracranial pressure and anti-seizure drugs are also

important drugs in the care of patients who experience seizures. The most commonly used drugs in the chemotherapy are temozolomide and the

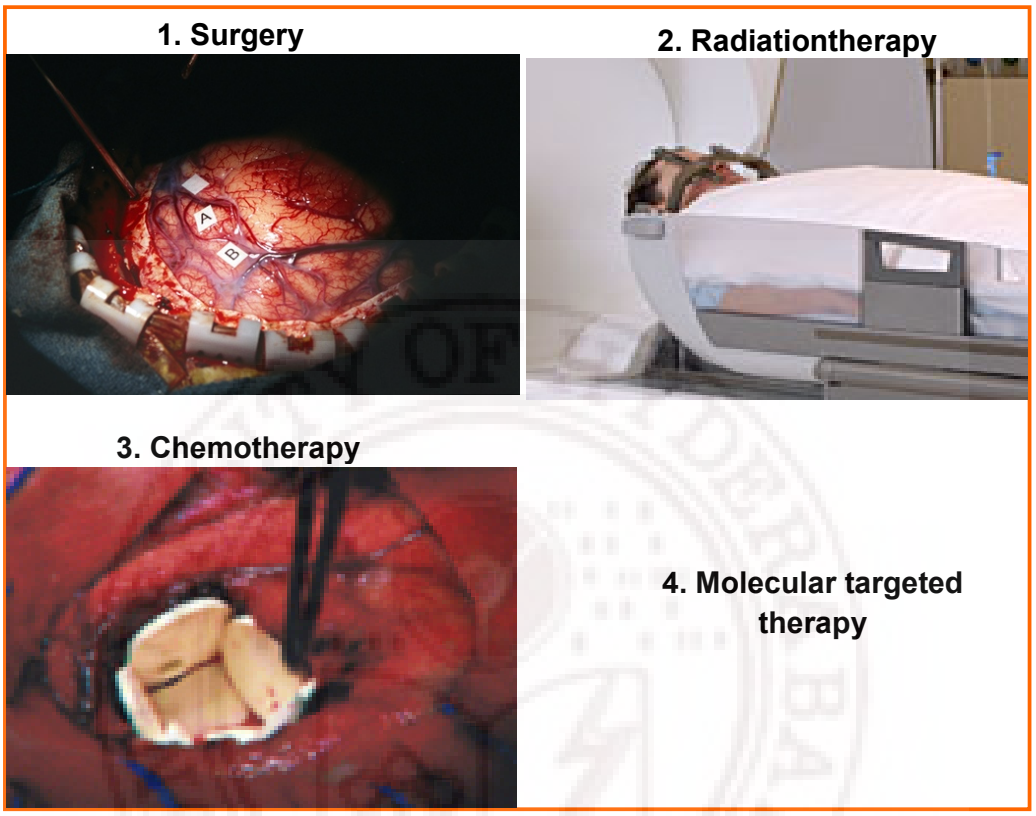


Figure 1.3. Treatment strategies for gliomas

combination of PCV (procarbazine, CCNU, and Vincristine). The alkylating agent temozolomide combined with radiation therapy and followed by adjuvant temozolomide, has become the standard care for patients with newly diagnosed GBM. Although, the development of new chemotherapeutic agents most of the GBMs display a high degree of resistance to chemotherapy. Investigations into the molecular mechanisms of drug resistance may give way to new therapeutic approaches. The patients with GBM containing a *MGMT* promoter benefited from radiation therapy and temozolomide and most of them survived at least 2 years, whereas those without a methylated promoter did not benefit to the same degree. These investigations indicate

that the methylation of the *MGMT* promoter may be a molecular marker for selecting patients who will respond to treatment with temozolomide and further studies of MGMT inhibitors could prove valuable.

Advances in the understanding of the cellular mechanisms involved in growth and progression of brain tumors has allowed the development of new drugs that block receptors on the tumor cell surfaces, affect intracellular chemical pathways (signaling pathways) or interact with proteins or DNA in the tumor cell nuclei. Critical gene amplifications, activating gene mutations, and over-expressed proteins in this respect may be useful targets for glioma therapy. Recently imatinib a specific receptor tyrosine kinase inhibitor used alone or in combination with hydroxyurea in treating GBM. Inhibitors that target EGFR, PI3K/AKT and mTOR signaling pathways are currently being

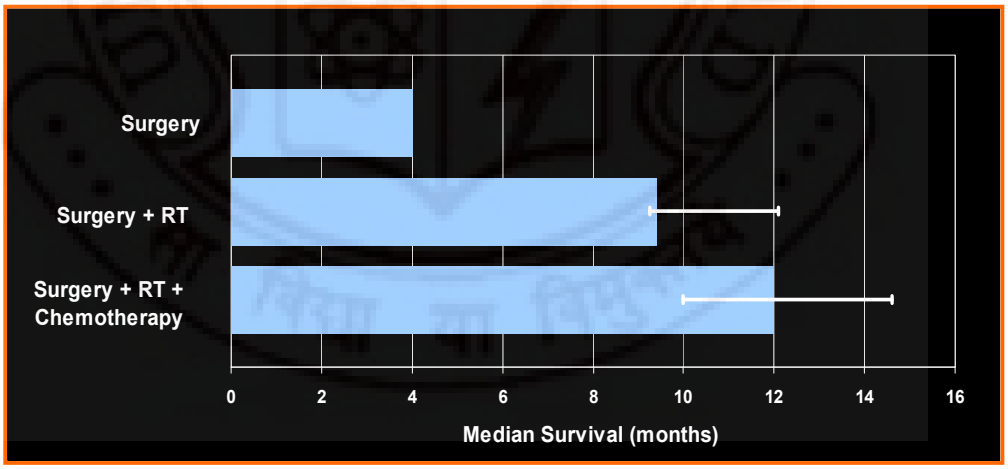


Figure. 1.4. Survival by treatment modality. Patients who are treated with surgery alone have survival duration of approximately 4 months. For those receiving surgery plus radiotherapy (RT), the survival duration improves to 9 months (range, 9.25-12.1 months). The addition of systemic chemotherapy provides modest benefit, extending survival to 12 months (range, 10-14.6 months). (Stewart, 2002)

assessed in the treatment of glioblastomas. Anti-angiogenic agents that inhibit VEGF signaling also have a therapeutic potential in the treatment of GBM (Nupponen et al, 2006). In spite of all these treatment options patients with GBM have a survival period of maximum one year. Various treatment strategies (figure 1.3) and the respective individual survival times were summarized in figure 1.4.

1.9. Cell of origin

The cell of origin of glioma has been subject to debate and many hypotheses were proposed on this issue. One hypothesis explains the morphological similarities of tumor cells to mature glial cells, and these considered as origin of gliomas and this could be done through the process of dedifferentiation. Most importantly glial cells are one of the few cells that are able to proliferate in the brain, but this hypothesis failed the explanation of origin of mixed gliomas (Dai et al, 2001). Another hypothesis suggests that neural stem cells are the origins of gliomas, because they persist in the subventricular zone of mature brain as well as their presence in the glial tumors. These hypothesis were summarized in figure 1.5.

Neural stem cells are immature progenitor cells with a long life span, and have been isolated from the subventricular zone, hippocampus, the lining of the lateral ventricles, and the dentate gyrus of an adult (Singh et al, 2004). The tumor initiating cells in glioma shares many similarities with normal neural stem cells such as expression of CD133, and glioblastomas, in particular may be seen as neoplasms of NPC (Beier et al, 2007). This suggest that the cell of origin to be a transformed neural stem cell. This hypothesis also explains the existence of mixed glial tumor such as oligoastrocytomas, since the NPC are

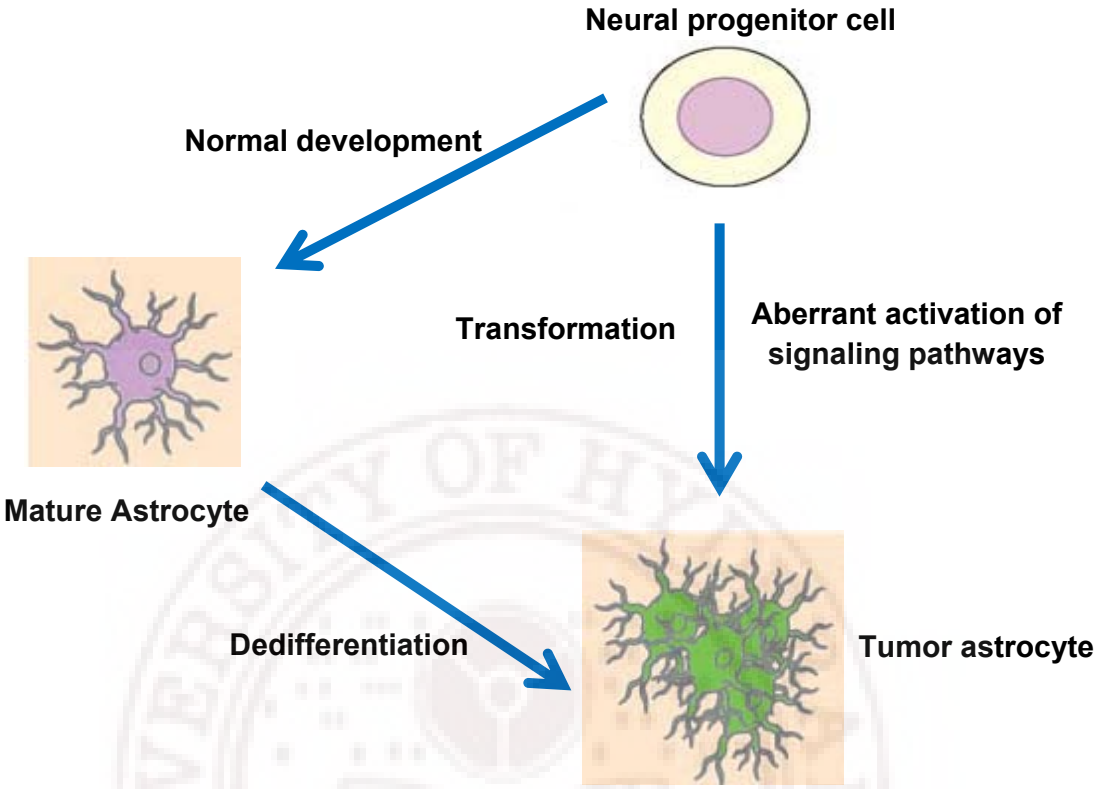


Figure 1.5. Cell-of-origin of gliomas

able to differentiate in to both the cell types, and transformed pluripotential cell may subsequently harbors some elements of either cell types (Gage, 2000; Sanai et al, 2005). Additionally, this offers a rationale for the resistance to treatment and recurrence, as the neoplastic progenitor may continue to seed the brain, despite local treatment to the tumor mass. Most recently it was reported that cell of origin for glioma may be a committed glial progenitor cell (Lindberg et al, 2009).

1.10. Experimental animal models

The gliomas are a large collection of primary brain tumors that have morphology and gene-expression characteristics similar to glial cells and their precursor cells. Many genetic alterations have been identified in human

gliomas, however, establishing unequivocal correlation between these genetic alterations and gliomagenesis requires accurate animal models for this disease. And there is a major need in experimental neuro-oncology for animal models that can be used to assess the efficacy of innovative approaches for the treatment of brain tumors. There are several reasons for modeling of gliomas, which include: 1) determining which genetic alterations identified in human gliomas are the actual etiological events, is to model the formation of gliomas by mimicking those genetic alteration in mice. 2) Accurate animal models are powerful tools to investigate important aspects of glioma biology that can not be studied in cell culture systems, such as angiogenesis, invasion, and metastasis.

Table 1.2. Different strategies of glioma models

Strategy	Principle
In vitro glioma models	
Migration assays a) Monolayer migration assay b) Spheroid migration assay **Invasion assays** a) Matrigel invasion assay b) C0-culture system	Cell motility and invasion
In vivo glioma models	
Mutagens	DNA Alkylation
Transplantation	Xeno or allograft, immunodeficient animals
Germline genetic modification	Transgene or gene targeting

There are several strategies for modeling gliomas. To date, four major strategies have been successfully employed to reach the goal: 1) chemical mutagen-induced models, germline genetic modification-induced models, 3) xeno or allograft transplantation-induced models, and 4) somatic genetic modification-induced models (table 1.2). Each of these strategies has characteristic features that are advantages or disadvantages depending on the application.

1.11. Chemical carcinogenesis

N-nitroso-compounds were first described as important environmental carcinogens (Magee and Barnes, 1967). Nitroso compounds and particularly nitrosamides are not only carcinogenic but also toxic, teratogenic and mutagenic (Magee, 1969). Highest exposure occurs in certain occupational situations including rubber industries, contaminated cutting fluids used in metal industries, life-style exposures such as smoking, tobacco chewing, certain cosmetics, house hold rubber products and drugs have shown to be containing low concentration of nitrosoamine impurities. Nitrosoamines display organ-specific and species specific carcinogenesis, and this organotropic action is predominantly governed by chemical structure of the compound, strain of the animal used, mode of application, dosage and the duration of exposure (Preussmann and Wiessler, 1977). Some of the agents were found to be relatively specific for tumors of the nervous system (Druckrey et al, 1972, 1973). Using the carcinogens, it was reported that simultaneous multiplication of cells of different origin would lead to glioma formation (Zimmerman, 1969). Among the different carcinogens methyl

nitrosourea (MNU), ethyl nitrosourea (ENU) and piperdine are most commonly used carcinogens to induce the nervous system tumors. Metabolism of these oncogens may ultimately result in carbonium ion and nitrogen ion formation (Druckrey et al, 1972). The sites of action may be upon –SH, –OH or –NH groups of proteins or nucleic acids. Alkylation of guanine in DNA has been observed with ethyl and methyl nitrosourea and observed to persist for longer time duration in the brain than in the liver (Wechsler et al, 1969; Goth and Rajewsky, 1974; Kleihues and Margison, 1974). Neoplasms produced by MNU and ENU have morphological and biological similarities to naturally occurring neural tumors in human and animals. An ethyl group in the nitrosourea results in a relative specificity of nervous system tumors by transplacental route of administration, where as methyl side chain results in high yields of these tumors after intravenous administration in the mature animal. MNU has to be given in the small repeated doses to adults to obtain highest incidence, whereas ENU exerts its maximal carcinogenic action transplacentally or in neonatal application.

1.12. ENU induced glioma rat model

Gliomas can be induced by treating rats with DNA alkylating agents, such as nitrosourea derivatives that generate point mutations. Histologically these tumors bear similarities to the equivalent tumors in humans. Chemical induction of gliomas by ethylnitrosourea has allowed the study of several aspects of tumor behavior, such as microvascular organization (Yoshimura et al, 1998), dedifferentiation (Jang et al, 2004), gene mutations (O'Neill, 2000), microcirculation, angiogenesis (Bulnes et al, 2007) and experimental therapeutic agents (Kish et al, 2001). Further, ENU-induced glioma rat model

is extensively utilized as experimental models of human gliomas because of their histopathological similarity to gliomas in humans. ENU has been used in a variety of paradigms to induce neural tumors including astrocytomas, oligodendroglioma, mixed glial tumors, schwannomas, meningiomas and medulloblastomas depending on dose, injection schedule, age of the animal, and species susceptibility (Bilzer et al, 1989; Diwan and Meier, 1974; Druckrey et al, 1966; Schlegel et al, 1994). Single intrauterine exposure to ENU may produce CNS tumors in rats, however, mice are relatively insensitive to this effect (Diwan and Meier, 1974; Wechsler et al, 1979). The ENU-induced glioma rat model is a well established animal model for studying the natural development of glioma and is representative model for human glioma because of its location and also of its similar molecular and genetic alterations. Transplacental neurocarcinogenesis induced by ENU, however, is an ideal model for the study of glial tumors, because essentially 100% of offspring develop neural tumors several months after a single maternal exposure in the late embryonic period (Druckrey et al, 1966; Koestner, 1990). Since ENU decomposes rapidly *in vivo* (Goth and Rajewsky, 1974), the interaction between the carcinogen and the relevant cellular macromolecules is brief and allows for a sequential study of tumor development. Several studies addressing the early stages of brain tumor development after *in utero* exposure to ENU have determined that hyperplastic lesions can be detected as early as 30 to 60 days of age (Ikeda et al, 1989; Lantos and Cox, 1976; Schiffer et al, 1978; Yoshino et al, 1985a,b).

1.13. Developmental signaling pathways

Morphogens play a critical role in many aspects of development. Major developmental pathways that regulate embryonic development and plays essential roles in pattern formation include 1) Wnt signaling pathway, 2) Hedgehog signaling pathway, 3) Notch signaling pathway, and 4) Bone Morphogenetic Protein/Transforming Growth Factor (BMP/TGF-β) signaling pathway. Particularly these pathways are crucial for proper development and

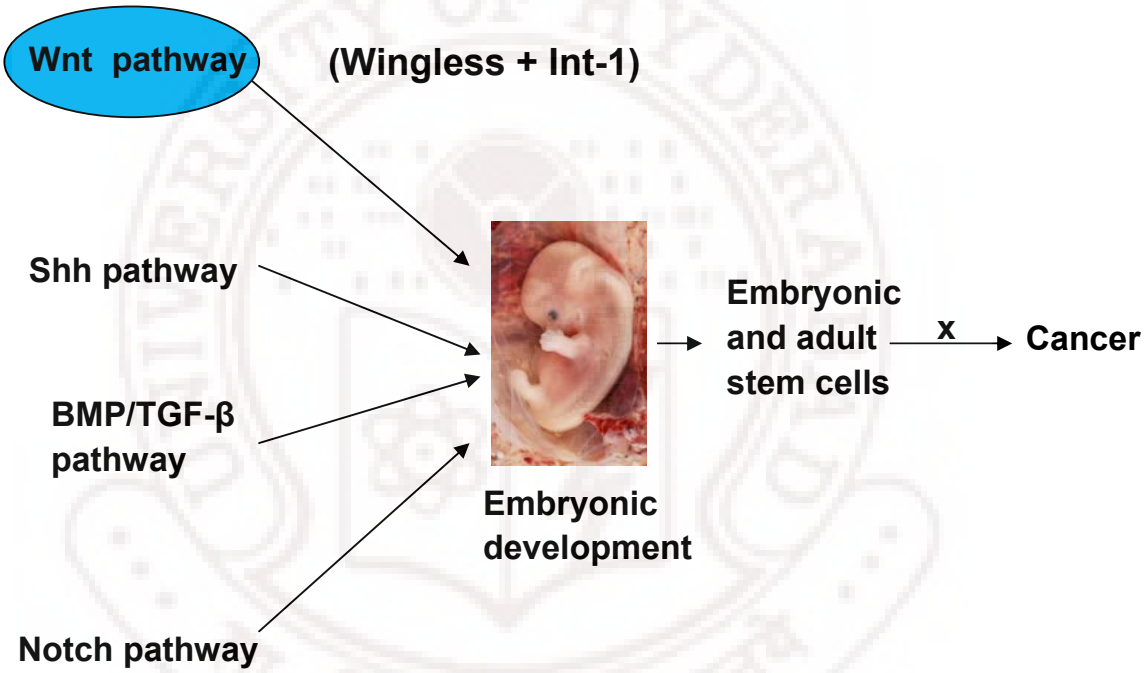


Figure 1.6. Major developmental signaling pathways in embryonic development and cancer

patterning of the central nervous system (figure 1.6). These pathways are essential in the maintenance of adult stem cells, particularly for neural stem cells that persist in the subventricular zone and other stem cell containing regions. The differentiation and self-renewal of stem cells and progenitor cells is tightly regulated phenomenon and understanding of how these critical steps

are regulated is challenging task. From embryological experiments, it is apparent that commitment to developmental lineages is a stepwise process, involving a succession of changes in cell state. The differentiation process is accomplished by supplying outside signals and extra-cellular factors. These signals and the micro environment maintain a balance between self-renewal and differentiation of stem cells. The best candidate factors that regulate cell fate decisions during development include, Wnt, Hedgehog, Notch, BMP and FGF molecules. Owing to their central role in highly regulated developmental processes, aberrant/deregulation of these pathways might leads to tumor formation. Indeed, increasing evidences suggest that these developmental pathways contribute to the growth and maintenance of a variety of human cancers. Among these, Wnt signaling pathway mediated functions are well described in CNS and its relation to carcinogenesis of several malignancies is well understood.

1.14. Wnt signaling pathway

During the development of nervous system neural precursor or progenitor cells (NPC) act as a source of various types of specialized cells in the brain. Several studies have suggested that these cells are able to self-renew, a hall mark of stem cells. Wnt signaling is a candidate pathway in controlling neural stem cells self-renewal and differentiation (Zechner et al, 2003). In addition, Wnt signaling is required at several stages of central nervous system development (figure 1.7) (Lee et al, 2000; Lie et al, 2005; Chenn et al, 2002). So, dramatic alterations in this pathway may leads to

development of CNS malignancies, and its oncogenic role was well studied in medulloblastoma development.

The Wnts comprise a large family of protein ligands that affect diverse processes such as embryonic induction, generation of cell polarity, and the specification of cell fate (Logan et al, 2004), tissue homeostasis, and cancer. The Wnt pathway activation in oncogenesis and its consequences has been extensively reviewed (Logan et al, 2004; Moon et al, 2004; Polakis, 1999; Reya and Clevers, 2005). It was originally described in Drosophila as Wingless pathway and is highly conserved among flies, frogs, and mammals. The combined effort of genetic, biochemical and developmental research has led to the comprehensive understanding of the Wnt pathway as it is known today.

The Wnt proteins (the name derived from mouse Int-1 and Drosophila wingless) are a family of secreted glycoproteins characterized by several conserved cysteine residues. To date 19 mammalian Wnt homologues are well characterized and expressed in temporal-spatial pattern. These Wnt molecules have the roles in regulating cell fate, differentiation, proliferation and potentially, tumor formation (Clevers, 2006; Polakis, 2000). A number of Wnt genes, including Wnt2, Wnt7b and Wnt 5a, have been associated with abnormal proliferation of human breast tissue and other tumors. Wnt10b and Wnt13 have been suggested to direct cell- growth regulation during development. In addition, several Wnt genes, including Wnt1, Wnt2, Wnt3a, Wnt5b and Wnt7b, have also been shown to induce cell transformation *in vitro*. To date, there are 10 human Frizzled receptors which are vary in length (537 to 706 amino acids) and all of them are seven pass transmembrane

receptors with an extra cellular N-terminal domain and an intracellular C-terminal domain.

Wnt signaling proteins binds specifically to Frizzled (Fzd) receptor complexes on the surface of target cells to activate distinct intracellular pathways that are broadly classified in to three different types (Pinson et al, 2000; Moon et al, 2002): they are canonical Wnt/β-catenin cascade, non-canonical planar cell polarity pathway (PCP) and Wnt/ca+2 pathway. In Wnt/ca+2 pathway Wnt proteins binds to a seven trans membrane Frizzled receptor which results in the activation of heterotrimeric G-proteins with subsequent mobilization of phospholipse C and phosphodiesterase. This results in a decrease in c-GMP, an increase in ca+2, and activation of protein kinase C (Kuhl et al, 2000). The PCP pathway activation determines polarity in select epithelial tissues, particularly along an axis perpendicular to the apical-basal border. In Drosophila, it is responsible for the orientation of cuticle hairs on wings. In vertebrates, it may contribute to the differentiation and orientation of inner ear hair cell stereo cilia, and direct the expansion of mesoderm and neuro-ectoderm during gastrulation. In this pathway, Wnt binding to Frizzled activates Dishevelled (Dvl), and recruits RhoA/Rac, which ultimately leads to JNK pathway activation. A major target of the JNK pathway is the AP-1 transcription factor (Heisenberg et al, 2000). The Wnt receptor complex that activates the canonical pathway contains two components: a member of the Frizzled family and either one of two single–span trans membrane proteins, low density-lipoprotein receptor related proteins (LRP5 and LRP6) (Jeannet and He, 2000). Once bound by their cognate ligands, the Fzd/LRP coreceptor complex activates the canonical signaling pathway

(Pinson et al, 2000; Tamai et al, 2000; Wehrli et al, 2000). Frizzled can physically interact with Dsh, a cytoplasmic protein that functions upstream of β-catenin. The binding of Axin to the LRP6 tail is promoted by phosphorylation of LRP6. Phosphorylation of LRP6 occurs on several clusters of serines and threonines, with a central PPPSP motif as a hallmark. Wnts are thought to induce the phosphorylation of the cytoplasmic tail of LRP, thus regulating the docking of Axin. GSK3 phosphorylates the PPP(S/T)P motif, whereas caseine kinase I-γ (CK1γ) phosphorylates multiple motifs close to the GSK3β sites. It remains presently debated whether Wnt controls GSK3β-mediated phosphorylation of LRP5/6 (Zeng et al, 2005) or whether CK1γ is the kinase regulated by Wnt (Davidson et al., 2005). When bound to their respective membrane receptors, Dsh and Axin may cooperatively mediate downstream activation events by heterodimerization through their respective DIX (Dishevelled-Axin) domains. Wnt signals are transduced by direct binding of Dishevelled to Axin. This results in the inhibition of GSK3β-dependent phosphorylation of β-catenin. Most likely this occurs through disintegration of the APC/Axin/GSK3β complex (Cliffe et al, 2003; Moon et al, 2004).

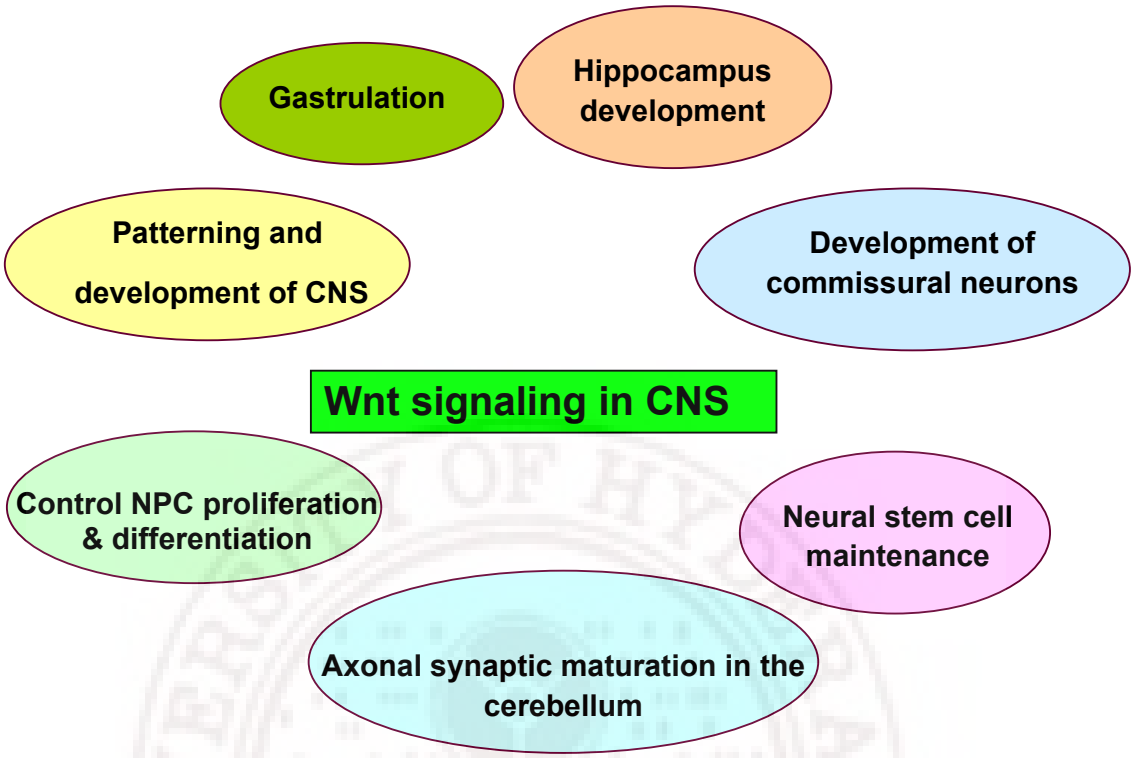


Figure 1.7. Wnt signaling key roles in central nervous system

The central player of the canonical signaling pathway is β-catenin, a cytoplasmic protein whose stability is regulated by the destruction complex. With in this complex the Axin and APC proteins form a scaffold that facilitates β-catenin phosphorylation by CK1α and GSK3β. Phosphorylated β-catenin is subsequently recognized and ubiquitinylated, resulting in its proteosomal degradation. The resulting low level of β-catenin allows the DNA binding Tcf/Lef proteins to interact with transcriptional co-repressors to block target genes expression in the nucleus (Brown and Moon, 1998; Wodarz and Nusse, 1998). Interaction of Wnt ligand with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dvl-Fzd complexes and the phosphorylation of LRP by CK1γ facilitating relocation of Axin to the membrane and inactivation of the destruction box. This allows β-

catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family. In the nucleus β-catenin converts the Tcf/Lef proteins into potent transcriptional activators by displacing groucho/TLE proteins and recruiting an array of co-activator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless, Mediator and Hyrax. This ensures efficient activation of Tcf target genes such as c-myc, n-myc, cyclinD1, c-jun, MMP7, VEGF, IL-8 etc (He et al, 1998; Shiina et al, 2003; Shtutman et al, 1999). The overview of Wnt signaling pathway is shown in figure 1.8.

In addition to its role in cancers, β-catenin has the role in cell-cell adhesion. It was first identified as counterpart of adherence junction. It is an essential binding partner for the cytoplasmic tail of E-cadherens. Newly synthesized β-catenin first saturates the pool that is part of the adherens junction, which never available for signaling. “Excess” free cytoplasmic β-catenin protein is then efficiently degraded by the APC complex.

Wnt antagonists can be divided into two functional classes, the sFRP (secreted Frizzled related protein) class and the Dickopf class: members of the sFRP class, which includes sFRP family (sFRP1, sFRP2, sFRP3, sFRP4, and sFRP5), WIF-1 and cerebrus, bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex. Certain dickopf family proteins inhibit Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex. Thus in theory those antagonists of the sFRP class will inhibit both canonical and noncanonical pathways, where as those of the dickopf class specifically inhibit the canonical pathways.

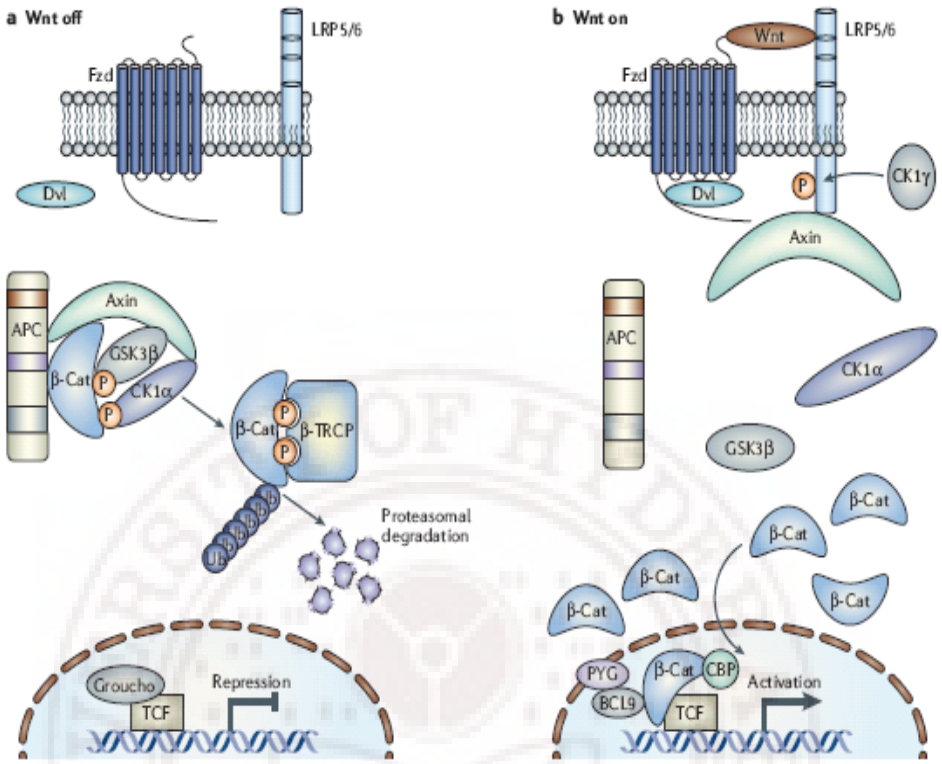


Figure 1.8. An overview of the Wnt signaling pathway. **(a)** In the absence of a Wnt signal, β-catenin is captured by APC and axin within the destruction complex, facilitating its phosphorylation by the kinases CK1α and GSK3β. CK1α and GSK3β then sequentially phosphorylate a conserved set of serine and threonine residues at the amino terminus of β-catenin. This facilitates binding of the β-TRCP, which subsequently mediates the ubiquitinylation and efficient proteasomal degradation of β-catenin. The resulting β-catenin 'drought' ensures that nuclear DNA-binding proteins of the Tcf/Lef transcription factor family (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoters and/or enhancers. **(b)** Interaction of a Wnt ligand with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dvl–Fzd complexes and the phosphorylation of LRP by CK1γ, facilitating relocation of axin to the membrane and inactivation of the destruction box. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family. In the nucleus, β-catenin converts the Tcf proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of coactivator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless, Mediator and Hyrax. This ensures efficient activation of Tcf target genes such as c-MYC, which instruct the cell to actively proliferate and remain in an undifferentiated state. Following dissipation of the Wnt signal, β-catenin is evicted from the nucleus by the APC protein and Tcf proteins revert to actively repressing the target gene program. β-TRCP, β-transducin repeat-containing protein; APC, adenomatous

polyposis coli; BCL9, B-cell lymphoma 9; CK1α, casein kinase 1α; CK1γ, casein kinase 1γ; CBP, CREB-binding protein; Fzd, Frizzled; Lef, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related protein; PYG, Pygopus; Tcf, T-cell factor. (Barker et al, 2006).

1.15. NSAIDs

Nonsterodial anti-inflammatory drugs (NSAIDs) are primarily used in the treatment of inflammation, pain and fever, specifically used in the treatment of headaches, arthritis, injuries, and menstrual cramps. The most commonly used NSAID aspirin is to inhibit the clotting of blood and prevent strokes and heart attacks. The term “nonsterodial” is used to distinguish these drugs from steroids, which have a similar eicosanoid-depressing and anti-inflammatory action. Prostaglandins acts as messenger molecules in the processes of inflammation, pain, fever, and support blood clotting by platelets and protects the stomach lining from the digestive juices. Prostaglandins and thromboxane are produced from the arachidonic acid catalyzed by the enzymes cyclooxygease-1 (COX-1) and cyclooxygenase-2 (COX-2). NSAIDs reduce pain by interfering with the production of prostaglandins from arachidonic acid. Prostaglandins, produced at a site of inflammation, sensitize pain receptors in the area. NSAIDs block prostaglandin production from arachidonic acid through the inhibition of the enzymes cyclooxygenase-1 and 2 (COX-1 and COX-2). Epidemiological studies have shown that long-term intake of aspirin is associated with a reduction in the incidence of colorectal cancer (Thun et al, 1991; Giovannucci et al, 1995) and breast cancer (Garcia Rodriguez and Gonzalez-Perez, 2004). In a clinical trial, celecoxib (Steinbach et al, 2000) was found to reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). The effects of several

nonsteroidal anti-inflammatory drugs (NSAIDs) on tumor growth have also been demonstrated in animal models of FAP (Oshima et al, 1996; McEntee et al, 1999) and chemical colon carcinogenesis (de Jong et al, 2000; Brown et al, 2001) and prostate cancer. These observations suggest that NSAIDs have a potent chemopreventive effect.

1.16. Mechanism of action of NSAIDs

NSAIDs inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2, which catalyze the conversion of arachidonic acid to prostaglandins. COX-1 is expressed constitutively and is required for physiological processes such as maintenance of gastrointestinal mucosa and platelet aggregation, whereas COX-2 is induced by cytokines, growth factors, and mitogens. NSAIDs vary in their abilities to inhibit COX-1 and COX-2 (Thun et al, 2002). Classic NSAIDs not only inhibit COX-2, but also inhibit COX-1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs, selective COX-2 inhibitors were developed (Cannon and Breedveld, 2001; Aisen, 2002). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon (Takahashi et al, 2006), stomach (Lazebnik et al, 2005), prostate and breast (Liu et al, 1998). These observations are consistent with the cancer chemopreventive effects of NSAIDs. Tumor inhibition by NSAIDs may be mediated by distinct cellular processes. These processes involve the ability of NSAIDs to restore apoptosis, induce cell cycle arrest, and inhibit angiogenesis (Chan, 2002; Subhashini et al, 2004). Though NSAIDs exert their anti-neoplastic activities by targeting COX enzymes, the underlying molecular

mechanisms remain poorly understood (Gupta and DuBois, 1998). Several reports suggest that NSAIDs inhibits tumor cell growth by acting on several COX-independent targets (Tegeder et al, 2001; Smith et al, 2000) such as β-catenin, NF-κB, PPAR delta etc.. Some *in vitro* studies provided evidence that different NSAIDs exert their action by acting on different molecular pathways (Herrmann et al, 1998; Kopp and Ghosh, 1994; He et al, 1999). Independent of the molecular pathway affected by NSAIDs, the common mechanism might be apoptosis. Among the COX independent targets, the most important pathways affected by NSAIDs are Wnt/β-catenin/Tcf signaling pathway (Dihlmann et al, 2001; Gardner et al, 2004) and NF-kB signaling pathway. Recently, NSAIDs together with standard therapies of GBM have entered phase I and phase II clinical trials and found that such combinations are safe and effective (Cerchietti et al, 2005; Tuettenberg et al, 2005; Reardon et al, 2005; Chuang et al, 2008). The effect of NSAIDs on GBM cell lines is not completely understood. However, recently it was identified that NSAIDs flurbiprofen and indomethacin inhibited the proliferation of glioma cells. Most importantly, celecoxib entered into phase I and Phase II clinical trials for the treatment of GBM. Recent findings reported that anti-tumor activity is enhanced by low-dose combination of the recombinant urokinase kringle domain and celecoxib in glioma models (Kim et al, 2009) and Phase II study of temozolomide, thalidomide, and celecoxib for newly diagnosed glioblastoma in adults (Kesari et al, 2008) have stimulated the use of NSAIDs and provides a desirable strategy for anti-glioma therapy.

1.17. Scope of the present study

Gliomas represent the most common primary tumors of the brain. Despite tremendous improvement in the various treatment techniques, these tumors and in particular GBM still carry a poor prognosis. Recently, there has been an intensive effort shedding light on gliomas to gain an understanding of the cellular and molecular mechanisms that contribute to the pathogenesis of gliomas as a first step toward the development of better treatments for the deadliest tumors. Elucidation of pathogenic or predisposing factors remains a high priority, as these are largely unknown. Understanding of such molecular features can help predict not only the natural history of glioma but also the likelihood that it will respond to conventional chemotherapeutic agents and novel molecular targeted therapy. Genetic modeling of gliomas in mice suggests that neural precursor cells represent preferred cellular substrates of gliomas or that wither glial cell or precursor cells constitute potential cells-of-origin of gliomas. During normal brain development, neural precursor cells including stem cells acts as a source of glial cells. As the mechanism/molecular pathways that control gliogenesis during normal brain development become better understood, it will be important to determine if deregulation of these mechanisms might contribute to the pathogenesis of gliomas. Wnt signaling pathway a major developmental pathway critically regulates the processes of self-renewal and dedifferentiation of neural precursor cells. So, deregulation or aberrant activation of this pathway may leads to development of gliomas. In addition, Wnt signaling pathway is required at several stages of CNS development during embryogenesis and adult life. Aberrant activation of this pathway has been implicated in variety of

human cancers including colon, breast, prostate, ovary and CNS malignancies such as medulloblastoma and subependymal giant cell astrocytomas. The present study is therefore carried out to unravel the relation between Wnt signaling with respect to malignancy of glioma in humans as well as in ENU-induced glioma rat model.

As, oncogenic role of Wnt signaling is evident in several cancers its specific inhibition by chemical agents may have an advantage in chemotherapy of tumors. Chemo preventive effects of NSAIDs are mainly mediated through COX-2 inhibition. However, recently several evidences suggest that alternative targets are also implicated in the tumor suppressive effects of NSAIDs which include Wnt and NF-κB signaling pathways. Accumulating evidences suggest that NSAIDs inhibited cell proliferation and induce apoptosis in several colon cancer cells and recently it was identified that NSAIDs flurbiprofen and indomethacin inhibited the proliferation of glioma cells. In the present study we investigated whether celecoxib and diclofenac have significant anti-proliferative effect on glioma cells via down-regulation of Wnt and NF-κB signaling pathways.

Objectives of the study

- Activation of Wnt/β-catenin/Tcf signaling pathway in human astrocytomas.
- Wnt/β-catenin/Tcf signaling pathway activation in malignant progression of rat gliomas induced by transplacental N-ethyl-N-nitrosourea exposure.
- Targeting Wnt/β-catenin/Tcf and NF-κB signaling pathways in gliomas by nonsterodial anti-inflammatory drugs celecoxib and diclofenac.

Chapter-2

Activation of Wnt/β-catenin/Tcf signaling pathway in human astrocytomas

2.1. INTRODUCTION

Astrocytomas are the most common primary brain tumors that are classified into four different malignancy grades according to World Health Organization (WHO) classification. GBM (glioblastoma multiforme; grade IV) is most common of these tumors and behaves as highly aggressive and fatal tumor, as indicated by a mean survival of less than 1 year. Patients with anaplastic astrocytoma (grade III) have the life expectancy of 2-3 years, whereas patients with low grade astrocytoma (Diffuse astrocytoma; grade II) have favorable outcome and can survive for as long as 10-15 years (Kleihues and Cavenne, 2000; Lacroix et al., 2001; Simmons et al., 2001; Holland, 2001). The treatment of patients with glioblastoma multiforme (GBM) is conventionally considered to be a palliative venture with no hope of cure. Traditionally, patients are treated with maximal surgical resection, although surgery is not a curative procedure, a major resection provides for a longer survival and better quality of life. Radiotherapy increases the duration of survival, but again is not a curative intervention. The role of chemotherapy, specifically focusing on a foundation of chloroethylating agents such as carmustine (BCNU) or lomustine (CCNU), has been controversial with an equal number of clinicians arguing in favor of or against this treatment. Meta-analysis makes it clear that there is a small increase in median survival associated with the addition of these agents, but a consensus was never reached regarding their use.

GBM include two subtypes, those, which develop *de novo*, are primary GBM and secondary GBM develop through progression from low grade diffuse or anaplastic astrocytomas (Kleihues and Ohgaki, 2007). Astrocytic

tumors develop as a result of stepwise accumulation of genetic alterations, which results in the activation of oncogenes and inactivation of tumor suppressor genes (Cavenee, 1992). These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. Mutation of the p53, RB and PTEN, deletion of p16INK4A, activation of the Ras and Akt pathways, and amplification of CDK4 and EGFR contribute to the development of astrocytomas (Ohgaki and Kleihues, 2007; Louis, 1997; Hayashi et al, 1997).

Wnt signaling pathway plays crucial roles in normal embryonic development and abnormal pathological processes in vertebrates (Robb and Tam, 2004; Zorn et al, 1999). Aberrant activation of Wnt signaling pathway has been implicated in variety of human cancers including colon, breast, prostate, and ovary. The Wnt pathway activation in oncogenesis and its consequences has been extensively reviewed (Logan and Nusse, 2004; Moon et al, 2004, Polakis, 1999; Reya and Clevers, 2005). Activation of canonical Wnt pathway requires the binding of Wnt ligands to frizzled (FZD) receptors together with the co-receptors LRP5 or LRP6 (Pinson et al, 2000; Tamai et al, 2000; Wehrli et al, 2000). Binding of Wnt proteins to FZD-LRP complex results in the activation and membrane recruitment of the phospho protein Dishevelled (Dvl). Dvl recruits Axin to the plasma membrane, where it binds directly to the cytoplasmic tail of LRP5-6, results in the inactivation of destruction complex (Cliffe et al, 2003; Moon et al, 2004). This allows the accumulation and translocation of unphosphorylated free β-catenin to the nucleus, where it interacts with members of the Tcf/Lef transcription factors

to induce expression of Wnt target genes like c-Myc, N-Myc, c-jun and cyclin D1, which instruct the cell to actively proliferate and remain in an undifferentiated state (He et al, 1998; Shiina et al, 2003; Shtutman et al, 1999). β-Catenin is the central hub of the Wnt signaling pathway. In the absence of Wnt signal, β-catenin is captured by adenomatus polyposis coli (APC) and Axin within the cytoplasmic destruction complex, which allows its phosphorylation by kinases CK1α and GSK3 β, facilitating its degradation via the ubiquitin-proteosome pathway (Brown and Moon, 1998; Wodarz and Nusse, 1998).

In the present study we set out to investigate the role of Wnt/β-catenin/Tcf signaling pathway in human astrocytomas and its correlation with the histological malignancy of astrocytomas.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection

Human astrocytic tumor samples were collected from patients who underwent surgical resection at Nizam Institute of Medical Sciences (Hyderabad, India) and followed the NIMS ethical guidelines. The tumors were classified histopathologically according to the WHO classification: 8 diffuse astrocytomas (grade II), 7 anaplastic astrocytomas (grade III), and 17 glioblastoma multiforme (grade IV) (Table 1). Peritumor tissues (1cm beyond the tumor margin) were also collected from the patients during the surgical resection. Two brain samples consisting of periventricular region were obtained from patients with epilepsy. These tissues were apparently normal histologically. All samples were obtained with informed consent. A part of the surgically removed samples were immediately snap frozen in liquid nitrogen

and then stored at -80°C until analysis. The remaining samples were fixed with formalin and embedded in paraffin for histological studies. Human glioblastoma derived cell lines A127, T98G (gifted by Dr. Ellora Sen, National Brain Research Center, India), GO-G-CCM, U373, and U87 and rat C6 glioma cell line were obtained from National Center for Cell science (Pune, India) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Biowhittaker, Lonza Pri Ltd, Mumbai, India), 10 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% CO_2.

2.2.2. Preparation of soluble cell lysates

Deep frozen samples were thawed gradually and further homogenized by using dounce homogenizer in 5 volumes of 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, 0.3 mM Na_3Vo_4 and 0.3 mM aprotinin. The lysates were sonicated for 2 min and then centrifuged at 14,000g for 15 min at 4°C. The supernatant (whole tissue lysate) was collected and frozen at -80°C before use. Protein concentrations were determined by Lowry method.

2.2.3. Western blotting

75 micrograms of cellular protein from human tissues and cell lines were mixed with SDS sample buffer, boiled for 5 min and then subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred on to nitrocellulose papers. After blocking the nitrocellulose paper in non-fat dry milk (5%) in Tris Buffered Saline (TBS) (10mM Tris (pH 7.5), 150 mM NaCl) for 1 h

at room temperature, the membranes were incubated for 12 h in primary antibodies at 4^0C. Blots were again incubated with secondary antibodies conjugated to alkaline phosphatase (ALP) (anti-rabbit and anti-mouse IgG conjugated to ALP obtained from Genei Pvt Ltd, Bangalore, India), for 1-2 h at room temperature. Before and after incubation of blots with secondary antibodies, blots were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivity was visualized by incubating the blots with BCIP-NBT solution (Genei Pvt Limited, Bangalore, India). Blots were analyzed quantitatively using scion image software (NIH).

The primary antibodies used in these experiments included rabbit polyclonal antibody against β-catenin, rabbit monoclonal antibody against Lef1, Tcf4, c-jun and cyclin D1 (obtained from Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal antibody against N-Myc (gifted by Dr. Robert Eisenman, Fred Hutchinson Cancer Research Center, Washington, USA), mouse monoclonal antibody against c-Myc (gifted by Naidu, Manipal Institute of Medical Sciences, India), and mouse polyconal β-actin (gifted by Prof. K. Anand Kumar, University of Hyderabad, India).

2.2.4. Co-immunoprecipitation

Co-immunoprecipitation was performed with anti-β-catenin antibody in 200 µg of nuclear protein from human GBM tissue and T98G cell lysates. The samples were pre-cleared using protein A-agarose (Sigma Chemicals, USA) and incubated with anti-β-catenin antibody over night at 4^0C. After the addition of protein A-agarose, samples were further incubated for 2-3 h at room temperature. Then washed thrice with phosphate buffered saline containing

0.1% NP-40 and the beads were resuspended in SDS sample buffer and subjected to western blot analysis.

2.2.5. Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded specimens. Briefly, sections were deparaffinized in xylene and passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by micro waving the sections for 10-14 min in 10 mM citrate buffer (pH 6.0). Sections were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase followed by incubation with serum for 1 h at room temperature in a humid chamber. The sections were then incubated overnight at 4^{o}C with primary antibodies against β-catenin, Lef1, Tcf4, c-jun, c-Myc, N-Myc and cyclin D1 diluted as per data sheet in blocking solution. Peroxidase conjugated secondary antibody was used for 1 h incubation time at room temperature followed by TBS washes (3x5 min each). Diaminobenzidine (DAB) in buffer was used till sections develop color. Then sections were counterstained using haematoxylin. Sections were washed with distilled water followed by dehydration in graded alcohols, xylene and mounted with DPX (kit obtained from Biogenex Pvt Limited, India). In each experiment, a negative control was included in which the primary antibody step was skipped and replaced by non-immune serum.

2.2.6. Immunofluorescence

As described in immunohistochemical analysis, after deparaffinization, rehydration and antigen unmasking, tissue sections were blocked with 5% serum for 1h at room temperature in a humid chamber. Then incubated the

sections in a cocktail of two primary antibodies (mouse polyclonal GFAP obtained from Sigma Chemicals, USA, and rabbit polyclonal β-catenin antibodies) overnight at 4^0C. The sections were subsequently washed and incubated in a mixture consisting FITC and TRITC secondary antibodies (Genei Pvt Limited, Bangalore, India) at 1:250 dilution for 1 h at room temperature. The sections were washed and mounted using 90% glycerol and visualized using a Leica confocal microscope.

2.2.7. RNA Isolation and semi quantitative RT-PCR

Total RNA was isolated from astrocytic tumor tissues (grade II-4, grade III-2 and grade IV-3) using TRI reagent (Sigma Chemicals, USA). Briefly, frozen tissues (50-100mg) were grounded in liquid nitrogen and homogenized in 1 ml of TRI reagent for RNA isolation following manufacturer's instructions. Purified total RNA was dissolved in DEPC treated water and stored at -80°C before use. 5 micrograms of RNA was treated with DNase I and reverse transcribed to cDNA using oligo $(dT)_{18}$ primer and M-mLV reverse transcriptase enzyme (Invitrogen, Sandiego, CA, USA) as per the protocol recommended by manufacturer. The amplification conditions were initial denaturatuion at 94°C for 3 min followed by 30 cycles of 94°C for 1 min; 58°C for 45 s; 72°C for 45 s. PCR amplification was conducted using the described primer sets (table 2.1). The house keeping gene *GAPDH* was used as an internal quantitative control.

2.2.8. Statistics

Statistical analysis was performed using sigma stat version 3.1. All data were represented as mean ± SD obtained from individual patients of each grade and were compared with control. The significant differences of the

data were determined using one-way ANOVA. The correlation of the data was determined by Pearson's test. Differences between tumor samples and controls were compared by paired student's *t*-test. Values of $p < 0.05$ were considered as statistically significant.

Table 2.1. Sequences of specific primers: F = forward primer; R = reverse primer

Gene	Primer sequence	Amplicon size (bp)
Dvl-1 F	5'-GTCAGGGCTACCCCTACCAG-3'	480
Dvl-1 R	5'- TGATGTCCACGAAGAACTCG-3'	
Dvl-2 F	5'-GCACCATTACATCTGGATCGT-3'	190
Dvl-2 R	5'-GGTAGAGCCAGTCAACCACAT-3'	
Dvl-3 F	5'-CACGTGGTTGCTTCACATTGC-3'	198
Dvl-3 R	5'-GACAAGTGGAAGTCGTCTAGG-3'	
β-catenin F	5'-CACAAGCAGAGTGCTGAAGGTGC-3'	189
β-catenin R	5'-AAGGAGGCCTTCCATCCCTTC-3'	
c-myc F	5'- CTGGAAGAAATTCGAGCTGA-3'	220
c-myc R	5'-ACATACAGTCCTGGATGATGA-3'	
cyclin D1 F	5'-GTGGCCTCTAAGATGAAGGAG-3'	281
cyclin D1 R	5'-GAACTTCACATCTGTGGCACAG-3'	
GAPDH F	5'-ACCACAGTCCATGCCATCAC-3'	186
GAPDH R	5'-ACACGGAAGGCCATGCCAGTG-3'	

Table 2.2. Clinical characteristics of astrocytoma patients

No.	Age	Sex	WHO grade	Location	Survival days	Out-come
1	34	F	II	Fronto- Temporal	1988	Alive
2	35	M	II	Frontal	663	Alive
3	27	M	II	Fronto-Temporal	1225	Alive
4	30	M	II	Frontal	1269	Alive
5	16	M	II	Frontal	1442	Alive
6	60	M	II	Parietal	412	Dead
7	37	F	II	Tempero-Frontal	389	Alive
8	40	M	II	Frontal	869	Alive
9	29	M	III	Frontal	345	Dead
10	62	F	III	Parietal	432	Alive
11	35	M	III	Frontal	326	Dead
12	37	M	III	Frontal	363	Alive
13	30	F	III	Frontal	419	Dead
14	45	F	III	Fronto-Temporal	986	Dead
15	43	M	III	Frontal	1021	Dead
16	45	M	IV	Parietal	431	Alive
17	36	F	IV	Frontal	446	Dead
18	16	M	IV	Frontal	321	Dead
19	52	M	IV	Frontal	496	Dead
20	20	M	IV	Frontal	462	Dead
21	19	M	IV	Tempero-Parietal	56	Dead
22	27	M	IV	Frontal	120	Dead
23	48	M	IV	Temporal	175	Dead
24	15	M	IV	Frontal	32	Dead
25	58	F	IV	Fronto-Tempero-Parietal	93	Dead
26	61	M	IV	Frontal	18	Dead
27	50	M	IV	Corpus Callosal	39	Dead
28	55	M	IV	Frontal	172	Dead
29	5	M	IV	Multiple intra cranial	113	Dead
30	30	M	IV	Frontal	269	Alive
31	40	M	IV	Parietal	59	Dead
32	34	M	IV	Temporal	213	Dead

2.3. RESULTS

2.3.1. Clinical characteristics of astrocytoma patients

For this study we collected 32 human astrocytoma patients representing different clinical grades. Among the patients there was no gender specificity but male predominance was observed. A wide variation in the clinical features among the patients also observed. Most affected site of tumor occurrence is frontal lobe and most of the GBM patients are died (15 out of 17) till to the date of analysis. Clinical features of astrocytoma patients are summarized in table 2.2. GBM is highly invasive tumor and it recurs even after applying various treatment strategies. The recurring ability of GBM is illustrated in figure 2.1.

2.3.2. Histological features of astrocytoma biopsies

H&E staining of pilocytic astrocytoma showed the presence of myxoid (hypo cellular) and piloid (cellular) areas and cells appearing as hair like structures (figure 2.2A, C). There is increased cellularity and marked heterogeneity and vasculature is prominent with the appearances of glomeruloid shapes (figure 2.2B, D). Diffuse astrocytoma sections showing the features of increased cellularity and moderate nuclear pleomorphism and the features of infiltration was also evident (figure 2.3A, B). In anaplastic astrocytoma there is further increase in cellularity along with nuclear pleomorphism. Mitoses were observed and the presence of glossy gemistocytes characteristic feature of anaplastic astrocytoma is also evident (figure 2.4A, B). In addition to grade III features GBM sections represented with coagulative or geographical necrotic features. In this dead necrotic area is surrounded by the densely packed tumor cell nuclei (figure 2.5A, C).

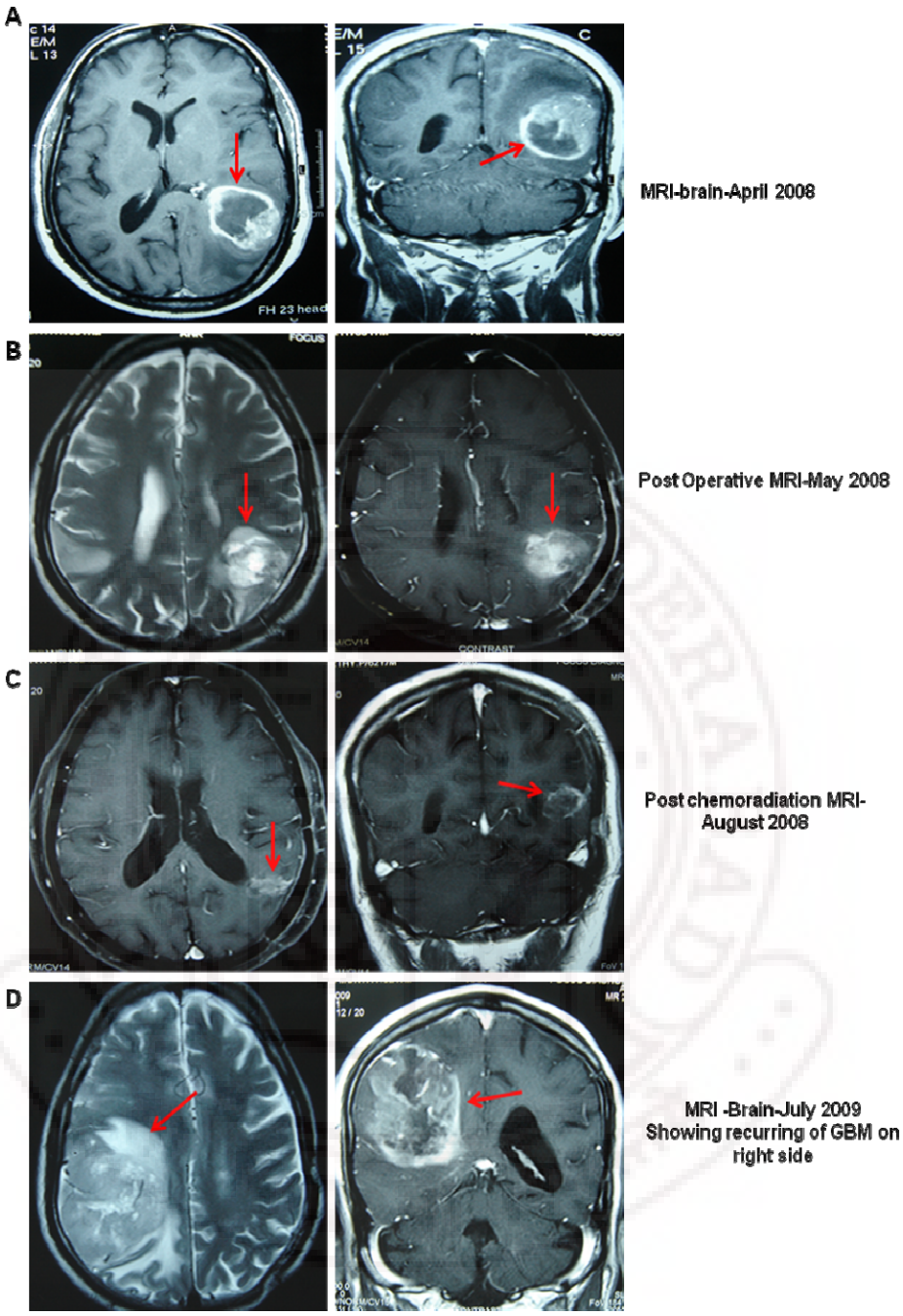


Figure 2.1. Recurring nature of GBM. A) MRI of patient diagnosed with GBM in April 2008 showing the tumor on left side of the brain. **B)** In May 2008, the post operative and post radiationtherapy MRI showing the resection of tumor. **C)** Then, in August 2008, post chemotherapy MRI showing great reduction in tumor size. **D)** After 11 months that is in July 2009 MRI showing the recurrence of tumor on right part of the brain with great increase in tumor mass. After, in August 2009 the patient was died.

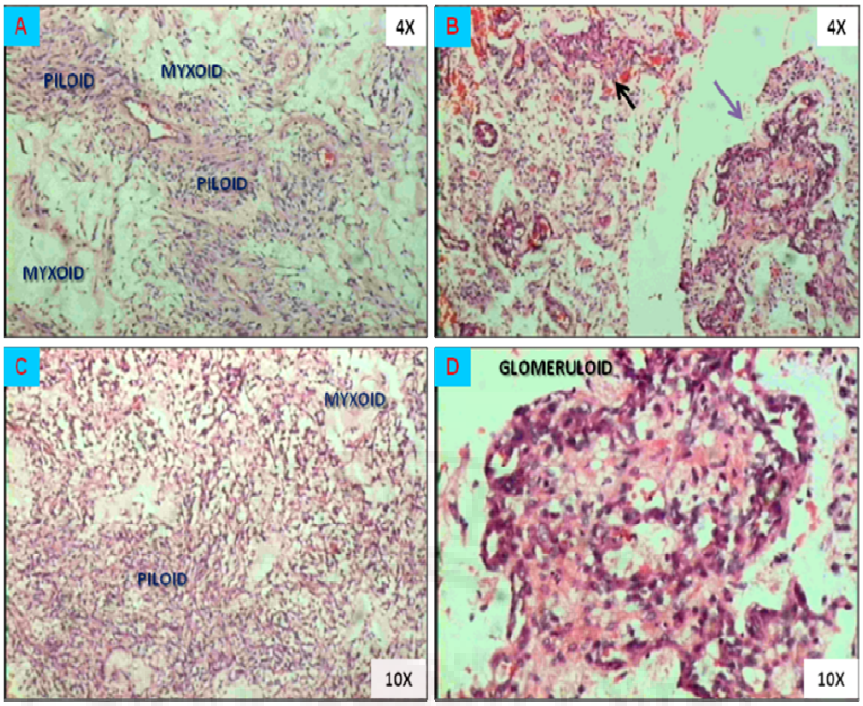


Figure 2.2. **H&E staining of pilocytic astrocytoma tissues.** Pilocytic astrocytoma (grade I) showing the myxoid (hypocellular) and piloid (cellular) areas and tumor cells appear as hair like structures **(A, C).** Pilocytic astrocytoma with features of angiogenesis **(B)** and at higher magnification showing the glomeruloid vasculature **(D).**

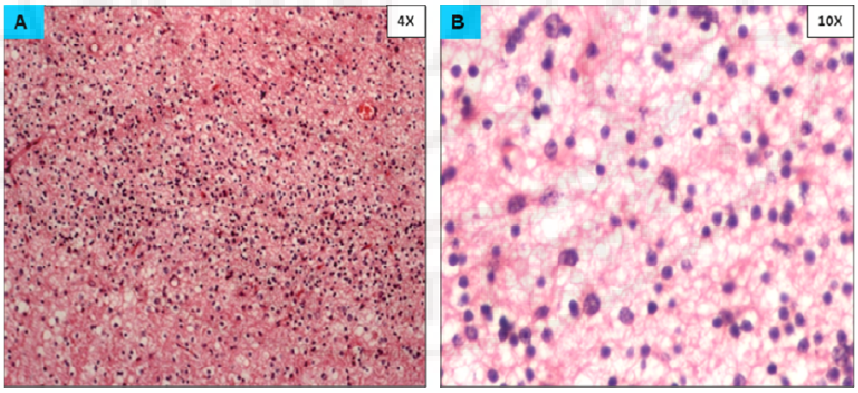


Figure 2.3. **H&E staining of diffuse astrocytoma sections.** Diffuse astrocytoma (grade II) showing the features of increased cellularity and atypia **(A)**, moderate nuclear pleomorphism and infiltration of astrocytes **(B).**

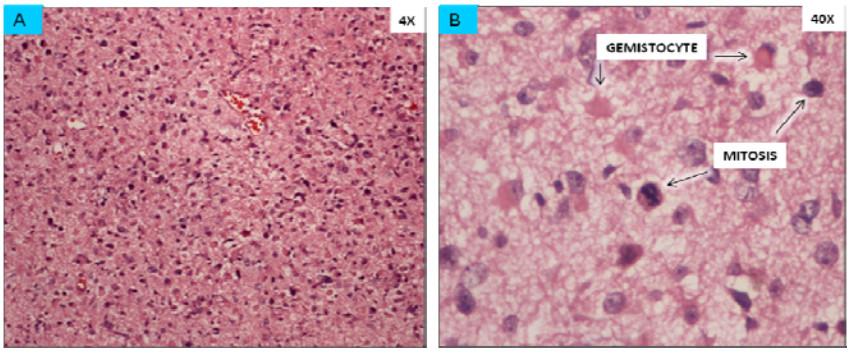


Figure 2.4. H&E staining of anaplastic astrocytoma sections. Anaplastic astrocytoma (grade III) showing the features increased cellularity, nuclear polymorphism **(A),** mitoses and gemistocytes **(B).**

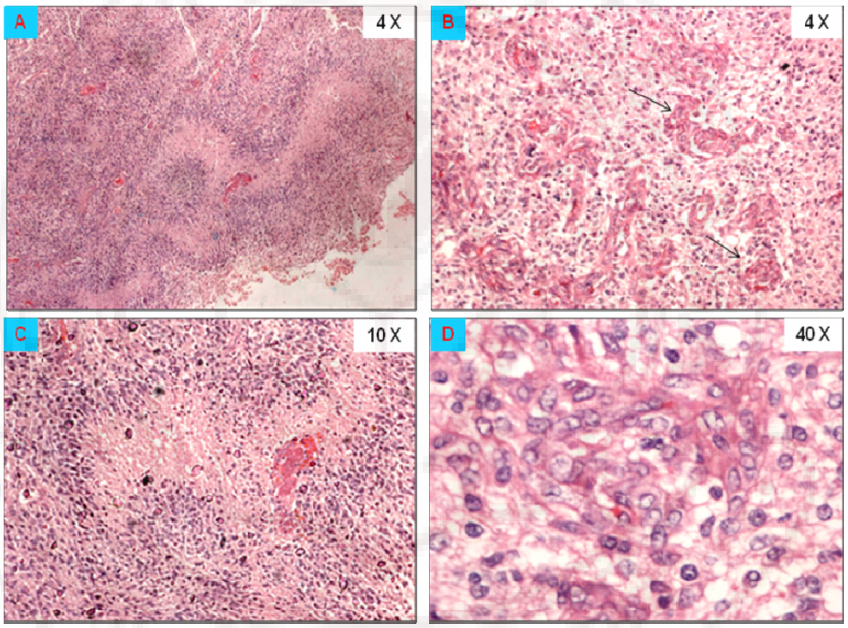


Figure 2.5. H&E staining of Glioblastoma Multiforme (GBM) grade (IV) sections. GBM sections showing the features of geographical necrosis and high grade morphology **(A, C)**, and extensive vascular endothelial cell proliferations **(B, D).**

Appearance of endothelial cell proliferations is evident with glomeruloid vascular proliferations (figure 2.5B, D). After confirming the histological features pathologically, astrocytoma tissues were used to investigate the status of Wnt signaling pathway components in their malignancy.

2.3.3. Overexpression of *Dvl-3* in astrocytic tumors

In order to examine *Dvl* expression in astrocytomas, we analyzed tumor tissues for variation in expression levels of *Dvl-1, Dvl-2* and *Dvl-3*, using total RNA for semi quantitative RT-PCR experiments.

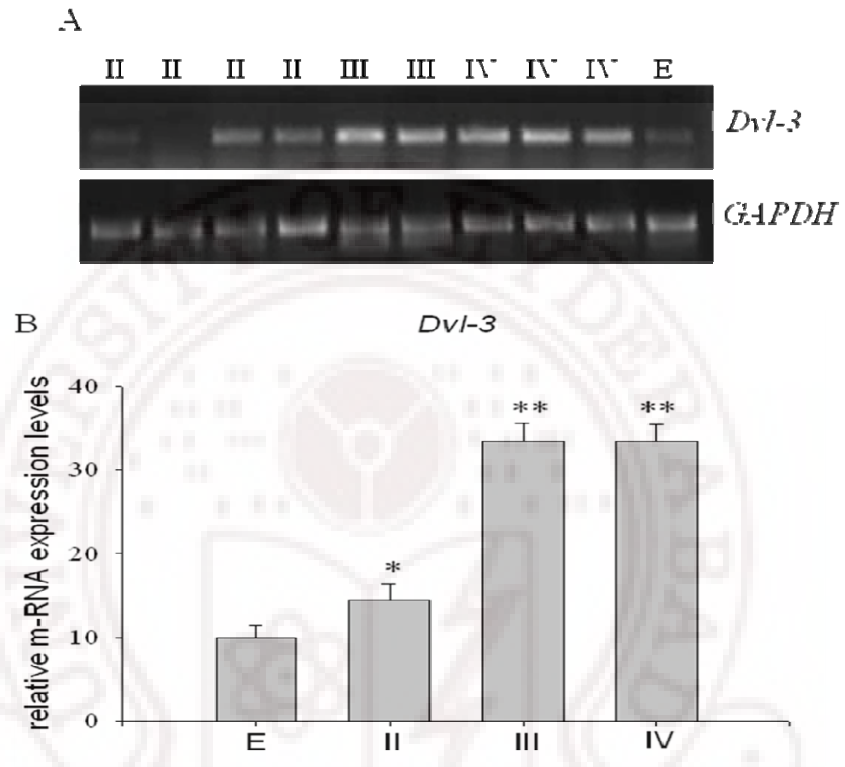


Figure 2.6. Overexpression of *DVL-3* in astrocytic tumors. A. Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *Dvl-3* specific primers. The house keeping gene *GAPDH* was used as an internal quantitative control (II- diffuse astrocytoma, III-anaplastic astrocytoma, IV-glioblastoma multiforme, E-control brain). **B.** Densitometric analysis of *Dvl-3* expression revealed the mRNA levels of *Dvl-3* were correlated with the histological grading of astrocytomas. Data are represented as mean ± standard error from three independent experiments (*p < 0.05 and **p < 0.001 indicate significant difference relative to the corresponding control).

The mRNA expression levels of *Dvl-3* in astrocytic tumors were increased significantly when compared to controls (figure 2.6A). Data represents mean expression levels of *Dvl-3* in grade II (14.5±1.8), grade III (33.5±2.1) and

grade IV (33.4±2.12) were statistically significant ($p < 0.05$) when compared to control brain (10±1.5) and positively correlated with the degrees of histological grading (figure 2.6B). In contrast, the expression of *Dvl-1* and *Dvl-2* were not found in astrocytomas.

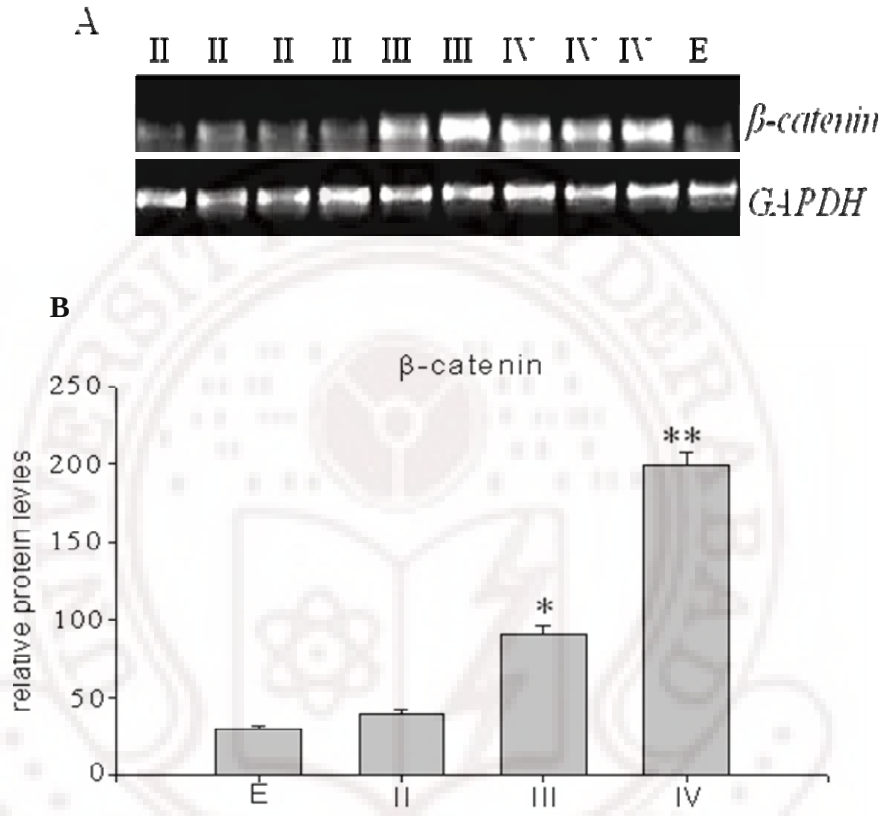


Figure 2.7. Overexpression of *β-catenin* mRNA in astrocytomas. A. Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *β-catenin* specific primers. The house keeping gene *GAPDH* was used as an internal quantitative control (II- diffuse astrocytoma, III-anaplastic astrocytoma, IV-glioblastoma multiforme, E-control brain). **B**. Quantitative densitometric analysis showing the expression levels of *β-catenin* were correlated with the histological malignancy. Data are represented as mean ± standard error from three independent experiments (**$p < 0.001$ indicate significant difference relative to the corresponding control).

2.3.4. Increased β-catenin levels correlates with histological malignancy

The mRNA expression profile of *β-catenin* was quantified by using RT-PCR in tumors of different grades. The relative mRNA levels of *β-catenin* were

significantly high in higher grade astrocytomas (III, IV), moderate in low grade astrocytomas (grade II), in comparison to control (figure 2.7A).

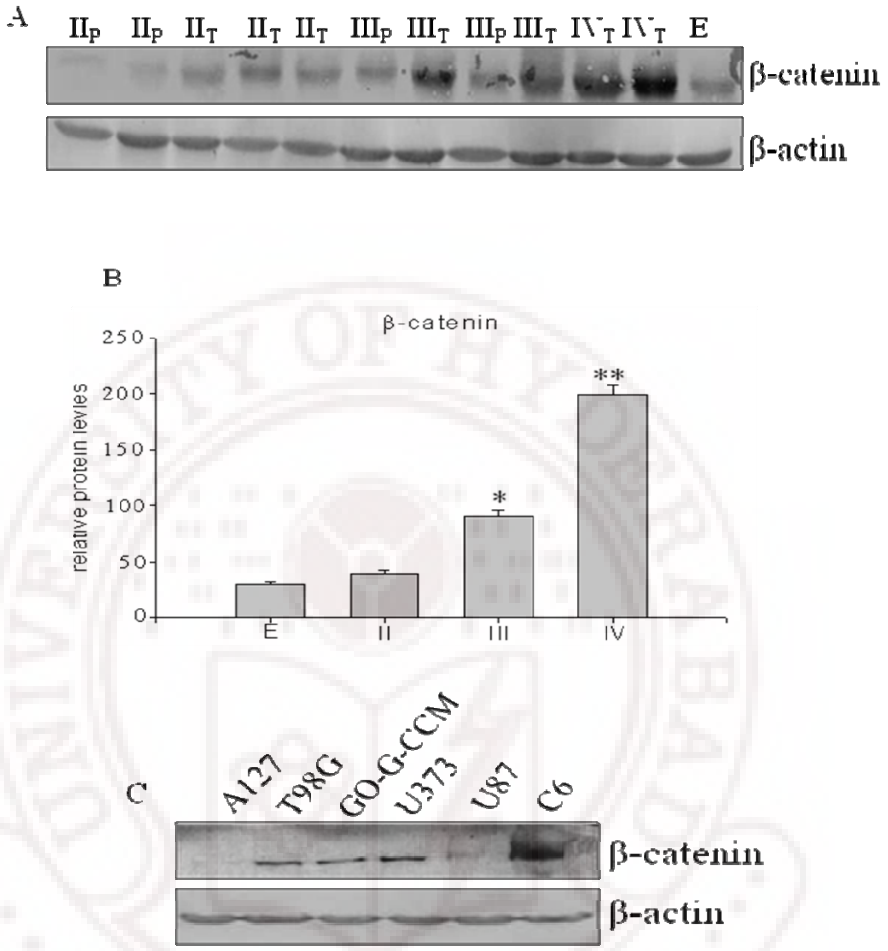


Figure 2.8. Upregulation of β-catenin in astrocytomas. A. Whole cell lysates extracted from human astrocytic samples separated on 10% SDS gels and transferred on to nitrocellulose membranes. Membranes were subjected to immunoblot analysis with a β-catenin specific primary antibody. The expression levels of β-actin were used as loading control. (II_P- diffuse astrocytoma peritumor tissue, II_T- diffuse astrocytoma core tumor tissue, III_P- anaplastic astrocytoma peritumor tissue, III_T- anaplastic astrocytoma core tumor tissue, IV_T- GBM core tumor tissue, E- normal control brain). **B**. Densitometric analysis showing the β-catenin levels were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments (*$p < 0.05$ and **$p < 0.001$ indicate significant difference relative to the corresponding control).**C**. Proteins were extracted from different GBM cell lines, separated on 10% SDS gel and transferred on to nitrocellulose membranes and immunoblots were detected with β-catenin specific primary antibody. T98G, GO-G-CCM, U373 and C6 cell lines bears more β-catenin expression, while A127 and U87 cell lines bear low β-catenin expression. β-actin used as a loading control.

The mean mRNA expression levels of *β*-catenin in grade III (67.5±3.2) and grade IV (68±3.4) were statistically significant ($p < 0.05$) when compared to control (18±1.6) and correlated with histological malignancy (figure 2.7B). Western blotting analysis demonstrated the relative protein levels of β-catenin were progressively increased from low grade (II) to higher grade (III, IV) astrocytomas. Moreover, the protein levels were higher in the core tumor tissues in comparison to peritumor and normal brain tissues (figure 2.8A). The mean protein expression levels of β-catenin in grade III (91.3±1.45) and grade IV (199±.6.06) were statistically significant ($p < 0.05$) when compared with that of control (28.6±1.45) and showed positive correlation with histological grading (figure 2.8B). But the mean mRNA and protein expression levels of β-catenin in grade II (28±1.7, 38.9±1.15 respectively) showed no significant difference with controls.

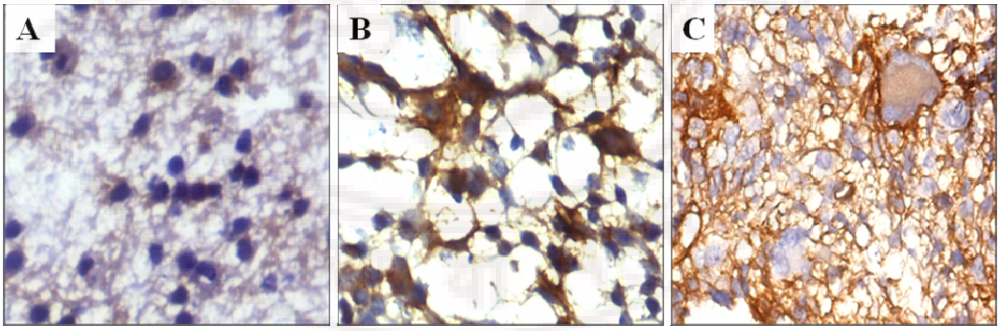


Figure 2.9. Cytoplasmic and nuclear accumulation of β-catenin. Paraffin-embedded sections were prepared from astrocytoma specimens. Sections were incubated with β-catenin specific primary antibody (1:100 dilution) overnight at 4°C and peroxidase conjugated secondary antibody was used for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Representative results showed cytosolic and nuclear positivity for β-catenin in tumor cells (**A**-diffuse astrocytoma; **B**-anaplastic astrocytoma; **C**-glioblastoma multiforme).

In addition, we also analyzed the β-catenin expression in different glioma cell lines (A127, T98G, GO-G-CCM, U87, U373 and C6). Representative western blotting results showed that T98G, GO-G-CCM, U373 and C6 cell lines were over-expressing β-catenin (figure 2.8C). Moreover, the growth inhibition of different GBM cell lines was demonstrated using non-steroidal anti-inflammatory drugs (NSAIDS) like diclofenac and celecoxib, which are the specific inhibitors of the Wnt pathway. Diclofenac and celecoxib suppressed the growth of GBM cell lines in culture and reduced the β-catenin dependent expression.

In order to confirm the cell type and sub-cellular distribution for the expression of β-catenin, immunohistochemistry and immunofluorescence experiments were performed. Immunohistochemical analysis showed a strong β-catenin expression in astrocytic tumors (DA, AA and GBM). The cytoplasmic accumulation and nuclear translocation of β-catenin, which is the hallmark of Wnt pathway activity was evidenced by the strong positive staining in the cytoplasm and nuclei of tumor cells (figure 2.9). Double immunofluorescence analysis also showed the cytoplasmic and nuclear localization of β-catenin in GFAP expressing tumor cells (figure 2.10).

2.3.5. Constitutive activation of Tcf4 and Lef1

Cytoplasmically accumulated β-catenin translocates to nucleus and activates the Tcf4 and Lef1 transcriptional factors. In order to know whether β-catenin interacted with Tcf4, we performed co-immunoprecipitation experiments. The corresponding results showed that β-catenin formed a complex with Tcf4 in human GBM tissue (figure 2.11A) and cultured T98G cell lysates (figure 2.11B). Further, to check the activation of Tcf4 and Lef1

transcriptional factors, we performed western blotting and immunohistochemical staining. Our results showed that the relative protein levels of Tcf4 and Lef1 were significantly higher in core tumor samples than in peritumor and control brain samples (figure 2.12A).

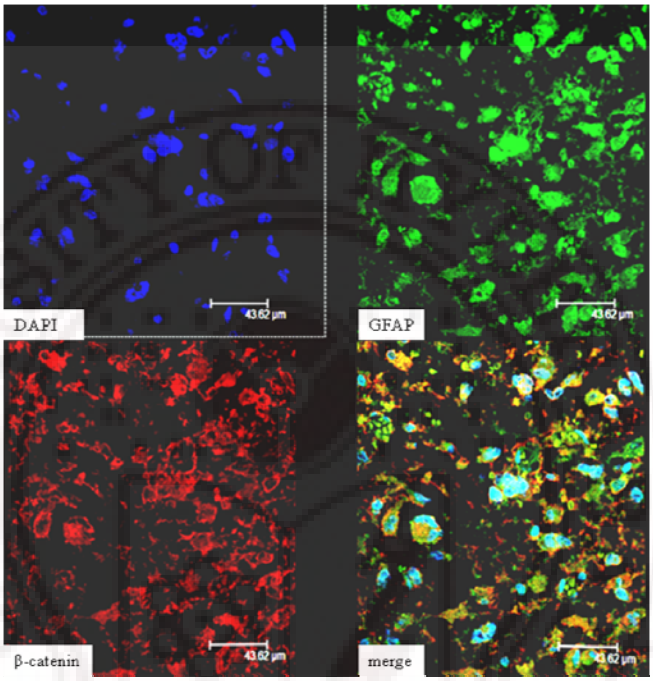


Figure 2.10. Colocalization of β-catenin and GFAP. Paraffin-embedded astrocytoma sections were prepared from astrocytoma specimens. Sections were incubated with anti-rabbit-β-catenin and anti-mouse-GFAP specific primary antibodies (1:100 dilution) overnight at 4°C and ant-rabbit-TRITC and anti-mouse-FITC secondary antibodies were used for 1 h at room temperature. DAPI was used for the detection of nuclei and fluorescence was captured under Leica confocal microscope. Representative figure showed the cytosolic and nuclear localization of β-catenin in GFAP positive tumor cells.

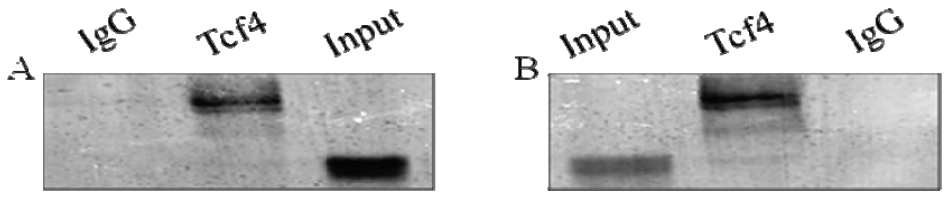


Figure 2.11. Binding of β-catenin with Tcf4. Nuclear extracts from human GBM tissue **(A)** and T98G cells **(B)** overexpressing β-catenin and Tcf4 were immunoprecipitated with anti-β-catenin antibody (normal IgG as a negative control). Proteins in the β-catenin complex were analyzed by western blotting with anti-Tcf4 antibodies.

The mean expression levels of Lef1 in grade II (52.6±1.45), grade III (61.3±2.02), grade IV (84.2±3.47) and Tcf4 in grade II (22.6±1.51), grade III (29.5±0.86), grade IV (40.52±2.59) were statistically significant ($p < 0.05$) when compared with that of controls (11±1.15, 12.3±1.76 respectively) and showed significant positive correlation with the degrees of histological malignancy (figure 2.12B, C). Further, immunohistochemical analyses of Lef1 and Tcf4 in DA, AA and GBM showed strong nuclear positivity in tumor cells (figure 2.13).

2.3.6. Upregulation of Wnt target genes: c-Myc, N-Myc, c-jun and cyclin D1

To assess the gene expression of c-Myc, N-Myc, c-jun and cyclin D1 in tumors as well as in controls we performed RT-PCR, western blotting and immunohistochemistry. RT-PCR analysis in astrocytic tumors showed the upregulation of *c-myc*, and *cyclin D1* in tumor samples than in controls (figure 2.14A). Quantitative densitometric analysis showed the expression levels of *c-myc* in grade II (18±1.43), grade III (44±2.14) and grade IV (45±2.4) and *cyclin D1* in grade II (25±1.16), grade III (50±2.24) and grade IV (58±2.7) were statistically significant ($p < 0.05$) when compared to controls (6±1.0, 11±1.1

respectively) and positively correlated with histological malignancy (figure 2.14B, C).

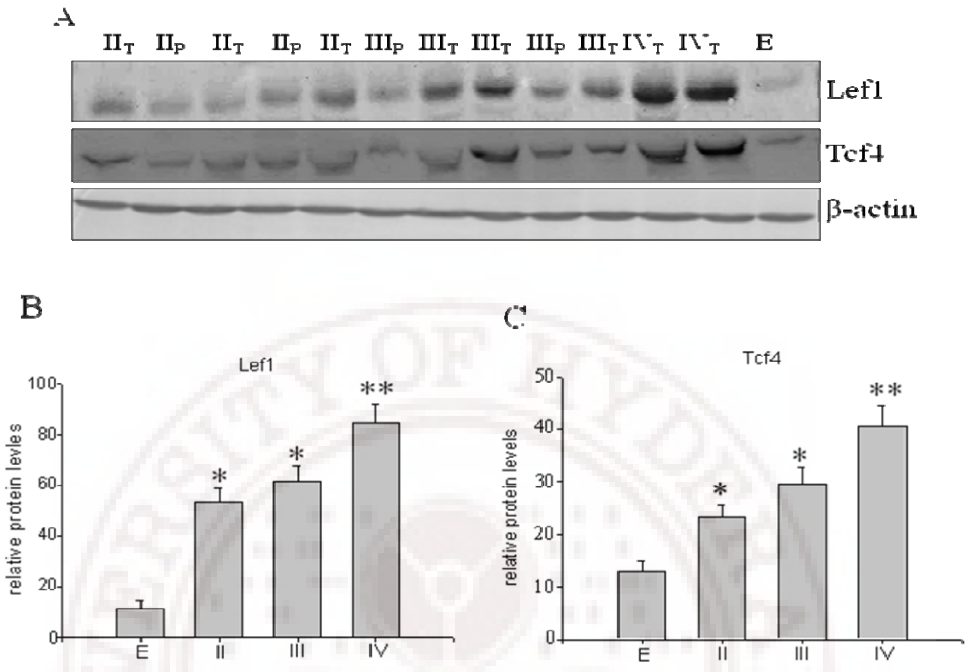


Figure 2.12. Overexpression of Lef1 and Tcf4. A. Whole cell protein extracted from human astrocytic tumor samples were separated on 10% SDS gels and transferred on to nitrocellulose membranes. Immunoblots were detected with Lef1 and Tcf4 specific primary antibodies. The expression levels of β-actin were used as loading controls (II_P- diffuse astrocytoma peritumor tissue, II_T- diffuse astrocytoma core tumor tissue, III_P- anaplastic astrocytoma peritumor tissue, III_T- anaplastic astrocytoma core tumor tissue, IV_T- GBM core tumor tissue, E- normal control brain). Densitometric analysis showing the Lef1 **(B)** and Tcf4 **(C)** levels were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments (*p < 0.05 and **p < 0.001 indicate significant difference relative to the corresponding control).

The mean protein expression levels of c-Myc (grade II-61.6±2.61; grade III-68.7±4.2; grade IV-114.25±.7.4; control-23.7±5.9) N-Myc (grade II-63±5.3; grade III-70±6.2; grade IV-103.5±8.0; control-21.5±3.6) c-jun (grade II-62.1±5.2; grade III-69.5±6.3; grade IV-90.5±.7.3; control-32.25±1.5) and cyclin D1 (grade II-171.3±15.4; grade III-213.25±18.8; grade IV-291.8±.19.54; control-128.6±3.2) were statistically significant when compared with that of

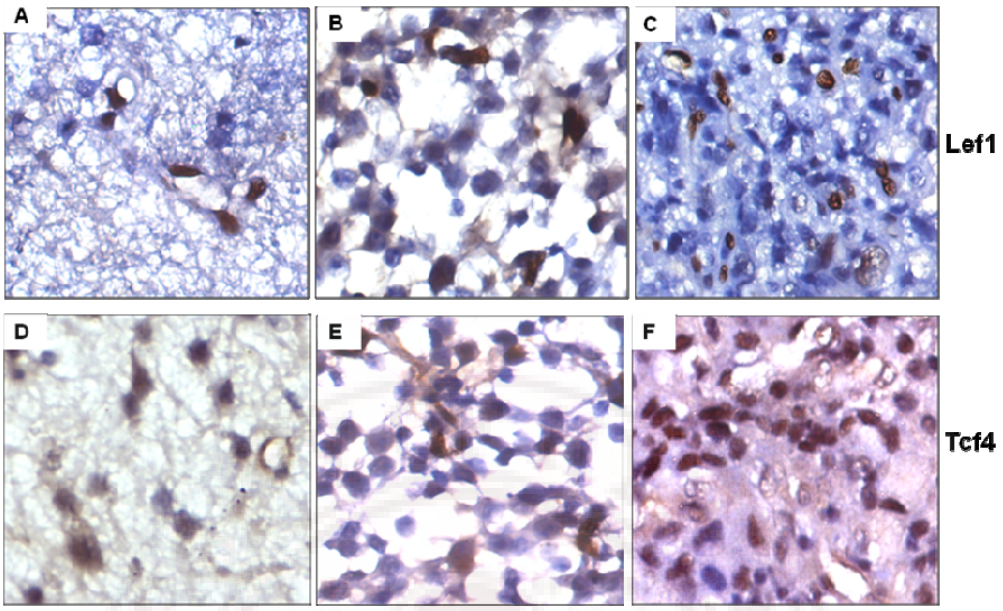


Figure 2.13. Immunohistochemical staining of Lef1 and Tcf4 in astrocytomas. Paraffin-embedded astrocytoma sections prepared from astrocytoma specimens. Sections were incubated with Lef1 and Tcf4 specific primary antibodies (1:200 dilutions) overnight at 4°C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positivite nuclear staining for Lef1 (**A, B, C**) and Tcf4 (**D, E, F**). A, D-Diffuse astrocytoma; B, E-Anaplastic astrocytoma; C, F-GBM.

controls ($p < 0.05$) and showed significant positive correlation with the progression of histological malignancy (figure 2.15B, C, D and E). Immunostaining of c-Myc, N-Myc (figure 2.16), c-jun and cyclin D1 (figure 2.17) showed strong nuclear positivity in tumor cells of DA, AA and GBM.

2.3.7. Correlations among Dvl-3, β-catenin, Lef1 and Tcf4 in astrocytomas

The expression levels of every two genes were compared. The mRNA levels of *β-catenin* and *Dvl-3* showed strong positive correlation ($n = 10$, $r =$

0.924, $p < 0.001$) and the protein and mRNA levels of β-catenin showed highly significant positive correlation ($n = 10$, $r = 0.887$, $p < 0.001$). Also, the

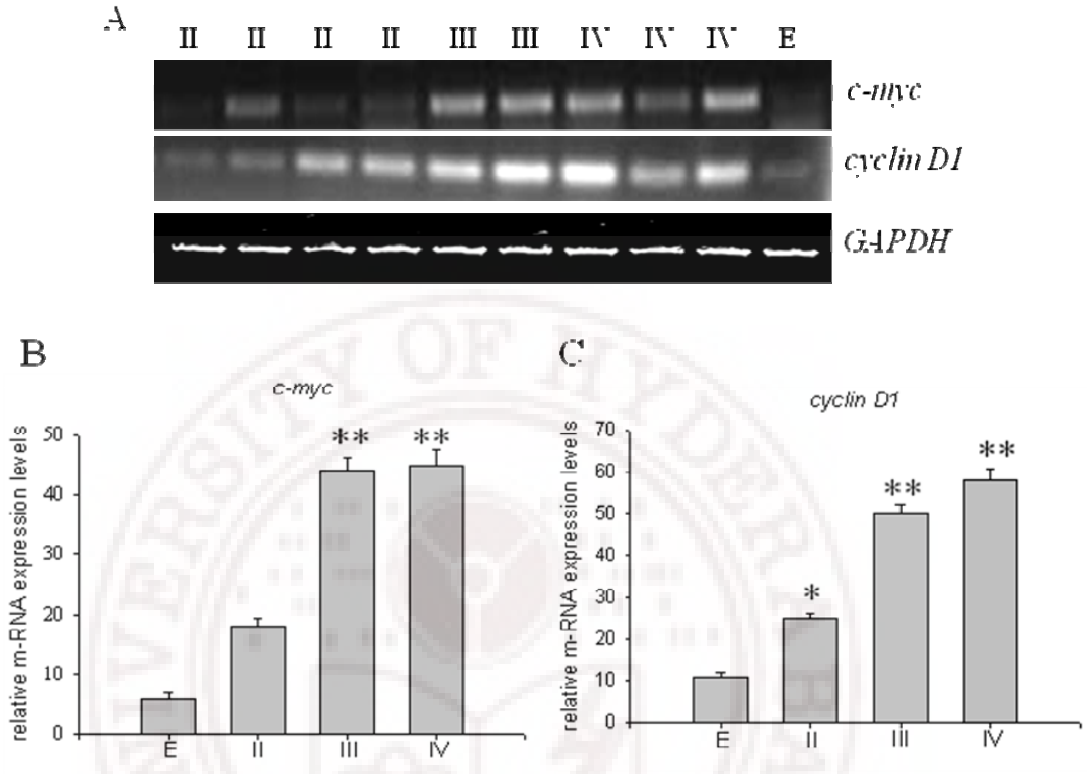


Figure 2.14. Overexpression of *c-myc* and *cyclin D1* mRNA in astrocytic tumors. **A**. Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *c-myc and cyclin D1* specific primers. Amplification of *GAPDH* served as an internal control (II- diffuse astrocytoma, III-anaplastic astrocytoma, IV-glioblastoma multiforme, E-control brain). Densitometric analysis showing the expression levels of *c-myc* **(B)** *and cyclin D1* **(C)** were positively correlated with the histological grading of astrocytomas. Data are represented as mean ± standard error from three independent experiments (*$p < 0.05$ and **$p < 0.001$ indicate significant difference relative to the corresponding control).

protein levels of β-catenin and Lef1 ($n = 32$, $r = 0.875$, $p < 0.001$), β-catenin and Tcf4 ($n = 32$, $r = 0.893$, $p < 0.001$), Lef1 and Tcf4 ($n = 32$, $r = 0.931$, $p < 0.001$) exhibited a highly significant positive correlation.

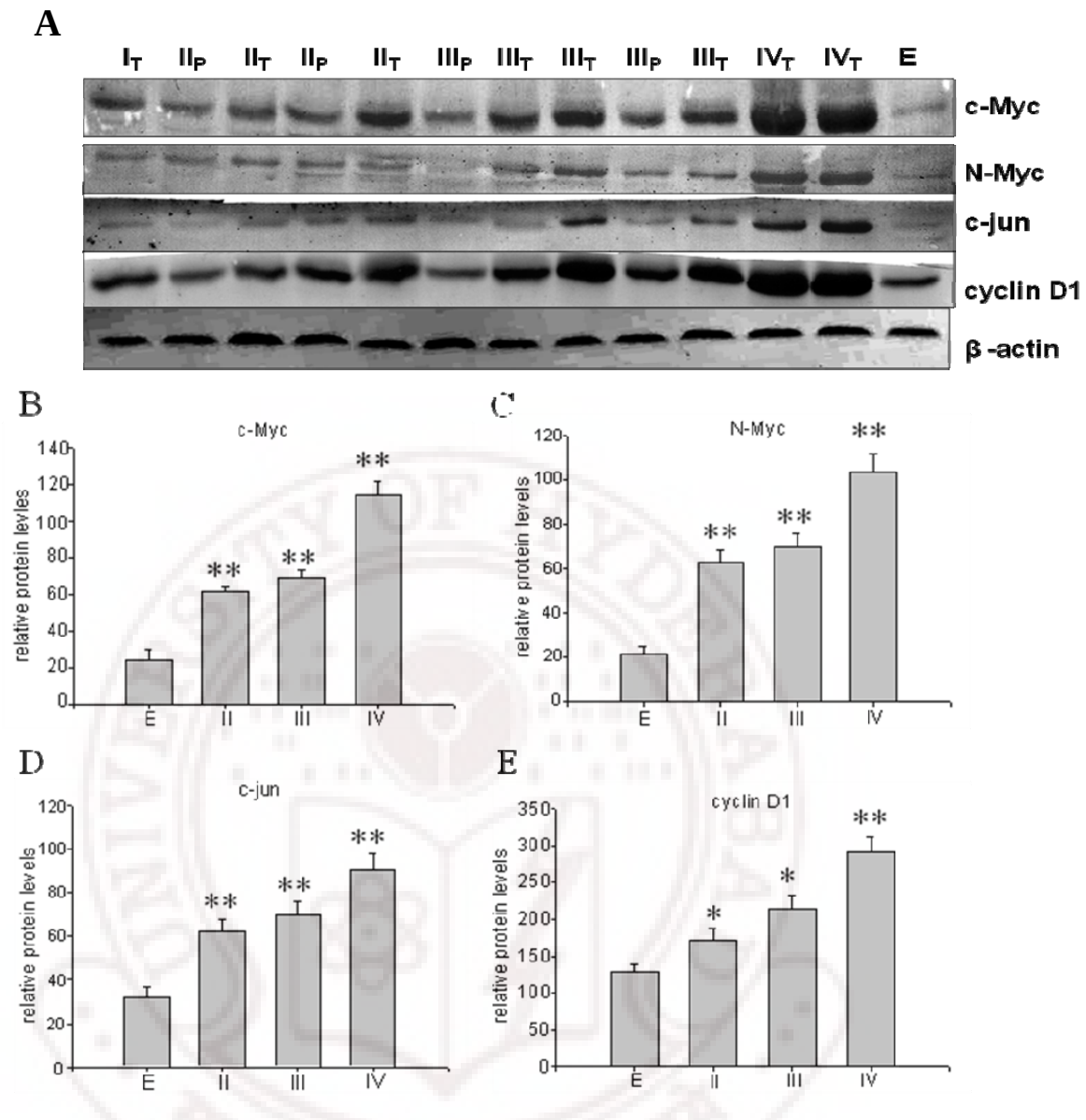


Figure 2.15. Upregulation of β-catenin target genes c-Myc, N-Myc, c-jun and cyclin D1. **A.** Whole cell protein extracted from human astrocytic samples separated on 10% SDS gels and transferred on to nitrocellulose membranes. Immunoblots were detected with c-Myc, N-Myc, c-jun and cyclin D1 specific primary antibodies. The expression levels of β-actin were used as loading controls (II_P- diffuse astrocytoma peritumor tissue, II_T- diffuse astrocytoma core tumor tissue, III_P- anaplastic astrocytoma peritumor tissue, III_T- anaplastic astrocytoma core tumor tissue, IV_T- GBM core tumor tissue, E- normal control brain). Densitometric analysis showing the protein levels of c-Myc **(B)**, N-Myc **(C)**, c-jun **(D)** and cyclin D1 **(E)** were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments (*p < 0.05 and **p < 0.001 indicate significant difference relative to the corresponding control).

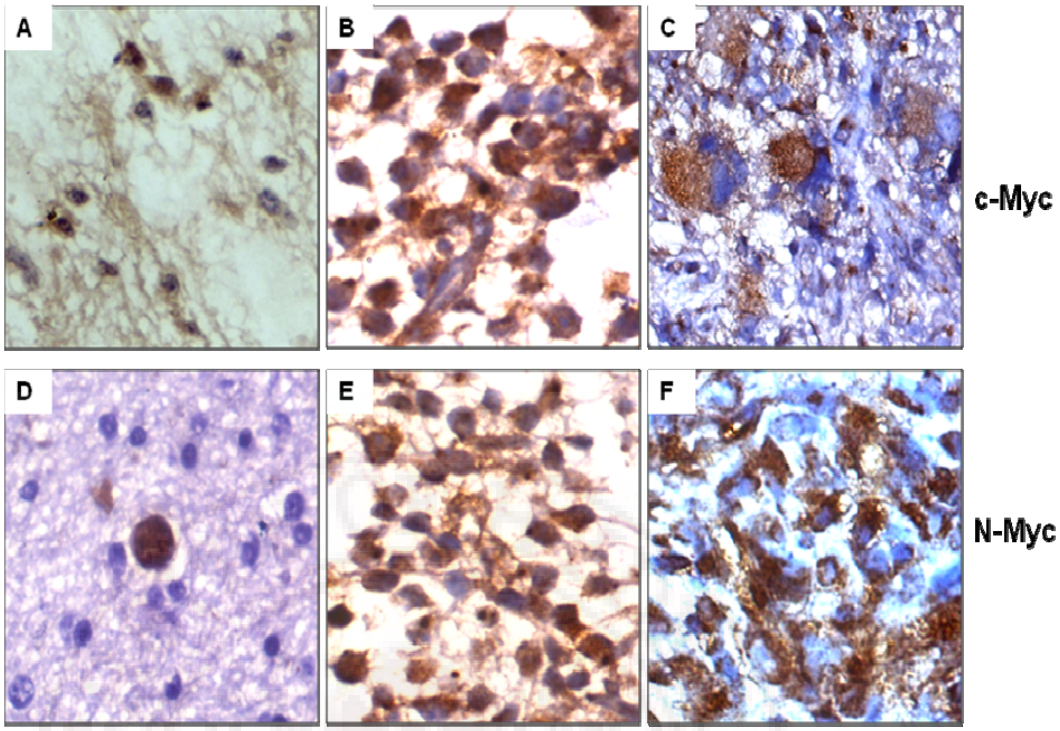


Figure 2.16. Paraffin-embedded astrocytoma sections were prepared from astrocytoma specimens. Sections were incubated with c-Myc and N-Myc (1:100 dilutions) specific primary antibodies overnight at 4^{o}C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positive nuclear staining for c-Myc **(A, B, C)** and N-Myc **(D, E, F)**. A, D-Diffuse astrocytoma; B, E-Anaplastic astrocytoma; C, F-GBM.

2.4. DISCUSSION

Cancer may arise because the developmental pathways that create the dramatic alterations in form and structure in embryonic development are potentially interrupted. The cells in our body retain memories of these pathways and cancer can occur later in life if imperfections occur in the fidelity of these pathways. Wnt signaling pathway is a major developmental pathway, which regulates CNS development during embryogenesis and also later in adult life (Fogarty et al, 2005). Activation of this pathway appears to play a critical role in carcinogenesis (Morin, 1999; Polakis, 2000). Its role as a critical

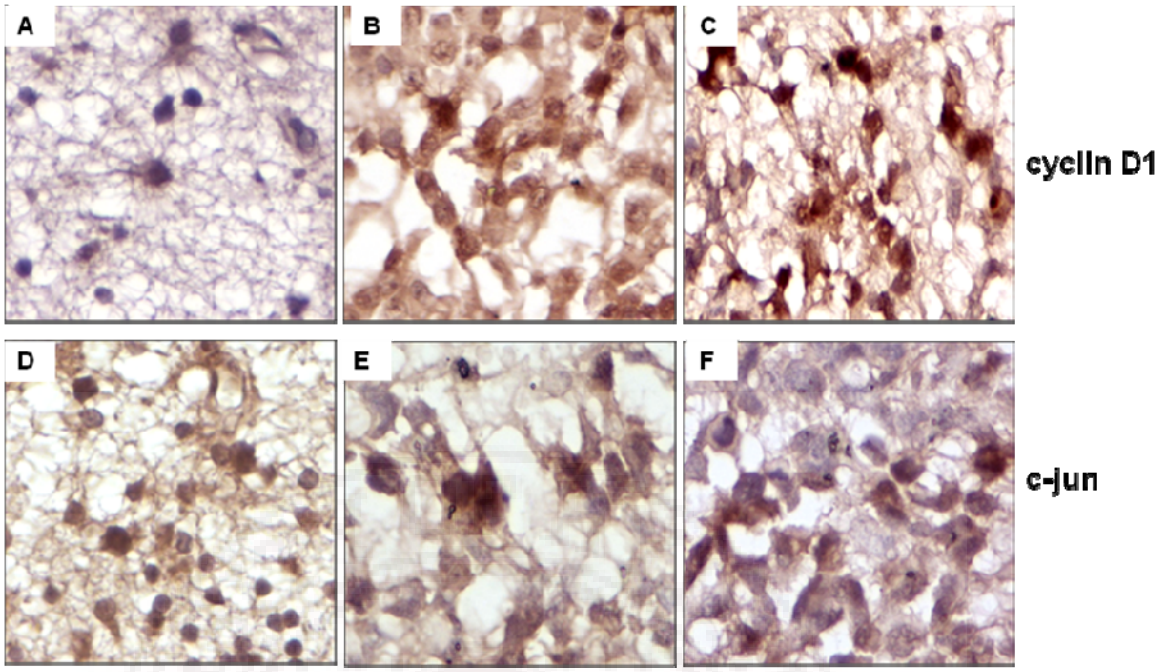


Figure 2.17. Paraffin-embedded astrocytoma sections prepared from astrocytoma specimens. Sections were incubated with c-jun and cyclin D1 (1:50 dilutions) specific primary antibodies for overnight at 4^{o}C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positive nuclear staining for c-jun **(A, B, C)** and cyclin D1 **(D, E, F).** A, D-Diffuse astrocytoma; B, E-Anaplastic astrocytoma; C, F-GBM.

mediator in carcinogenesis was evident in many cancers (Giles et al, 2003), including CNS malignancies like medulloblastoma (Huang et al, 2000), a major childhood brain tumor, and subependymal giant cell astrocytomas (Jozwiak et al, 2007). Expression of Wnt signaling cascade genes in human astrocytic tumors has not been investigated so much, only few reports showed that Wnt5a, Wnt10b and Wnt13 ligands (Yu et al, 2007; Howng et al, 2002), and frizzled 9 receptor were upregulated in GBM (Zhang et al, 2006) and also little has been studied on correlation between the histological malignancy of human astrocytic tumors and expression profile of the Wnt pathway components. To our knowledge, this is the first report showing the

upregulation of Wnt signaling cascade genes in the malignant progression of astrocytomas.

Dvl genes are upstream key mediators of β-catenin, activated in response to Wnt binding to frizzled receptor complex. Dvl activates β-catenin by suppressing the inhibitory activities of GSK3β and relocating Axin to plasma membrane. Upregulation of different *Dvl* homologs *Dvl-1* and *Dvl-3* was evident in many cancers (Okino et al, 2003; Nagahata et al, 2003; Uematsu et al, 2003). The present study has shown an enhanced expression of *Dvl-3* mRNA in astrocytic tumors and its positive correlation with astrocytoma malignancy. However *Dvl-1* and *Dvl-2* were not expressed in astrocytomas. Activated Dvl-3 transduces signals to β-catenin in response to the binding of Wnt to Frizzled and LRP. In this study we observed the relative β-catenin protein and mRNA levels were moderate in low grade tumors, higher in high grade tumors, compared to control brain samples. The mRNA and protein levels of β-catenin showed positive correlation with histological malignancy. The evidence from immunohistochemistry and immunofluorescence studies suggested nuclear and cytoplasmic accumulation of β-catenin in astrocytomas which is the hallmark of active β-catenin/Tcf signaling. In the absence of β-catenin, Tcf/Lef factors suppress the Wnt target gene expression by binding with members of the Groucho (Grg/TLE) family of transcriptional co-repressors (Brantjes et al, 2001). Translocation of β-catenin converts Tcf family proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of co-activator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless, Mediator and Hyrax (Hecht et al, 2000; Takemaru and Moon, 2000;

Hoffmans et al, 2005; Kramps et al, 2002; Barker and Clevers, 2006). β-Catenin does not have a DNA binding domain, but it has a potent transcription activation domain. In general, Lef/Tcf transcription factors do not have a strong transcription activation domain, but they have a good DNA binding/bending domain (Waterman et al, 2004). Thus, when β-catenin binds to Lef/Tcf proteins, a potent transcription regulatory complex is formed. The present study has shown elevated protein levels of Tcf4 and Lef1 in astrocytomas and their positive correlation with histological grading. Immunohistochemical staining showed Tcf4 and Lef1 were prominent in nuclei of tumor cells. With co-immunoprecipitation experiment it has been demonstrated that β-catenin interacted with Tcf4 in the nuclear fractions of GBM tissue and T98G cell line. The effects of Wnt signaling are mediated by the expression of their targets like c-jun, c-Myc, N-Myc and cyclin D1. We observed over expression of *c-myc* and *cyclin D1* mRNA in cancerous tissues than controls. In addition, protein levels of c-Myc, N-Myc, c-jun, and cyclin D1 were up regulated in astrocytic tumors and correlated with histological malignancy as well.

Astrocytic tumors develop and progress as a result of occurrence of genetic alterations in low grade lesions, which further acquire additional mutations or genetic alterations when progress towards more malignant lesion. These genetic alterations results in the activation of oncogenes and inactivation of tumor suppressor genes. These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. In the present study, we observed the elevated protein and mRNA profile of Wnt signaling pathway

components in tumors of low grade as well in high grades and their expression patterns significantly correlated with histological malignancy of astrocytomas. This clearly indicates that the Wnt/β-catenin/Tcf signaling activation is implicated the development and malignant progression of human astrocytomas. Future studies utilizing conditional expression of β-catenin and/or dnTcf4 *in vivo* at different stages of carcinogenesis will be required to corroborate our current data suggesting the involvement of the β-catenin/Tcf signaling pathway in astrocytoma development.

Chapter-3

Wnt/β-catenin/Tcf signaling pathway activation in malignant progression of rat gliomas induced by transplacental N-ethyl-N-nitrosourea exposure

3.1. INTRODUCTION

The gliomas are a large collection of primary brain tumors that have morphology and gene-expression characteristics similar to glial cells and their precursor cells. Many genetic alterations have been identified in human gliomas, however, establishing unequivocal correlation between these genetic alterations and gliomagenesis requires accurate animal models for this disease. Moreover, glioma animal models are powerful tools to investigate important aspects of tumor biology that cannot be studied in cell culture system such as angiogenesis, invasion, metastasis, and to observe effect of therapeutics under multifactorial system.

3.1.1. N-ethyl-N-nitrosourea (ENU) induced glioma rat model: exclusive suited to study stage specific alterations associated with the tumor progression

For many decades, gliomas were considered to arise from dedifferentiation of glial cells (Rubinstein, 1987). Recently much interest has been directed toward the possibility that these tumors arise from multipotent neural stem cells including cells that persist in the sub ventricular zone (SVZ) (Seyfried, 2001; Conover and Allen, 2002). Expression of neural stem cell-like characters in a novel model system generated from human malignant glioma comprising two cell lines including human neural glial cell lines-1 and 2 (HNGC 1 and HNGC2) is reported earlier (Shiras et al, 2003). Intrauterine exposure of neuronal precursor cells to ENU, synergistically interact with specific genetic abnormalities (eg. P53 deficiency) to produce glial neoplasms expressed in the adult brain. These results have lead to hypothesize that glial tumors in the adult brain arise from mutated neuronal precursor cells that

have escaped apoptotic deletion during the course of development (Leonard et al, 2001). Higher expression of a laminin receptor α6, β4 integrin proteins and mRNA in ENU induced model was reported to be crucial in controlling the migration, proliferation and other neoplastic transformations (Previtali et al, 1999).

ENU is a potent resorptive neuro carcinogen able to induce tumors in various organs of mammals (Shibuya and Morimoto, 1993). ENU has referential transplacental and neonatal action, a single intra peritoneal injection inducing almost 100% incidence of neural neoplasms (Lantos, 1986) and the incidence is higher in prenates and neonates than adults (Slikker et al, 2004). ENU induce tumors by acting on the NPC which persists in the ventricular and subventricular region (Leonard et al, 2001). During brain development, neuronal proliferation and differentiation precede the glial development. Hence, ENU injected at 18th day of gestational period is likely to show effect on glial cells that are actively proliferating and makes them susceptible to ENU action. ENU induced glioma rat model is a appropriate model to study stage specific alterations during the tumor progression and it also provides latent period, which gives the lucid perception of pathological changes in tumor development (Schiffer et al, 1980; Trosko, 2001).

The chemical mechanism of tumor induction by ENU is well known ENU-induced glioma model is well established animal model to study the tumor biology. The long latency between the administration of ENU which is cleared immediately and the appearance of brain tumors after *in utero* suggest that tumors develop through a multi step process. The short term effects observed immediately with in 6-48 h are necroses, nuclear pyknosis

and temporal cell cycle arrest. But the tumor lesions morphologically observed only after 2nd month of extra uterine life. From birth to one month of extra uterine life there is no difference between treated and control rats in the ventricular region, cortex, periventricular white matter and subeppendymal layer. After 30th day diffused cell hyperplasia can be observed in the paraventricular white matter and reactive astrocytes appear. These lesions further grow and become early neoplastic proliferation (ENP) (Schiffer et al, 1980). These ENP centers or oligodendroglial foci appear at the end of second month of extra uterine life and continue to appear till $3^{1/2}$ months and subsequently progress to "micro tumors" and then "tumors" at different stages of tumor development (Koestner et al, 1971). Circumscribed necrosis and endothelial cell proliferations appear at $5^{1/2}$ months (Schiffer et al, 1980; Schiffer et al, 1978). General scheme of tumor development after transplacental ENU administration is represented in figure 3.1 and tumor emergence over time as assessed using MRI imaging is shown in figure 3.2. In contrast to sections of untreated rats, H&E staining sections of ENU induced glioma rats show cells with apoptotic features and marked cellularity including mitotic figures and bizarre pleomorphic cells and increased cellularity in sub ventricular region.

Wnt proteins are a large group of secreted glycoproteins, which play a critical role in regulating cell fate, differentiation, proliferation and potentially tumor formation (Moon et al, 1997; Cadigan and Nusse, 2004). Aberrant activation of Wnt signaling pathway has been implicated in a variety of human cancers including colon, breast, prostate, and ovary. The Wnt pathway activation in oncogenesis and its consequences has been extensively

reviewed (Moon et al, 2004; Polakis, 1999; Reya and Clevers, 2005). Activation of canonical Wnt signaling pathway requires the binding of Wnt ligands to frizzled (FZD) receptors together with the co-receptors LDL receptor-related protein 5 (LRP5) or LRP6 (Pinson et al, 2000; Tamai et al, 2000; Wehrli et al, 2000).

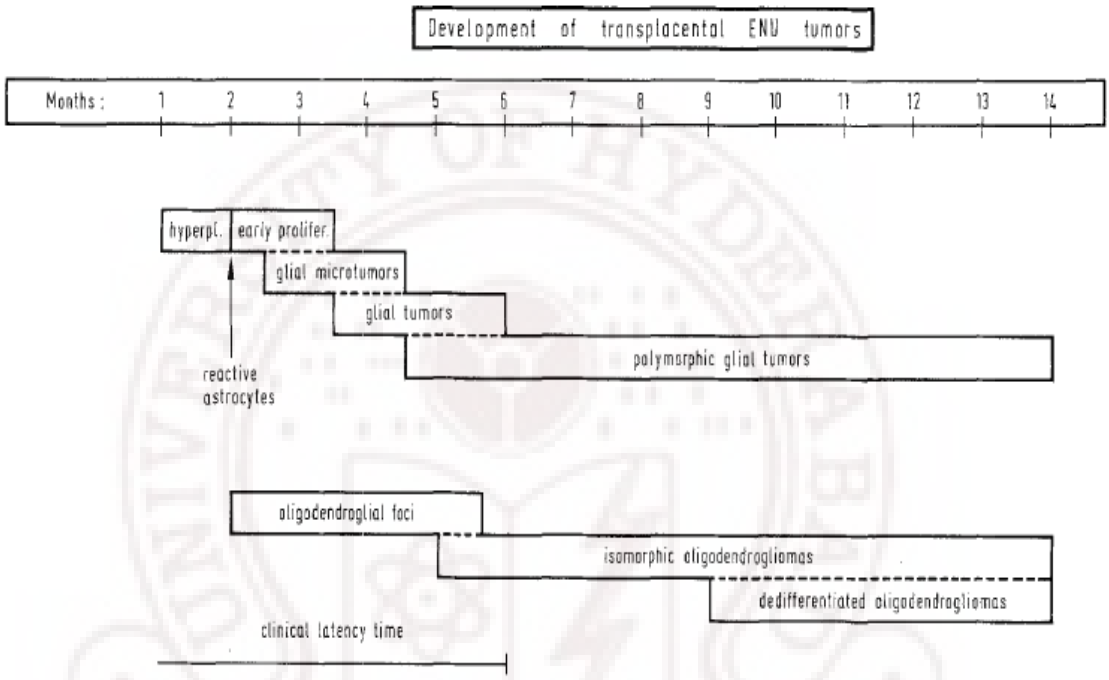


Figure 3.1. General scheme of tumor development after transplacental ENU. (Schiffer et al, 1980)

It is well documented that in the absence of Wnt signaling, β-catenin is captured in the destruction complex consists of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by GSK3β and CK1α results in its ubiquitination and subsequent proteosomal degradation (Brown and Moon, 1998; Wodarz and Nusse, 1998). Eventually, the resulting β-catenin drought ensures that the Tcf/Lef transcription factors (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their

promoter and enhancers. Binding of Wnt ligands to their cognate receptors FZD and to co-receptors LRP at the membrane results in the formation of Dishevelled (Dvl)-Fzd complex and relocation of Axin from the destruction complex to the cell membrane (Cliffe et al, 2003; Moon et al, 2004). This allows β-catenin to accumulate in the cytosol and enter the nucleus, where it interacts with members of the Tcf/Lef transcription factors to induce expression of Wnt target genes like c-Myc, N-Myc, and cyclin D1. (He et al, 1998; Shiina et al, 2003; Shtutman et al, 1999).

Though there are several reports which evident the involvement of Wnt/β-catenin signaling in several malignancies, little is known about the role of Wnt/β-catenin signaling pathway in glioma development. To begin addressing this question, we have been working with a rat model of neurocarcinogenesis in which gliomas invariably develop several months after a single prenatal exposure to N-ethyl-N-nitrosourea (ENU) (Druckery et al, 1966; Druckery et al, 1970; Shibuya and Morimoto, 1993; Swenberg et al, 1972). This model is uniquely suited to address this question because ENU itself is rapidly cleared with short half life, after which tumors are not observed before 90 days of age in the offspring. This creates a large temporal window in which one can safely assume that pathological changes are because of developing tumor and not from continued exposure to mutagens (Jang et al, 2006).

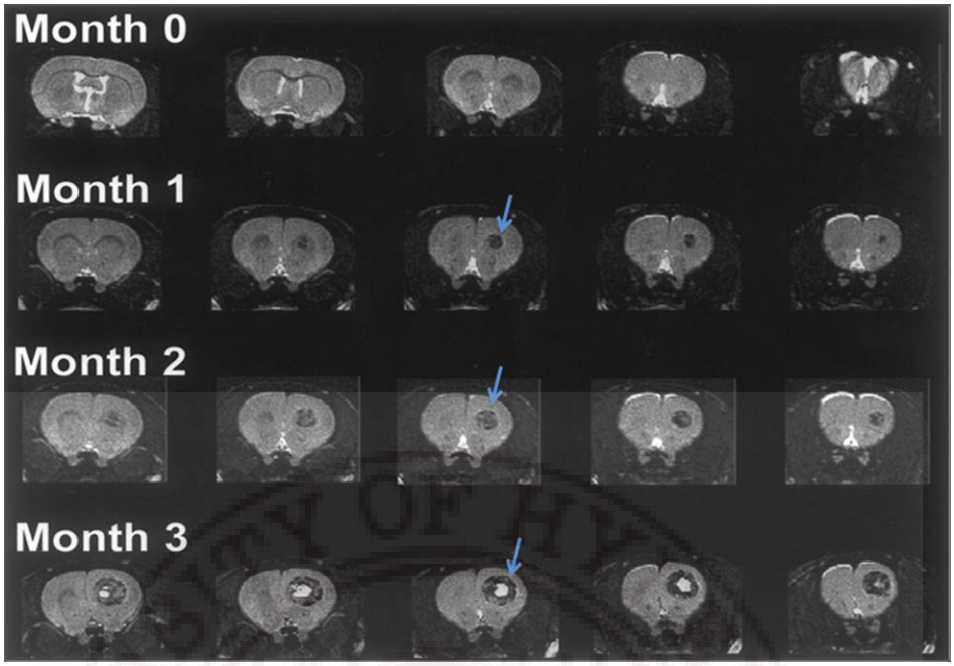


Figure 3.2. Glioma emergence over time as assessed using MR imaging. Tumors were appeared at the age of 1 month and progressively increased in size over a period of time. (Kish et al, 2001).

3.2. MATERIALS AND METHODS

3.2.1. Screening of the vaginal smears for time specific gestation in rats

Prior to the experimentation, institutional ethical clearance was obtained as per the national ethical committee guidelines (CPCSEA-2003). Female Wistar rats weighing about 250 g were screened regularly at daily interval by using methylene blue dye method for the pattern of vaginal smears to detect the different stages of estrus cycle. When the rats show estrus smear pattern, they were allowed for mating and the day following mating with the appearance of diestrus pattern of vaginal smear will indicate zero-day of pregnancy.

3.2.2. Tumor induction

The animal model used in the present study is approved by Institutional Animal Ethical Committee (IAEC). ENU (Sigma Aldrich, St Louis, MO, USA)

was freshly prepared by dissolving 10mg/ml in 0.9% saline and adjusted pH to 4.5 with crystalline ascorbic acid. A single transplacental dose of (75 mg/kg body weight) of ENU was administered intraperitonially to pregnant Wistar rats (n=4) on 18th day of gestational period and control rats received saline. All the ENU treated pregnant rats were normal. 34 (out of 38) offspring rats with glioma symptoms (corneal erosion observed from birth, limb paresis, shaggy and loss of for along with discoloration of skin, depressive or aggressiveness, loss of weight and other neurological symptoms) were used for experimentation (Bhaskara et al, 2006). Ten rats at postnatal day (P) 90, 12 rats at P135 and 12 rats at P180 were used for western blot and immunohistochemical analyses.

3.2.3. Haematoxylin and eosin (H & E) staining

Glioma rats were anesthetized with excess ketamine-Hcl and perfused transcardially with saline, followed by 4% paraformaldehyde solution. Then rat brains were collected and fixed in 4% paraformaldehyde overnight in the phosphate buffer saline and subsequently tissues were dehydrated in a series of graded alcohols and cleared by using chloroform. Finally tissues were infiltrated and fixed in paraffin to make tissue blocks. Sections of 5μm thickness were taken and transferred onto poly-L-lysine (Obtained from Sigma Aldrich; Bangalore, India) coated slides. Sections were first deparaffinized by using xylene (two washes for 7 min each) and cleared in absolute acetone for 2 min. Sections were then washed with 95% ethanol for 2 min followed by tap water and double distilled water for 5 min each. Sections were incubated in haematoxylin (Harries) for 5 min and then washed with tap water and double distilled water for 5 min each. Then the sections were incubated with the

eosin reagent for 1 min. The sections were washed with 95% ethanol two times for 1 min and followed by acid alcohol wash (1% acid in 70% alcohol) for few seconds. Acetone wash was followed by acetone and xylene mixture wash for 2 min each. Finally, stained sections were cleared in xylene two times for 2 min each. Mounting was done by using coverslips and DPX mount to view the section under microscope.

3.2.4. Western blotting

Rat brain tissues were homogenized in 5 volumes of 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, 0.3 mM Na3Vo4 and 0.3 mM aprotinin. The lysate was sonicated for 2 min and centrifuged at 14,000g for 15 min at 4^{o}C. The supernatant was collected as whole tissue lysate and frozen at -80^{o}C before use. Protein concentrations were determined by Lowry method. 75 µg of cellular protein were mixed with SDS sample buffer, boiled for 5 min and subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking the nitro cellulose paper in non-fat dry milk (5%) in Tris Buffered Saline (TBS) (10mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature, membranes were incubated for 12 h with primary antibodies. Then membranes were incubated with secondary antibodies conjugated to alkaline phosphatase (ALP) (anti-rabbit and anti-mouse IgG conjugated to ALP obtained from Genei Pvt ltd, Bangalore, India), for 1-2 h at room temperature. Before and after incubation with secondary antibodies,

membranes were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivity was visualized by incubating the membranes with BCIP-NBT solution (Genei Pvt Limited, Bangalore, India). Membranes were analyzed quantitatively using image J software (NIH).

The primary antibodies used in this experiments included rabbit polyclonal antibody against β-catenin, rabbit monoclonal antibody against Lef1, Tcf4, and cyclin D1 (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal antibody against N-Myc (gift of Dr. Robert Eisenman, Fred Hutchinson Cancer Research Center, Washington, USA), mouse monoclonal antibody against c-Myc (gift of Naidu, Manipal Institute of Medical Sciences, India), and mouse polyconal β-actin (gift of Prof. K. Anand Kumar, University of Hyderabad, India).

3.2.5. Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded specimens. Briefly, sections were deparaffinized in xylene and passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by micro waving the sections for 10-14 min in 10mM citrate buffer (pH 6.0). Sections were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase followed by incubation with blocking serum for 1 h at room temperature in a humid chamber. The sections were then incubated overnight at 4^{o}C with primary antibodies against β-catenin, Lef1, Tcf4, c-Myc, N-Myc and cyclin D1 diluted as per data sheet in blocking solution. Peroxidase conjugated secondary antibody was used for 1h incubation time at room temperature followed by TBS washes (3x5 min each). Diaminobenzidine (DAB) in buffer was used till

sections develop color. Sections were counter-stained using haematoxylin. Sections were washed with dH_2O followed by dehydration in graded alcohol, xylene and then finally mounted by using DPX (kit obtained from Biogenex Pvt limited, India). In each experiment, a negative control was included in which the primary antibody step was skipped and replaced by non-immune serum.

3.2.6. Statistics

Densitometric data from quantitative western blotting was expressed as means ± SD. Differences between tumors and controls were compared by paired Student's *t*-test (n=34) .Values of $p<0.05$ were considered statistically significant.

3.3. RESULTS

3.3.1. Identification of vaginal smears pattern representing different stages of the estrus cycle

Time specific gestation is essential for the administration of ENU during the critical gestation time period of the rats. Appearance of different patterns of vaginal cells followed in a cyclic manner among the rats. Estrus stage was indicated by the presence of large cornified (degenerative cells which lost nuclei) epithelial cells with few nucleated cells. Metestrus was observed after ovulation and consists of large cornified epithelial cells mixed with polymorphonuclear leukocytes. Presence of polymorphonuclear leukocytes exclusively along with few nucleated epithelial cells was classified as diestrus stage, which was known as period of inactivity. Mixture of round nucleated epithelial cells and presence of few leukocytes in the smear will indicate proestrus stage. Proestrus stage is known to be as preparatory period of next

estrous cycle. Vaginal cell patterns of different stages are presented in the figure 3.3.

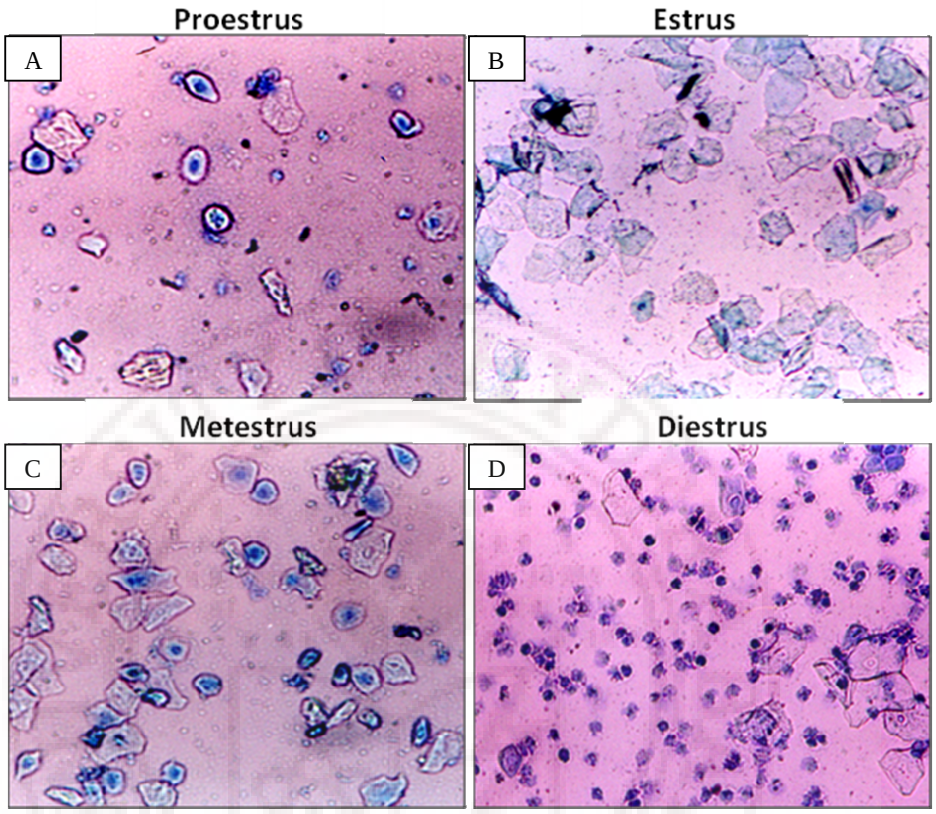


Figure 3.3. A. Rat vaginal smear showing mixture of round nucleated epithelial cells and with few leukocytes, which lasts for 12 h. This pattern is referred to as proestrus stage of the cycle.
B. Rat vaginal smear showing presence of exclusively a large cornified epithelial cells with few nucleated epithelial cells, which lasts for 12 h. This pattern is referred to as estrus stage of the cycle.
C. Rat vaginal smear showing presence of large cornified epithelial cells mixed with polymorphonuclear leukocytes, which lasts for 21 h. This pattern is referred to as metestrus stage of the cycle.
D. Rat vaginal smear showing presence of exclusively a large number of polymorpho nuclear leukocytes through the smear, which lasts for 60–70 h. This pattern is referred to as diestrus.

3.3.2. Screening the glioma rats with neurological symptoms indicating growth of brain or spinal cord tumors

Clinical examination should include observation on state of consciousness and behavior, grooming state, gait, head posture, visual and tactile placing

reactions, bladder function, appearance of eyes, response of limbs to stimuli, and palpation of cranium and spine. In the present study, rats were suffering due to the corneal erosion observed from the birth, weight loss, shaggy and loss of fur along with discoloration of skin, rats were also observed to be aggressive or depressive. Animals with neurological symptoms indicating growth of brain tumors were used for the present experimentation.

3.3.3. Histopathological studies by using haematoxylin and eosin (H & E) staining

For histological studies we examined the coronal section of glioma rat brains encompassing the anterior corpus callosum to the posterior hippocampus. Haematoxylin and Eosin staining of tissue sections of P90 glioma rats have shown the presence of early neoplastic proliferation (ENP) centers in the form of nodules and showing the features of increased cellularity. These were observed in the periventricular white matter of the cerebral hemispheres (figure 3.3A) and no tumors were found before the age of 90 days. These early neoplastic centers resemble oligodendroglial foci continued increase in size as a function of age (figure 3.3A, B, C). Where as in control rats there is marked distinction between white and grey matter (figure 3.4A). P90 glioma rats showed increased cellularity (figure 3.4B) and these features were further increased in P135 glioma rats and also showed the nuclear pleomorphism and signs of angiogenesis and mitotic figures (figure 3.4C, D). Further all these features were markedly increased to the progression stage (P180).

In addition P180 glioma rats showed the endothelial cell proliferations and hemorrhage was also observed in these rats (figure 3.5 A, B). Ki 67 is an ideal nuclear proliferation marker used to identify the labeling index of the

malignant cells in relation to their normal cell proliferation rates. Significant nuclear positive cells for ki67 were observed in the lesions of P180 glioma rats (~30% nuclear positivity) (figure 3.5C).

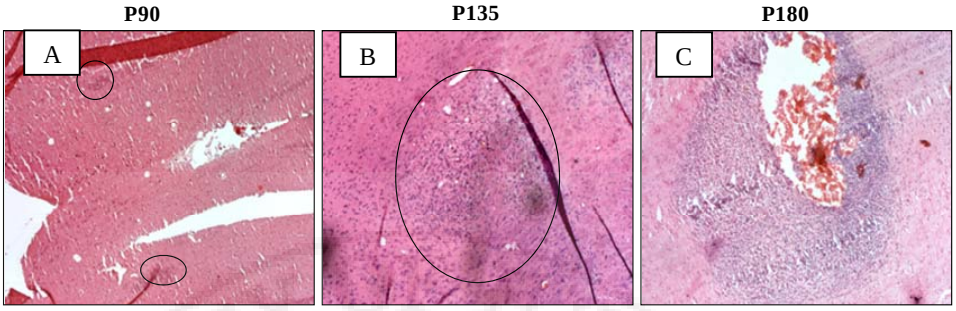


Figure 3.3. H&E staining of brain sections of ENU-induced glioma rats. A. P90 (initiation stage) glioma rat showing the presence of ENP centers in the form of nodules. **B.** P135 (promotion stage) glioma rat showing the presence of increased tumor mass with increased cellularity. **C.** P180 (progression stage) glioma rat showing the profound increase in tumor mass, angiogenesis, and cellularity. Original magnification 10X. "P" indicates postnatal in days.

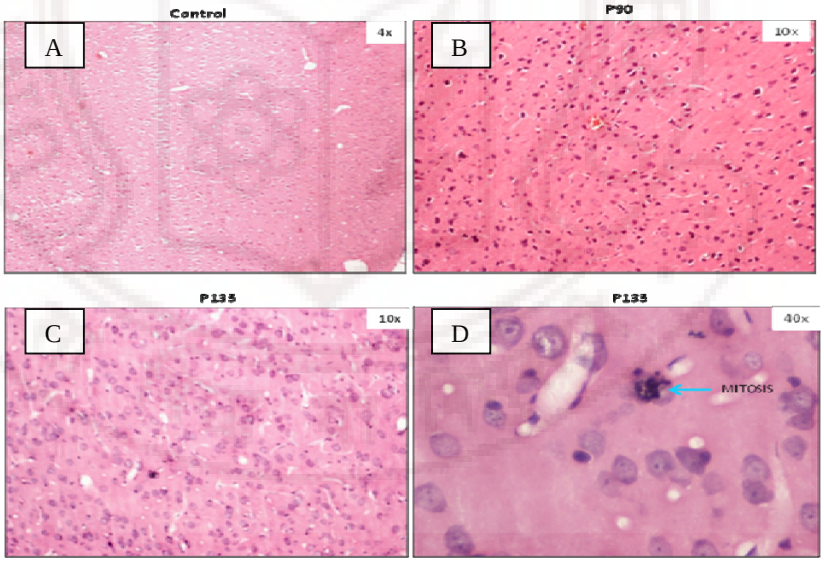


Figure 3.4. Histological characteristics of glioma rat sections. A. control rat brain section showing the white and grey matter distinctively. **B**. In P90 glioma rat increase in cellularity was observed and no marked separation of grey and white matter and cells are diffusing to the surrounding tissue. **C**. Further increase in cellularity was observed in P135 glioma rats. **D**. P135 glioma rat sections showing the mitotic figures.

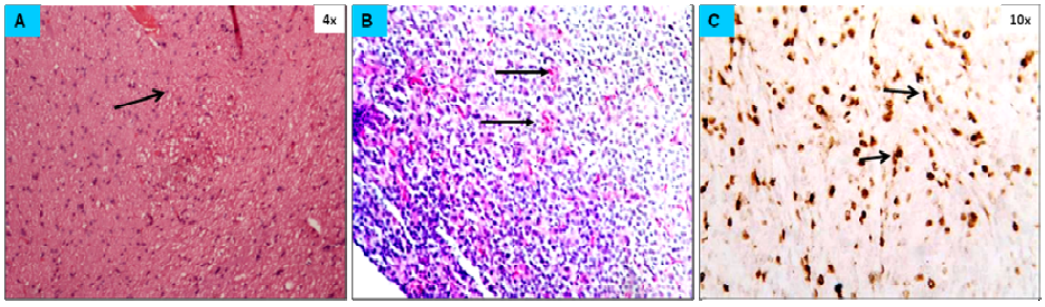


Figure 3.5. Brain sections of ENU induced glioma rats after six months of its extra uterine life. A. P180 glioma rat showing the signs of hemorrhage and to some extent necrotic features. **B**. Tumor showing the profound increase in angiogenesis. **C.** Proliferation index by Ki 67 labeling showing proliferation index (~30%) among P180 glioma rats.

3.3.4. Analysis of Wnt/β-catenin/Tcf signaling pathway components in glioma rats

In order to examine whether aberrant activation and accumulation of β-catenin in ENU-induced gliomas, P90, P135 and P180 glioma rat samples were examined using western blot analysis and saline-treated rats were used as controls. Glioma rats showed a significant increase in β-catenin levels in comparison to control (figure 3.6A), and expression levels were progressively increased from P90-P180 as evident from densitometric analysis (figure 3.6B). In order to confirm the cell type and sub-cellular distribution for the expression of β-catenin, immunohistochemical staining was performed. Representative results showed that β-catenin accumulation in P90, P135 and P180 glioma rats and immunoreactivity observed in cytoplasm and nuclei of tumor cells (figure 3.6C). Cytoplasmically accumulated β-catenin that are translocated to the nucleus there it activates the Tcf4 and Lef1 transcriptional factors. So, in order to reveal the activation of Tcf4 and Lef1 transcriptional factors, we performed western blotting and immunochemical staining.

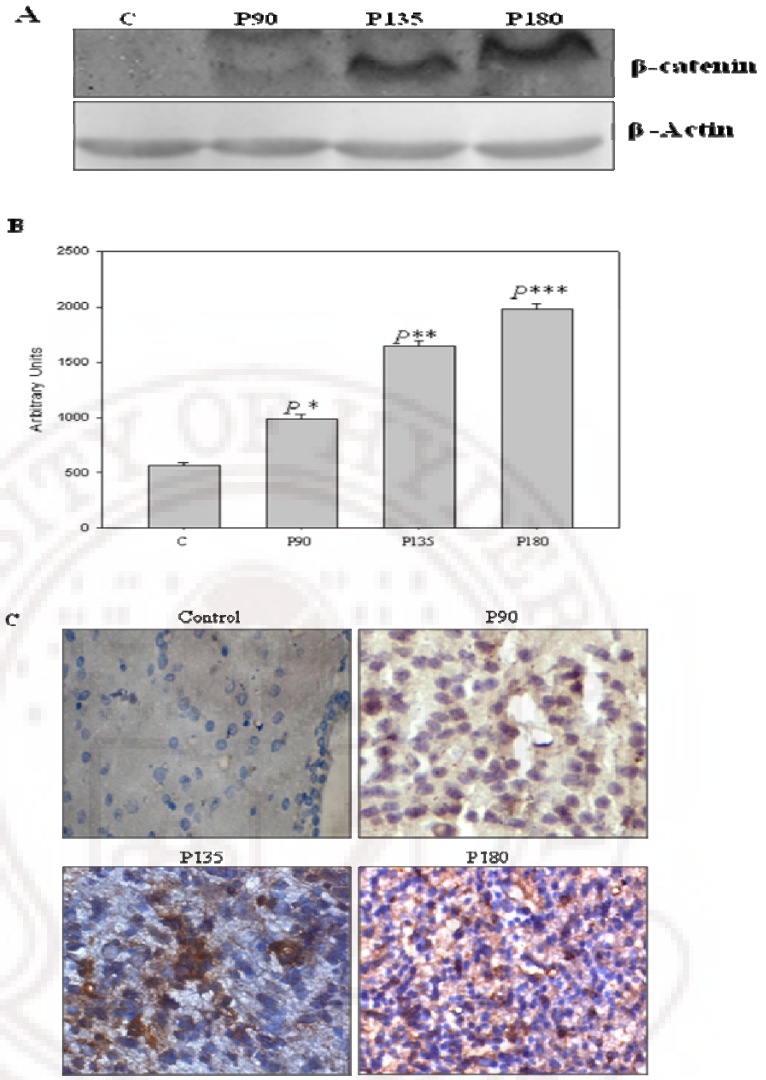


Figure 3.6. Over-expression of β-catenin in ENU-induced gliomas. A. Western blotting analysis demonstrating β-catenin overexpression in glioma rats, in comparison to saline-treated controls. The protein levels were progressively increased from P90-P180. β-actin used as a loading control. **B.** Quantitative comparison of β-catenin protein levels between glioma and control rats. The protein levels, determined by western blotting analysis, were analyzed by densitometry and normalized with expression levels of β-actin. The data were presented as mean ± SD of relative protein expression levels in tumors and control. Statistical significance is calculated using paired *t*-test between control (n=4) and glioma rats of P90 (n=6) (* represents $P<0.001$), P135 (n=8) (** represents $P<0.0002$) and P180 (n=8) (*** represents $P<0.0001$). **C.** Representative immunohistochemical staining for β-catenin in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited cytoplasmic and nuclear staining for β-catenin. Original magnification 40X. (n=4 for each age group).

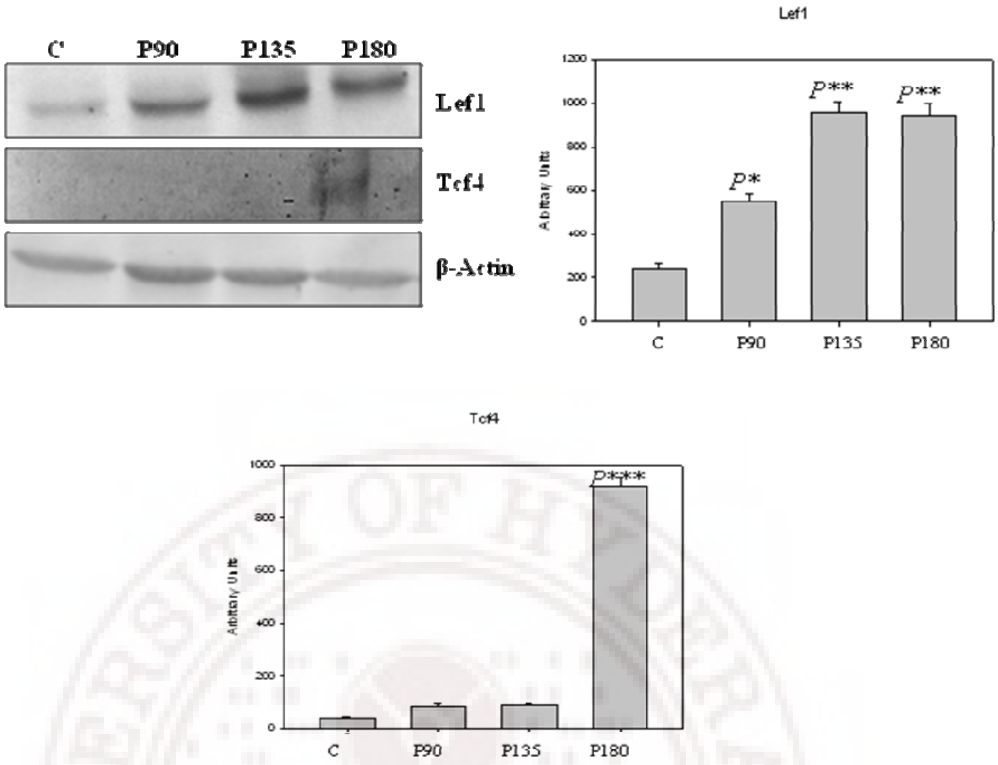


Figure 3.7. Expression levels of Lef1 and Tcf4 in glioma and control rats determined by western immunoblotting analysis. Lef1 expression levels progressively increased from P90-P180 glioma rats, while Tcf4 expression was evident only in P180 rats. Expression levels of β-actin used as loading controls. Data are shown as the means ±SD of independent densitometric measurements among different glioma rats. Statistical significance is calculated using paired *t*-test between control (n=4) and glioma rats of P90 (n=6) (* denotes $P<0.001$), P135 (n=8) (** denotes $P<0.0006$) and P180 (n=8) (** denotes $P<0.0007$, *** indicates $P<0.00001$).

Representative results showed the relative protein levels of Tcf4 and Lef1 were significantly higher in tumors than control. We observed a significant increase in Tcf4 expression at P180 only. Lef1 expression was observed in all glioma rats and relative protein levels were significantly increased from P90-P180, while very low expression was observed in controls (figure 3.7). Further, immunohistochemistry of Lef1 and Tcf4 showed their nuclear positivity in P90, P135 and P180 glioma rats (figure 3.8). We studied the gene expression of c-Myc, N-Myc, and cyclin D1 that are activated in

response to constitutive activation of Tcf4, Lef1 and β-catenin using western blot and immunohistochemistry. Western blot results showed significantly elevated levels of c-Myc, N-Myc, and cyclin D1 in gliomas when compared to

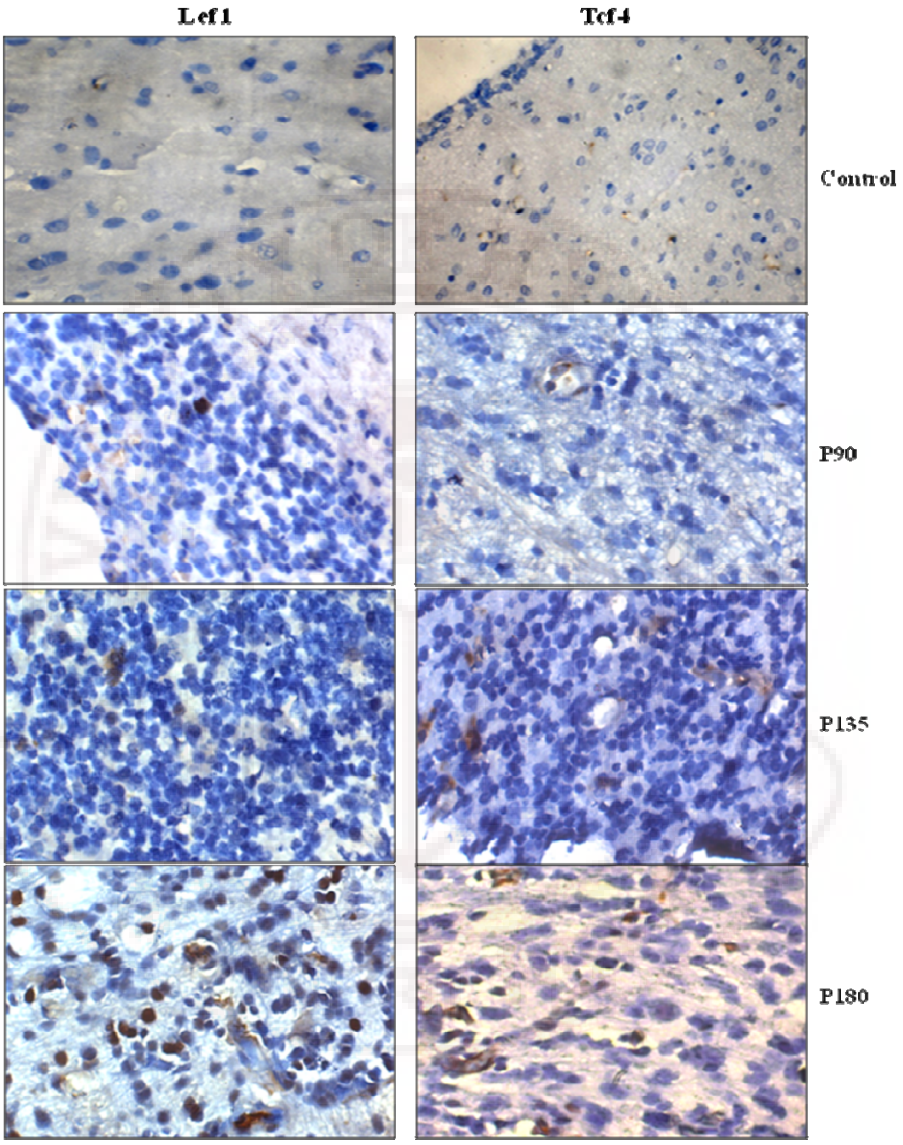


Figure 3.8. Immunohistochemical analysis of Lef1 and Tcf4 in control (C), P90, P135 and P180 glioma rats demonstrated their nuclear positivity in tumor cells. In control rats no immunoreactivity was observed. Original magnification 40X. (n=4 for each age group).

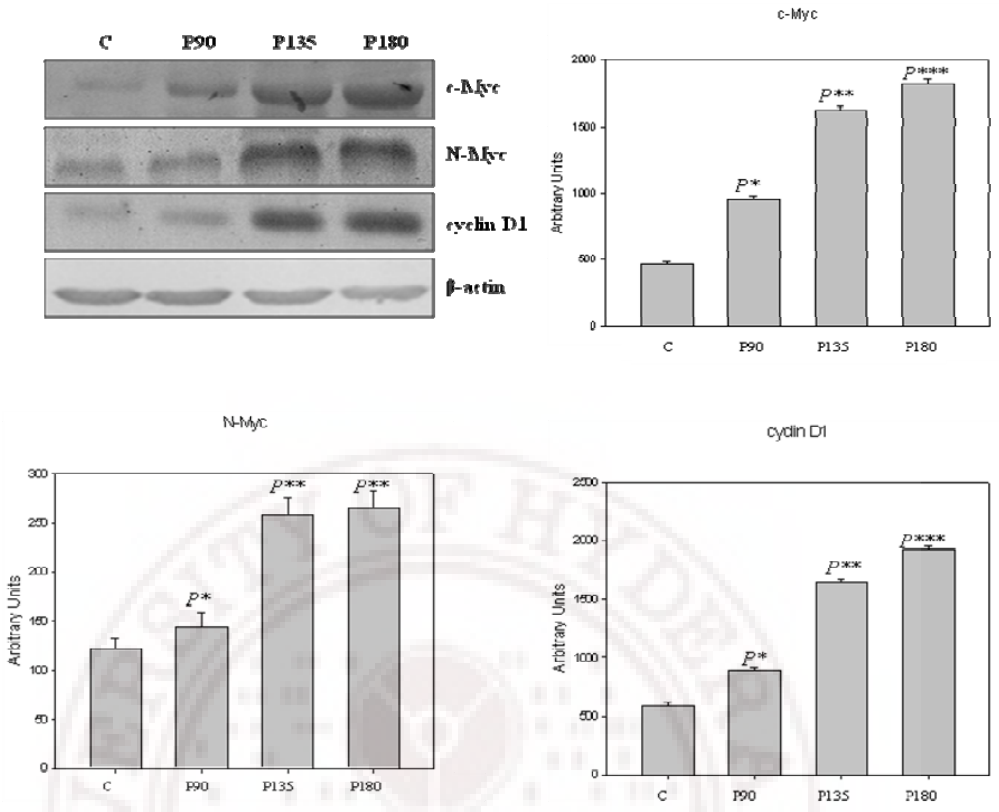


Figure 3.9. Upregulation of β-catenin target genes c-Myc, N-Myc, and cyclinD1. Western Immunoblotting demonstrating elevated protein levels of c-Myc, N-Myc, and cyclin D1 in glioma rats and protein levels were significantly increased from P90-P180. The expression levels of β-actin were used as loading controls. The data are presented as mean ± SD of relative protein expression levels in tumors and control. Statistical significance is calculated using paired *t*-test between control (n=4) and glioma rats of P90 (n=6), P135 (n=8) and P180 (n=8). In c-Myc histogram *, ** and *** indicates *P*<0.0001, 0.0002 and 0.00006 respectively. For N-Myc * and ** indicates *P*<0.005 and 0.001 respectively. For cyclin D1 *, ** and *** represents *P*<0.0005, 0.0001 and 0.00002 respectively.

controls from P90-P180 (figure 3.9). Further immunohistochemical analysis demonstrated the nuclear immunoreactivity for c-Myc, N-Myc (figure 3.10) and cyclin D1 (figure 3.11) in tumors of P90, P135 and P180 glioma rats.

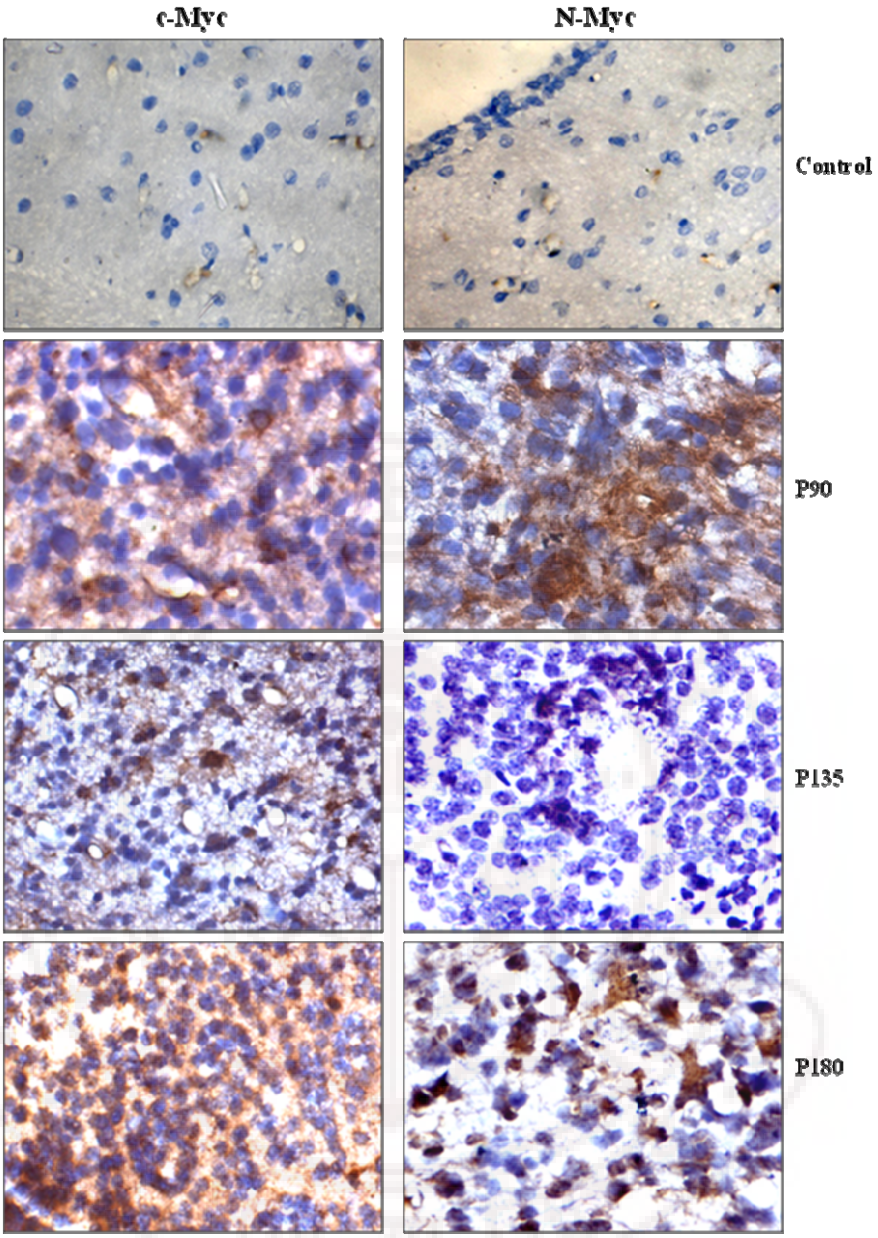


Figure 3.10. Representative immunohistochemical staining for c-Myc and N-Myc in control, P90, P135 and P180 glioma rats. The tumor cells exhibited nuclear staining for c-Myc, N-Myc and cyclin D1. Original magnification 40X. (n=4 for each age group).

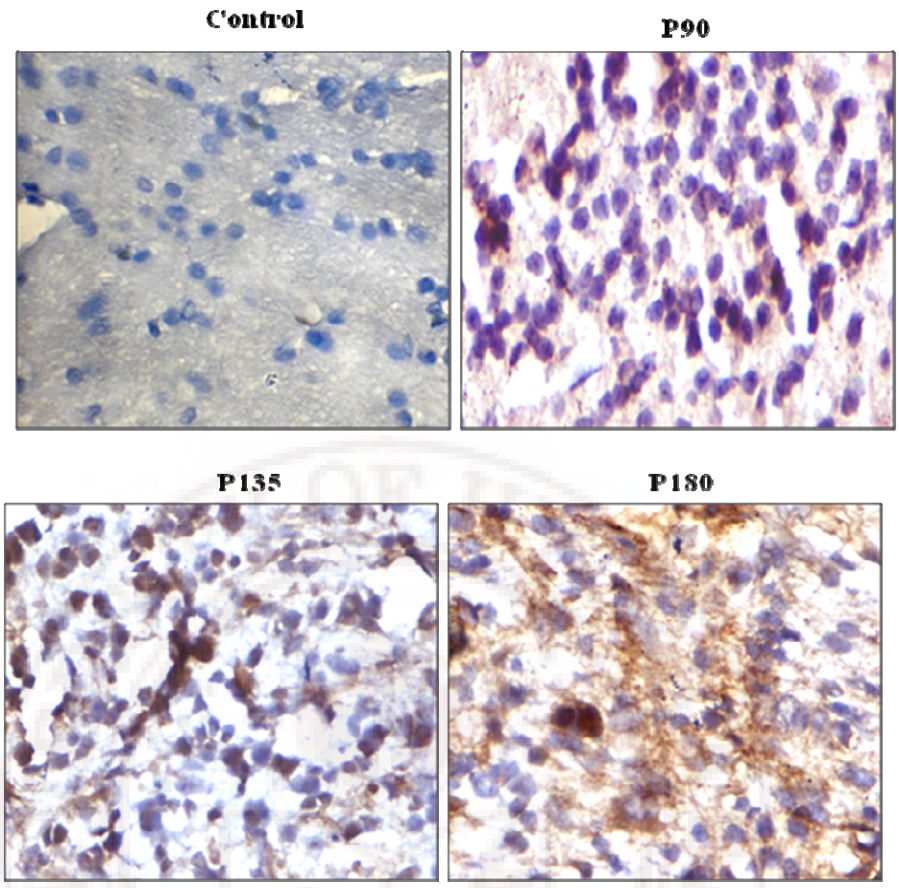


Figure 3.11. Immunohistochemical analysis of cyclin D1 in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited nuclear staining for cyclin D1. Original magnification 40X. (n=4 for each age group).

3.4. DISCUSSION

Although the prognosis for patients with malignant glioma remains poor, it is not significantly different from that of other cancers diagnosed at advanced stages. What does distinguish patients with CNS tumors from those of other cancers is the inability to detect and treat them at early, curable stages. Unfortunately, there is no effective information on whether gliomas develop through specific stages. An early detection strategy would be much more likely to be effective and this is not a problem that can be studied using human clinical samples. Although used extensively in the 1970s and 1980s,

the ENU model has been abandoned for long time by glioma biologists on the basis that ENU is not a common cause of gliomas (which are spontaneously), produces nonspecific genetic damages, and results in a diverse number of glioma types that creates too much heterogeneity for systemic study. However, each of these points can be answered. Thus, although the vast majority of gliomas appear to rise “spontaneously”, this does not exclude the possibility that the process was initiated by a prior exposure. As, in fact, it was shown by several epidemiological studies that exposure to chemical and radiological agents, as important risk factors in glioma development (Wrensch et al, 2002). The ENU model accurately reflects the scenario of the apparent spontaneous development of gliomas because there is no way to distinguish the ENU-induced rat from an uninduced littermate until the former suddenly becomes symptomatic of glioma in middle age. Interms of the chromosomal aberrations produced, ENU produces N- and O-ethylation that damages DNA. Targeted cells then apply repair mechanisms. Subsequently, the insult and repair mechanisms results in a random mutagenesis including deletions, substitutions, and translocations (Shibuya et al, 1993; Alvarez et al, 2003; Beranek, 1990). Thus a wide range of abnormalities can be noted, several of which are quite representative of the clinical situation. Finally although many types of primary tumors occur after ENU exposure, they resemble those encountered clinically. The WHO classification lists >17 tumors that can be clinically classified as gliomas based on their morphological characteristics (Kleihues et al, 2000). The ENU-induced tumors are composed of similar glial type cells that can be fit within such subgroups by using clinical neuropathological strategies. Furthermore, macroscopic tumors frequently

contain hemorrhage and necrosis that are characteristic of the most malignant GBM (Zook et al, 2000). Thus, although a variability of glioma types is encountered in this model, they all have analogies to human histological findings (Jang et al, 2008).

Cancer may arise because the developmental signaling pathways that regulate embryonic development in form and structure are potentially interrupted. The cells in our body retain memories of these signaling pathways and cancer can occur later in life if the signaling pathways are interrupted (Kelleher et al, 2006). Wnt signaling pathway is one of the major developmental pathway, which regulates CNS development during embryogenesis and also later in adult life (Fogarty et al, 2005). Activation of this pathway appears to play a critical role in carcinogenesis (Morin, 1999; Polakis, 2000), and its role as a critical mediator for carcinogenesis was evident in many cancers (Giles et al, 2003). Activation of Wnt/β-catenin signaling cascade genes in glial tumors has hardly been investigated, only a few reports showed that Wnt5a, Wnt10b and Wnt13 ligands (Yu et al, 2007; Howng et al, 2002), and FZD 9 receptor were upregulated in GBM (Zhang et al, 2006) and also little has been studied on the association between malignant progression and expression profile of the Wnt/ β-catenin pathway components.

ENU is a potent resorptive neuro-carcinogen that has been found to be mutagenic in a wide variety of mutagenicity test systems and carcinogenic in various organs of mammals (Shibuya and Morimoto, 1993). ENU has referential transplacental and neonatal action, a single intraperitoneal injection induced almost 100% incidence of neural neoplasms and the incidence is

higher in prenates and neonates than adults (Slikker et al, 2004). Multistage nature of carcinogenesis through initiation, promotion and progression stage in tumor development was reported earlier (Trosko, 2001). Characterization of different phases may be significant in the identification of effective molecular targets. This ENU-induced glioma rat model is a suitable model to study stage specific alterations during the tumor progression (Jang et al, 2006). In the present study, we studied the stage specific alterations of β-catenin/Tcf signaling at initiation (P90), promotion (P135) and progression stages (P180).

As a first step to analyze the activation of β-catenin/Tcf signaling during the development of gliomas, we studied the expression profile of β-catenin. The relative protein levels of β-catenin were higher in glioma rats compared to controls and significantly increased from 3 months to 6 months. Consistent with this, nuclear and cytoplasmic accumulation of β-catenin was observed in sections of gliomas, which is the hallmark of active β-catenin/Tcf signaling. Another illustration of β-catenin/Tcf activation during glial carcinogenesis is the overexpression of β-catenin counterparts Tcf4 and Lef1. We observed the elevated protein levels of Tcf4 and Lef1 in gliomas and their levels were significantly higher in 6 months glioma rats. Immunohistochemical analysis has shown Tcf4 and Lef1 immunoreactivity in nuclei of tumor cells. Activated β-catenin and its transcriptional counter parts Lef1 and Tcf4 mediate Wnt pathway activity by enhancing the expression of their targets like, c-Myc, N-Myc and cyclin D1. We observed the protein levels of c-Myc, N-Myc, and cyclin D1 were upregulated in gliomas and increased from initiation stage (P90) to progression stage (P180) as well.

β-Catenin levels are regulated by the ubiquitin-dependent proteolysis system and β-catenin ubiquitination is preceded by phosphorylation of its N-terminal region by the GSK-3β/Axin/APC complex. In the absence of the Wnt signal, GSK-3β constitutively phosphorylates β-catenin, leading to low expression levels as a result of ubiquitin-mediated proteolysis of β-catenin. Mutations of the adenomatous polyposis coli (*APC*) tumor suppressor gene are the most common genetic events in colorectal cancers (Ilyas and Tomlison, 1997). A recent study showed that β-catenin is a target for the ubiquitin–proteasome pathway and that phosphorylation of serine/threonine residues at positions 29, 33, 37, 41 and 45 by GSK-3β appears to be a prerequisite for ubiquitination (Aberle et al, 1997). Abnormal accumulation of β-catenin resulting from deregulation of the proteolytic machinery via phosphorylation/ubiquitination is the most likely cause of tumorigenesis of several cancers.

Gliomas develop and progress as a result of occurrence of genetic alterations in low grade gliomas, which further acquire additional mutations or genetic alterations when progress towards more malignant tumor. Secondary GBM develop through progression from low grade glioma or anaplastic glioma, while primary GBM develop *de novo* with no signs of precursor lesion. These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. The understanding of the signal transduction pathways associated with cell proliferation and cell death have the great importance in finding new drug targets against glial tumors.

In this study, we observed the up regulation of β-catenin/Tcf signaling in ENU-induced rat gliomas compared to controls. Further, the relative expression of β-catenin/Tcf signaling pathway components was significantly increased from initiation phase to progression phase and correlating with glioma malignancy. This clearly indicates that the Wnt/ β-catenin/Tcf signaling pathway may play a significant role in gliomagenesis and its malignant progression to higher grades. Further studies require analyzing the Wnt pathway activity with respect to tumor behavior, and inhibition of this pathway at early stage may promise a reliable therapy for gliomas. Together, these findings suggest that Wnt/β-catenin/Tcf signaling is significantly activated in the development of glial tumors.

Chapter-4

Targeting Wnt/β-catenin/Tcf and NF-κB signaling pathways in gliomas by nonsterodial anti-inflammatory drugs celecoxib and diclofenac

4.1. INTRODUCTION

Epidemiological data, rodent studies and in vitro experiments have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) have anti-cancerous properties against colon cancer and also widely used in the treatment of various cancers and other anomalies like rheumatoid arthritis, prophylaxis against cardiovascular disease and injuries and headaches (Dubois et al, 1998). NSAIDs exert their anti-neoplastic activities by specifically inhibiting the prostaglandin synthesis by inactivating cyclooxygenase enzymes COX-1 and COX-2 which catalyses the rate liming step, synthesis of prostaglandins from arachidonic acid substrates (Thun et al, 2002; Howe and Dannenberg, 2002). COX-1 is constitutively expressed in most tissues and is essential for homeostatic functions, whereas COX-2 is an inducible enzyme expressed in response to inflammation and cancer (Vane et al, 1998; Smith et al, 2000). Further, COX-2 overexpression was observed in several cancers including gliomas (Deininger et al, 1999; Joki et al, 2000), and its expression is associated with poor prognosis of gliomas (Shono et al, 2001).

The traditional NSAIDs, such as aspirin, flurbiprofen, indomethacin, diclofenac and sulindac inhibit COX-1 as well as COX-2 activities, whereas new generation drugs, such as celecoxib and rofecoxib, selectively inhibit COX-2 activity. Despite convincing evidence from clinical investigations, as well as from animal studies, that both, traditional NSAIDs and specific COX-2 inhibitors exert potent anti-neoplastic properties by targeting COX enzymes, the underlying molecular mechanisms remains poorly understood (Gupta and Dubois, 1998). Several reports suggesting that NSAIDs inhibits tumor cell

growth by acting on several COX-independent targets (Tegeder et al, 2001; Smith et al, 2000). Some *in vitro* studies provided evidence that different NSAIDs exert their action by acting on different molecular pathways (Herrmann et al, 1998; Kopp and Ghosh, 1994; He et al, 1999). Independent of the molecular pathway affected by NSAIDs, the common mechanism might be apoptosis. Among the COX independent targets, the most important pathways affected by NSAIDs are Wnt/β-catenin/Tcf and NF-κB pathways (Dihlmann et al, 2001; Gardner et al, 2004). Oncogenic activity of Wnt/β-catenin pathway is well described in several human malignancies (Reya and Clevers, 2005). The activity of this pathway is dependent on the β-catenin activation. In unstimulated state β-catenin is captured in complex composed of APC, GSK3β and Axin and phosphorylated by GSK3β and CK1α. Phosphorylated β-catenin is ubiquitinylated and further degraded in 26S proteosomes. Due to loss of APC function or mutations in phosphorylation sites on β-catenin results in the release of β-catenin into cytosol and its translocation to nucleus. In the nucleus β-catenin binds to its transcriptional counterparts Lef1 and Tcf4 and modulates the expression of their target genes.

Constitutive activation of extracellular signal regulated kinase 1/2 (ERK 1/2) (Bhaskara et al, 2005) c-Jun N-terminal kinase (JNK) frequently occurs in primary glial tumors (Antonyak et al, 2002; Tsuiki et al, 2003; Potapova et al, 2000). The transcription factor nuclear factor κB (NF-κB) is constitutively expressed in GBM and is associated with enhanced growth, cell cycle progression, and inducible chemo resistance (Wang et al, 1999; Ansari et al, 2001; Nagai et al, 2002). Moreover, as NF-κB and MAP kinases are

preferentially overexpressed in GBM, but not in the normal brain, these factors may represent attractive therapeutic targets for GBM tumor cells, but not normal brain tissue.

The recurring nature of GBM to standard therapies signifies a major clinical impediment that ruthlessly limits life expectancy and is yet to be poorly understood phenomenon. Many factors supports the highly resistance and persistence nature of GBM to therapies: genetic abnormalities can defend the tumor from conventional therapies, the blood-brain barrier limits the entry of chemotherapeutic agents to tumors, and GBM is highly angiogenic and has highly invasive potentials (Giese et al, 1996; Jansen et al, 2004). The environmental and obscure genetic factors underlying gliomagenesis remain poorly understood. Recently, NSAIDs together with standard therapies of GBM have entered phase I and phase II clinical trials and found that such combinations are safe and effective (Cerchietti et al, 2005; Tuettenberg et al, 2005; Reardon et al, 2005; Chuang et al, 2008). Accumulating evidences suggested that NSAIDs inhibited cell proliferation and induce apoptosis in several colon cancer cells (Smith et al, 2000). The underlying molecular mechanisms involved in NSAIDs-induced growth inhibition in GBM are not fully elucidated. Recently it was identified that NSAIDs flurbiprofen (King and Khalili, 2001) and indomethacin (Wang et al, 2005) inhibited the proliferation of glioma cells. In the previous chapters we reported the deregulation of Wnt/β-catenin/Tcf signaling and in human and ENU-induced rat gliomas and its positive correlation with histological malignancy.

The present study is undertaken to examine the growth inhibitory effects of the nonsterodial anti-inflammatory drugs, celecoxib and diclofenac

on GBM cells and also target the Wnt and NF-κB signaling pathways in GBM cells lines by celecoxib and diclofenac.

4.2. MATERIALS AND METHODS

4.2.1. Reagents

C6, U373, GOG-C-CM cell lines were obtained from National Center for Cell Science (Pune, India) and T98G and A127 cell lines were provided by Dr. Ellora Sen (National Brain Research Center, India). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (CA, USA). Diclofenac Sodium, TNF-α, Trypsin-EDTA, protease inhibitor cocktail, Caspase-3 and -8 substrates (Ac-DEVD-AFC, Ac-IETD-AMC respectively) and BCIP/NBT were purchased from Sigma Chemicals (St Louis, MO, USA). β-catenin, Tcf4, cyclin D1, p65, pERK1/2, pJNK, pAkt, JNK, cleaved-PARP, HDAC 1 and β-tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipofectamine^{TM}2000 were obtained from Invitrogen (CA, USA). VECTA SHIELD mounting medium was from Vector Laboratories (Burlingame, CA, USA). All secondary antibodies were purchased from Genei Pvt Ltd (Bangalore, India). Ready-To-Glow^{TM} NF-κB secreted Luciferase Reporter System was purchased from Clontech (USA) and pRL-TK vector was purchased from Promega (Madison, WI, USA). Celecoxib was provided by Prof. P. Reddanna (University of Hyderabad, India). Dual-Luciferase reporter assay system was purchased from Promega Corporation (USA). The Tcf-responsive reporter constructs pTOPFLASH and pFOP-FLASH was kindly provided by H Clevers (University of Utrecht, The Netherlands). Control plasmid pRL-TK was obtained from Upstate Biotech (USA). The expression plasmid pCMVΔ45, containing a mutant β-catenin

cDNA was kindly provided by KW Kinzler (Johns Hopkins Oncology Center, Baltimore, MD, USA).

4.2.2. Cell culture

C6, U373, GOG-C-CM, T98G, and A127 glioma cell lines were cultured in RPMI media, supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

4.2.3. MTT assay

Inhibition of cell proliferation was measured by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. C6, U373, A127, T98G and GOG-C-CM cells were seeded ($1x10^4$ cells/well) in 96 well plates. After over night incubation cells were treated with varying concentrations of celecoxib (25 µM, 50 µM, 75 µM and 100 µM, 150 µM and 200 µM) and diclofenac (25 µM, 50 µM, 75 µM, 100 µM, 200 µM, 400 µM and 500 µM) or vehicle (0.1% DMSO) alone for 48 h. Then MTT reagent was added to each well and incubated for 4 h at 37^0C. Then, reduced formazan crystals were solubilized in acidic iso-propanol and absorbance was then measured at 540 nm on micro plate ELISA reader. All treatments were performed in triplicate and results expressed as mean ± S.D.

4.2.4. Cell viability and clonogenic assays

The growth inhibitory effect of celecoxib and diclofenac was assessed by trypan blue exclusion assay. Briefly, C6, U373, A127, T98G and GOG-C-CM cells were plated ($1x10^5$/well) in a 6-well plate and treated with different concentrations of celecoxib and diclofenac or vehicle (0.1% DMSO) for 24 h, 48h and 72 h. Then, number of trypan blue-excluding viable cells was counted using light microscope. Clonogenic assays were performed by plating 500

U373 cells in a 100-mm dish and treated with varying concentrations of celecoxib and diclofenac or vehicle (0.1% DMSO) for 48 h. Then cells were allowed to grow for 7 days and stained with 0.5% methylene blue in 50% methanol. Colonies that contain more than 50 cells were counted (Du et al, 2004). All treatments were performed in triplicate and results expressed as mean ± S.D.

4.2.5. Flow cytometry analysis

U373 cells were seeded in 6 well plates ($1x10^5$ cells/well) and after over night incubation cells were treated with celecoxib (100 µM) and diclofenac (400 µM) or vehicle (0.1% DMSO) for 48 h. Cells from each plate were then harvested with trypsin, fixed in 70% ethanol, stained with 50 µg/ml propidium iodide (PI) and incubated for 20 min in the dark at room temperature. Then, PI stained cells were subjected to flow cytometry using a FACS Calibur (BD Biosciences). Cell cycle parameters were analyzed using Modfit software.

4.2.6. Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear fractions were performed as reported previously (Kesanakurti et al, 2009). Briefly, U373 cells were pretreated with varying concentrations of celecoxib and diclofenac and stimulated with TNFα (10 ng/ml) for 30 min. Cells were then harvested and washed in ice-cold PBS, lysed in 400 µl of cold buffer A [HEPES 10 mmol/L (pH 7.9), KCl 10 mmol/L, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1 mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L]. After 15 min incubation on ice, 0.1% NP-40 was added to the lysates and the tubes were vigorously rocked for 1 min and centrifuged

(20,800g, 5 min) at 4°C. The supernatant was collected as cytosolic fraction. Then, nuclear pellets were washed with cold buffer A, then resuspended in 50 µl of cold buffer B (HEPES 20 mmol/L (pH 7.9), NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/ L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L) and vigorously rocked for 30 min at 4°C followed by centrifugation at 20,800g for 5 min and the supernatant was collected as nuclear fraction.

4.2.7. Western blotting

Protein samples were mixed with SDS sample buffer boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis and resolved proteins were transferred on to nitrocellulose membranes. Then membranes were blocked with 5% non-fat dry milk in Tris Buffered Saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature. After blocking, membranes were incubated for overnight in primary antibodies at 4^0C. Membranes were then incubated with respective secondary antibodies for 1-2 h at room temperature and immunoreactivity was detected using BCIP/NBT solution. Before and after incubation of membranes with secondary antibodies, membranes were washed with TBS and TBST (TBS containing 0.1% Tween-20).

4.2.8. Immunofluorescence

To examine the effect of celecoxib and diclofenac on p65, U373 cells were seeded on sterile cover slips in 24-well plates and treated with celecoxib (100 µM) and diclofenac (400 µM) for 48 h. Cells were then stimulated with TNFα for 30 min, and fixed with 4% paraformaldehyde for 10 min at room

temperature followed by permeabilization with 0.2% Triton X-100. After blocking with 5% goat serum for 1 h, cells were incubated with p65 primary antibody for 1 h at room temperature. After three washes with PBS, cells were incubated with anti-FITC conjugated rabbit secondary antibody for 1 h at room temperature. Cells were washed thrice in PBS and cover slips were mounted with VECTA SHIELD mounting medium and fluorescence was captured under a Leica confocal microscope.

To know the effect of celecoxib and diclofenac on subcellular alteration of β-catenin, C6 cells were seeded on to glass cover slips in 12-well plates and treated with celecoxib (100 μM) and diclofenac (400 μM) for 48 h. Cells were then washed with PBS and fixed with 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100. After blocking with 5% goat serum for 1 h, cells were incubated with β-catenin primary antibodies for 1-2 h and anti-TRITC conjugated secondary antibodies (Genei Pvt Ltd, Bangalore, India) for 1 h at room temperature. Cells were washed thrice in PBS and cover slips were mounted with VECTA SHIELD mounting medium (Vector Labs, CA, USA) and fluorescence was captured under a Leica confocal microscope.

4.2.9. Transient transfection and Luciferase assays

To examine the effect of celecoxib and diclofenac on TNF-α induced NF-κB reporter activity U373 cells were seeded in 24 well plates and transiently transfected with 1μg of pNFκB-MetLuc2-Reporter, pMetLuc2-Control and 0.5 μg of pRL-TK (for normalization of transfection efficiency) vectors by using lipofectamine 2000. After 6 h, cells were treated with

celecoxib (100 μM) and diclofenac (400 μM) for 48 h followed by TNF-α (10 ng/ml) treatment. The cell culture medium was harvested after 24 h and subjected to metridia luciferase activity according to the manufacturers protocol (Clontech, USA). For renilla luciferase activities cells were lysed in passive lysis buffer and measured the luciferase activities with luminometer (Promega, Madison, WI, USA). NF-κB metridia luciferase activities were normalized with that of renilla luciferase values.

To assess the efficacy of celecoxib and diclofenac on alterations in β-catenin localization, C6 cells were seeded in 24 well plates and transiently transfected with 1 μg of pTOPFLASH, pFOPFLASH and 0.5 μg of pRL-TK (for normalization of transfection efficiency) vectors by using lipofectamine 2000 (Invitrogen, USA) (Korinek et al, 1997). For expression of mutant β-catenin, 1 μg of pCMVΔ45 plasmid was transfected together with TOPFLASH, FOPFLASH and pRL-TK plasmids. After 3 h of transfection, cells were treated with celecoxib and diclofenac. After incubation for 48 h, cells were washed with PBS, lysed with Passive Lysis Buffer and Dual-Luciferase reporter assays were performed as per the manufacturer's instructions (Promega, USA).

4.2.10. Caspase-3 and Caspase-8 assays

Caspase-3 and Caspase-8 activities were performed as described earlier (Kesanakurti et al, 2009). Briefly, after treating with celecoxib and diclofenac for 48 h, cells were lysed in the lysis buffer and 50 μg of the cellular protein and 8 μM of fluorogenic substrate (Ac-DEVD-AFC for Caspase-3, Ac-IETD-AMC for Caspase-8) were added to 1 ml of the assay buffer and

incubated for 1 h at 37°C. Measurements were made on a spectrofluorimeter with a λ_{ex} of 380 nm and a λ_{em} of 460 nm for Caspase-8 and with a λ_{ex} of 400 nm and λ_{em} of 480–520 nm for Caspase-3.

4.2.11. Statistics

All data were expressed as mean ± standard deviation (SD) obtained from at least three independent experiments. Statistical comparisons among two groups were carried out by student's t-test. Differences were considered to be statistically significant at a P value of <0.05.

4.3. RESULTS

4.3.1. Celecoxib and diclofenac suppress the growth of GBM cells

NSAIDs inhibit the proliferation of several cancer cell lines and affecting the different molecular pathways. Independent of the molecular pathway affected by NSAIDs the common mechanism might be the apoptosis. A series of experiments were conducted to determine the effect of celecoxib and diclofenac on proliferation of GBM cell lines. First, to know whether celecoxib and diclofenac inhibits the proliferation of GBM cells, MTT assays were performed. Celecoxib and diclofenac significantly reduced the proliferation of C6, A127, T98G, U373 and GOG-C-CM cells in a dose dependent manner (figure 4.1 and figure 4.2). Further to determine the effect of celecoxib and diclofenac on growth rate, GBM cells were treated with celecoxib and diclofenac for 24 h, 48h and 72 h and the number of viable cells were counted at each time period. Celecoxib and diclofenac significantly retarded the growth rate of various GBM cells in a dose and time dependent manner (figure 4.3 and figure 4.4).

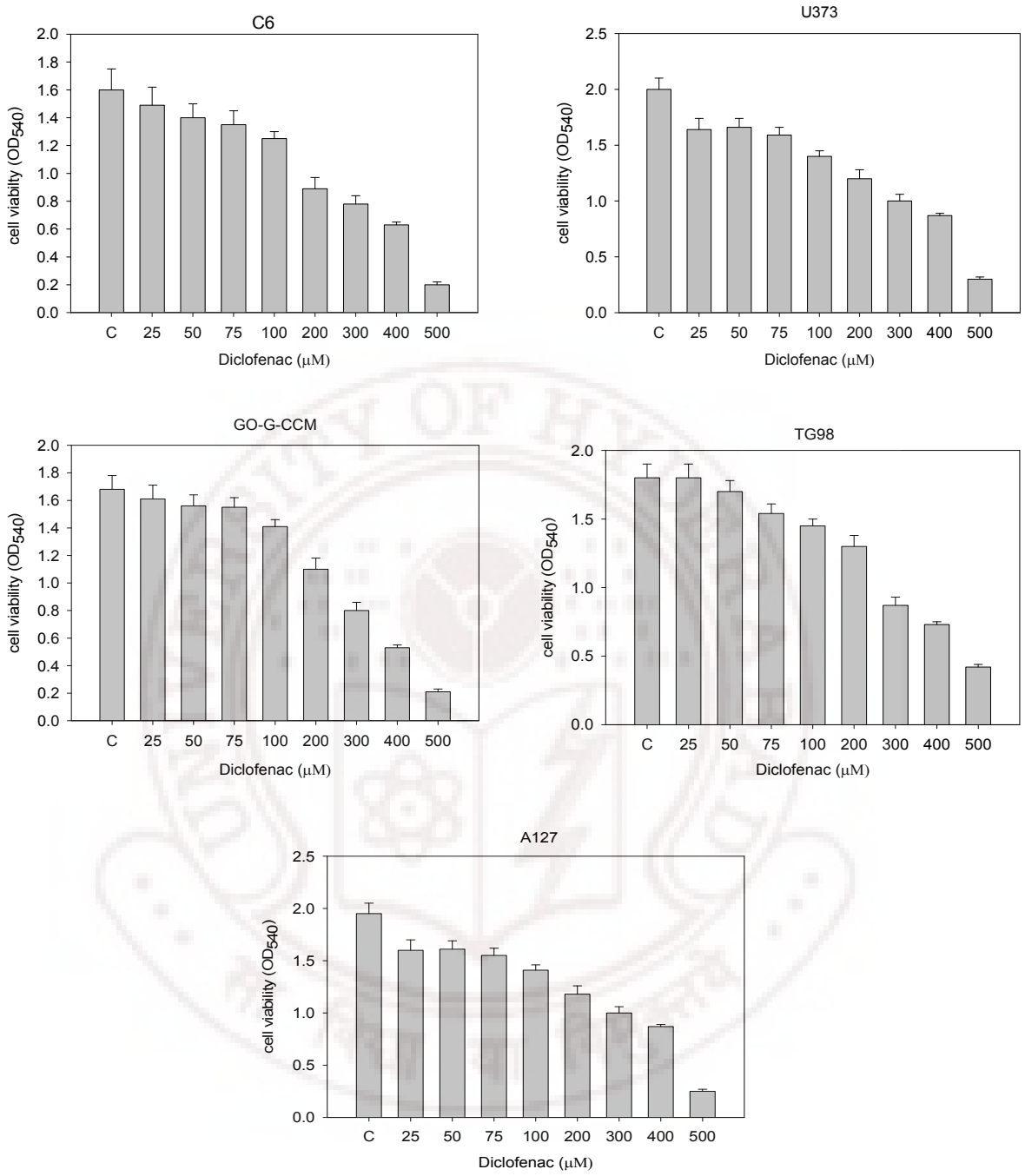


Figure 4.1. Effect of diclofenac on proliferation of GBM cells. C6, U373, T98G, A127 and GOG-C-CM cells were treated with vehicle (0.1% DMSO) or indicated concentrations of diclofenac for 48 h and subjected to MTT assay. All data presented are the mean + SD and are representative of three independent experiments. 'C' denotes the vehicle treated control cells.

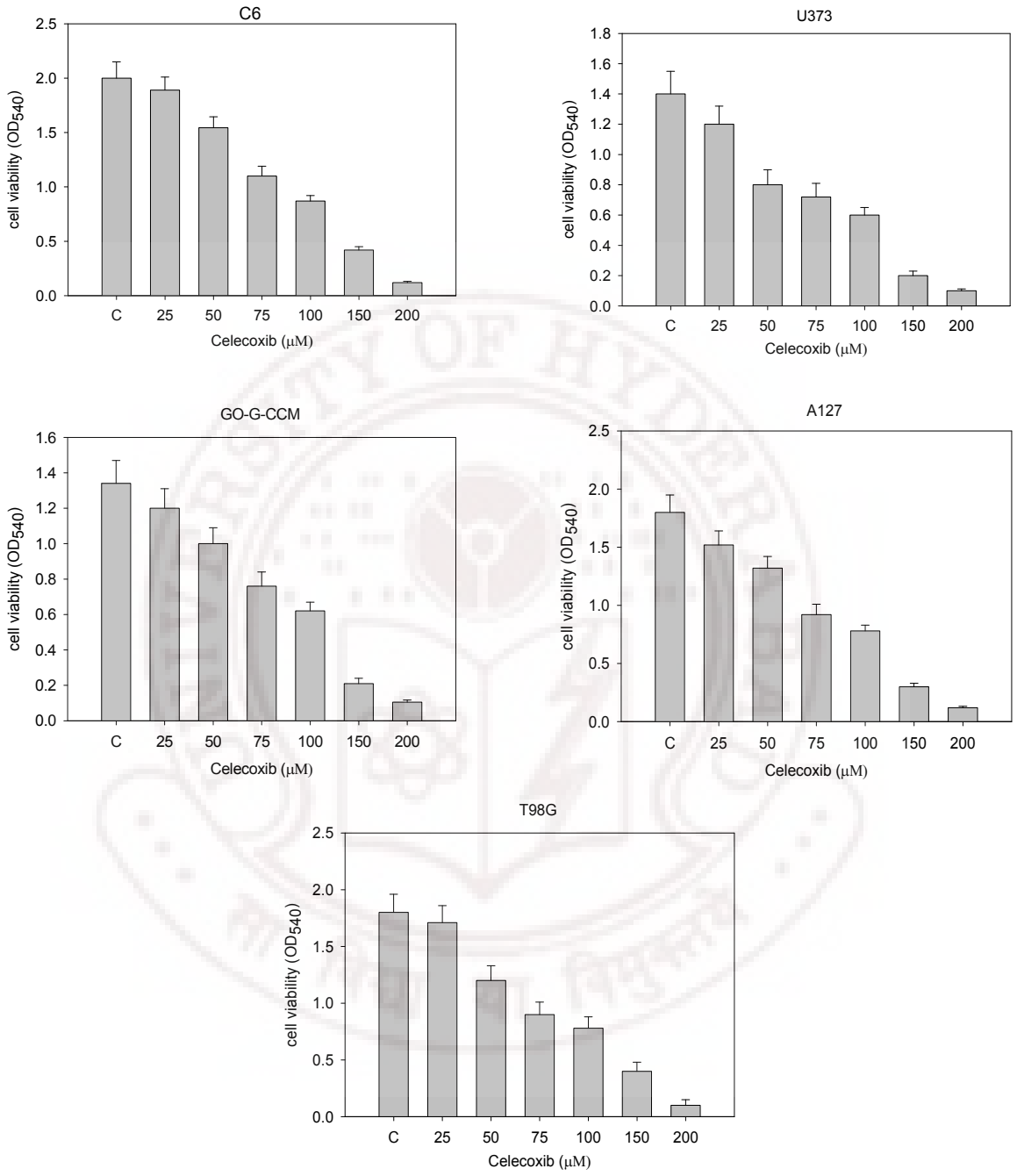


Figure 4.2. Effect of celecoxib on proliferation of GBM cells. C6, U373, T98G, A127 and GOG-C-CM cells were treated with vehicle (0.1% DMSO) or indicated concentrations of celecoxib for 48 h and subjected to MTT assay. All data presented are the mean + SD and are representative of three independent experiments. 'C' denotes the vehicle treated control cells.

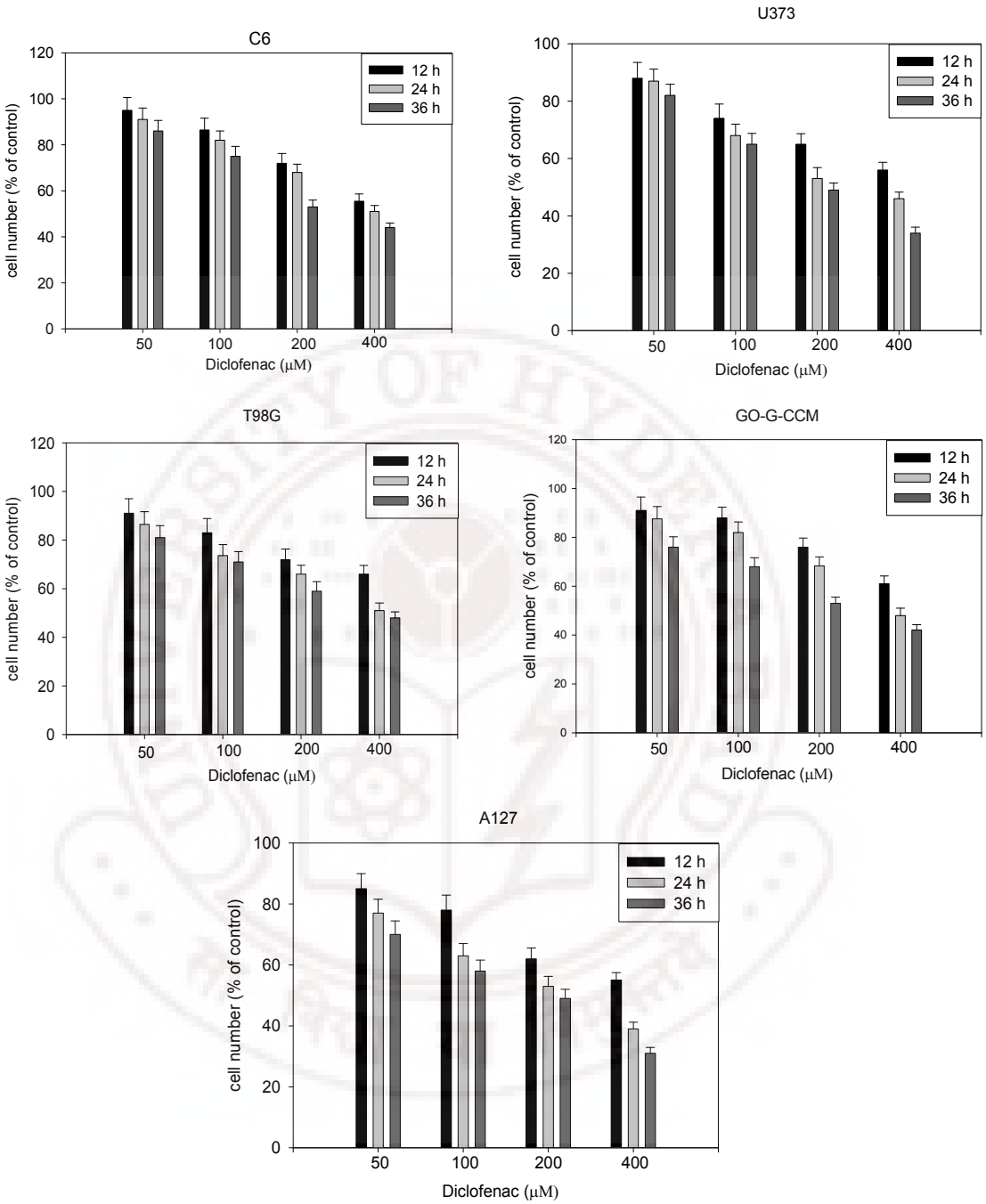


Figure 4.3. Effect of diclofenac on growth rate of GBM cells. C6, U373, T98G, A127 and GOG-C-CM cells were seeded in 6 well plates and treated with vehicle (0.1% DMSO) or indicated concentrations of diclofenac for 24 h, 48 h and 72 h and total viable cell numbers were counted. Data expressed as mean + SD and are representative of three independent experiments. 'C' denotes the vehicle treated control cells.

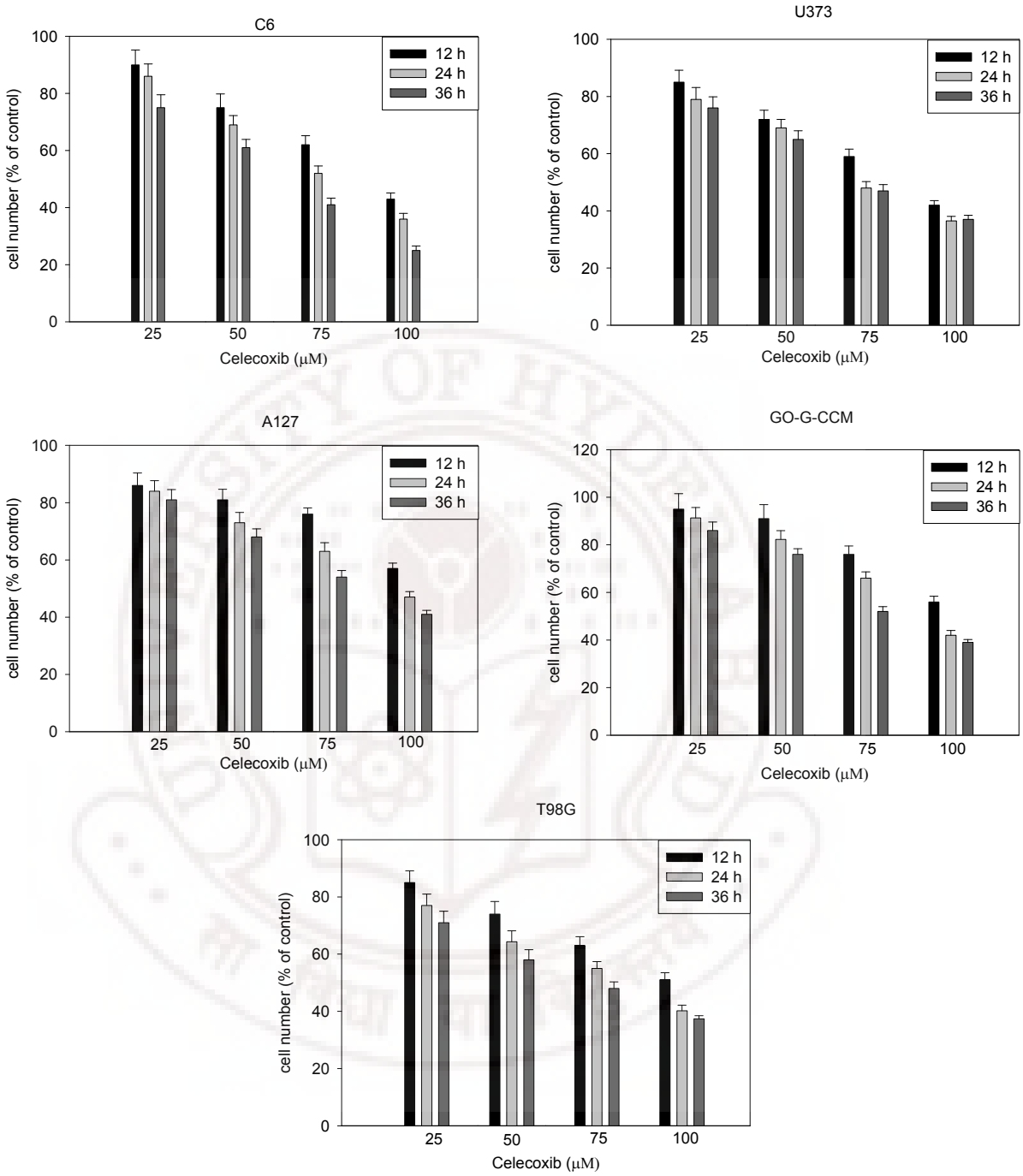


Figure 4.4. Effect of celecoxib on growth rate of GBM cells. C6, U373, T98G, A127 and GOG-C-CM cells were seeded in 6 well plates and treated with vehicle (0.1% DMSO) or indicated concentrations of celecoxib for 24 h, 48 h and 72 h and total viable cell numbers were counted. Data expressed as mean + SD and are representative of three independent experiments. 'C' denotes the vehicle treated control cells.

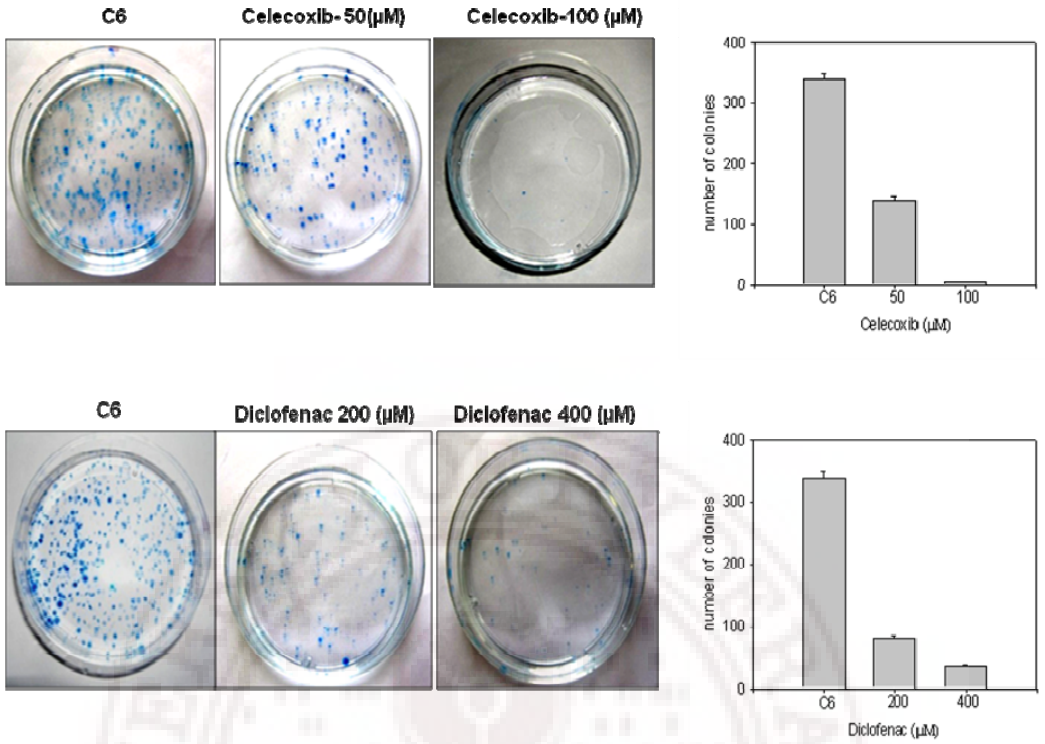


Figure 4.5. Effect of celecoxib and diclofenac on cloning efficiency. 500 C6 cells were plated in 100-mm culture dishes and after 24 h cells were treated with vehicle (0.1% DMSO) or indicated concentrations of celecoxib and diclofenac for 48 h. After 7 days colonies were stained with methylene blue and colonies that contain ≥50 cells were counted. All data presented are the mean + SD and are representative of three independent experiments. 'C' denotes the vehicle treated control cells.

To further characterize the effect on cell proliferation, clonogenic assays were performed. Untreated GBM cells attain around 80% of cloning efficiency, whereas celecoxib and diclofenac treatment significantly reduced the cloning efficiency of GBM cells in a dose dependent manner (figure 4.5). Altogether, these experiments demonstrated that celecoxib and diclofenac suppress the growth of GBM cell lines.

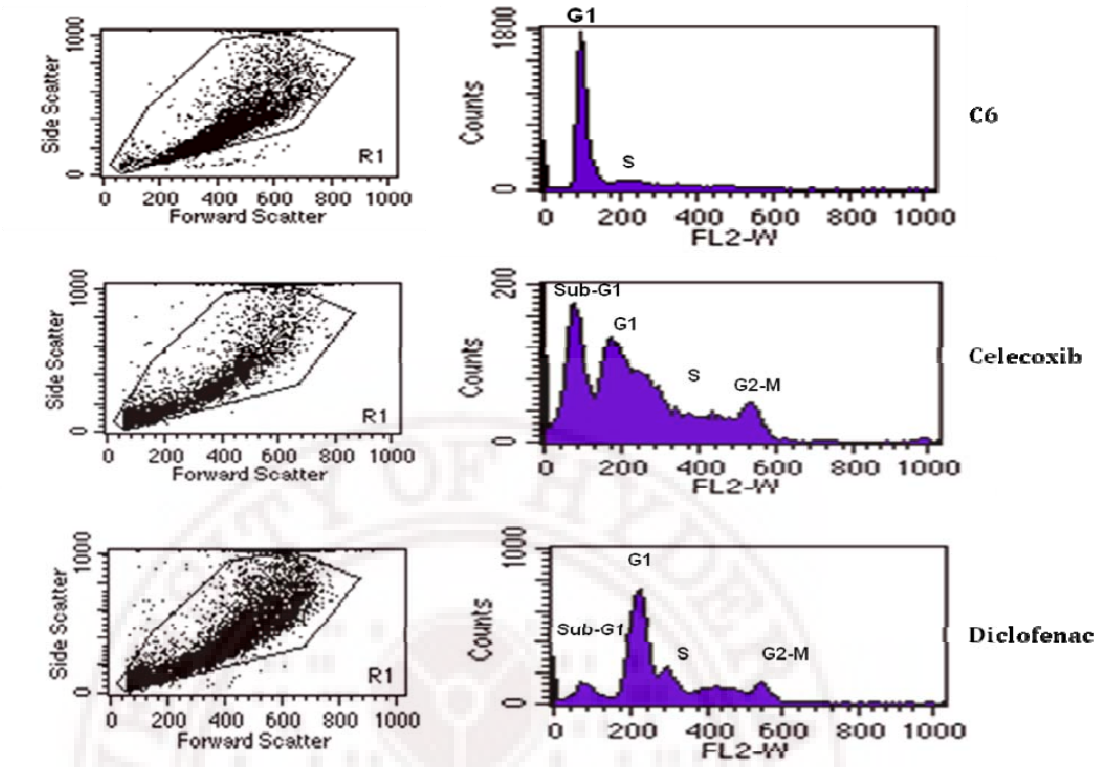


Figure 4.6. Effect of celecoxib and diclofenac on cell cycle distribution. C6 cells were treated with vehicle (0.1%) **(A)** or celecoxib (100μM) **(B)** and diclofenac (400 μM) for 48 h, fixed in 70% ethanol and stained with propidium iodide and subjected to FACS analysis. Non-apoptotic cell population in different phases of cell cycle is specified, together with sub-G1apoptotic cell population.

Table 1. Cell cycle distribution of C6 cells after treatment with celecoxib and diclofenac

	C6	Celecoxib (100 μM)	Diclofenac (400μM)
Sub-G1 (%)	0.54	33.34	16.74
G1 (%)	78.21	35.78	49.25
S (%)	17.21	21.85	24.93
G2/M (%)	2.98	6.99	6.94

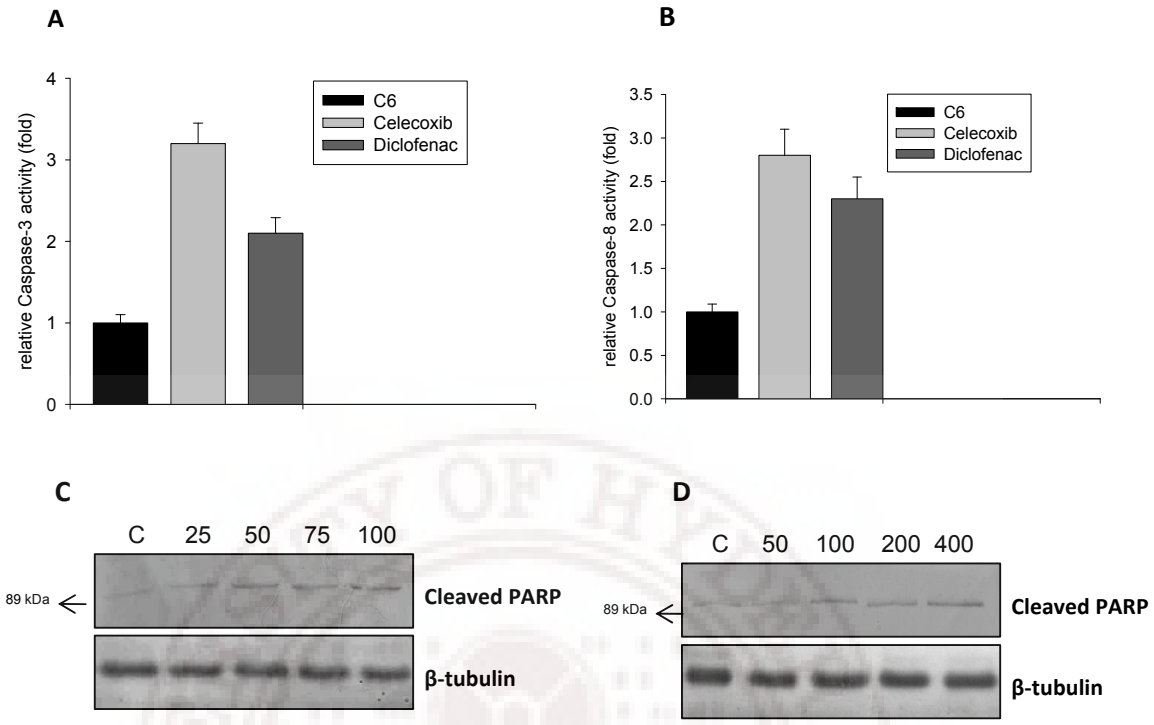


Figure 4.7. Effect of celecoxib and diclofenac on Caspase-3 and -8 activities and PARP cleavage. Effect of celecoxib and diclofenac on Caspase-3 **(A)** and Caspase-8 **(B)** activities in C6 glioma cells. Caspase-3 and Caspase-8 substrates were added after 48 h incubation of C6 cells with celecoxib (100μM) and diclofenac (μM) and enzyme activities were measured by fluorescence intensity examination. C6 cells were treated with indicated concentrations of celecoxib **(C)** and diclofenac **(D)** for 48 h and cell lysates were prepared and subjected to western blotting for cleaved PARP. β-tubulin used as loading control and the blots were representatives of three independent experiments. 'C' denotes the carrier control.

4.3.2. Celecoxib and diclofenac alter the cell cycle, increases Caspase-3 and Caspase-8 activities and induce PARP cleavage

Uncontrolled cell cycle is one of the prominent characteristic of GBM cells. To ascertain the effect of celecoxib and diclofenac on cell cycle distribution, C6 cells were treated with celecoxib and diclofenac for 48 h and subjected to FACS analysis. Celecoxib and diclofenac increased the G2/M cell population at the expense of G1 fraction, suggesting that celecoxib and diclofenac arrest the cell cycle at G2/M phase (table 4.1). Moreover, celecoxib

and diclofenac significantly increased the apoptotic cell population (sub G1), indicating that celecoxib and diclofenac promoted apoptosis in GBM cells (figure 4.6A, B, C). To further confirm the ability of celecoxib and diclofenac on apoptosis, Caspase-3 and Caspase-8 activities and PARP cleavage were examined. The Caspse-3 and Caspase-8 activities were significantly increased in a dose dependent manner ($P<0.005$) (figure 4.7A, B) significantly increased the cleaved PARP levels in a concentration dependent manner (figure 4.7C, D). These results clearly suggested that celecoxib and diclofenac arrest the cell cycle and induce the apoptosis in GBM cells.

4.3.3. Celecoxib and diclofenac inhibits Wnt/β-catenin/Tcf signaling activation

In previous chapters, we showed that the expression of β-catenin and its target genes including cyclin D1 were up regulated in gliomas and correlated with degree of histological malignancy. In addition, we also checked the status of β-catenin levels in different glioma cell lines, among those C6 cell line bear more β-catenin levels than remaining glioma cell lines. So, we have chosen this cell line for analyzing β-catenin signaling in response to NSAIDs treatment. To date several reports provided the evidences that NSAIDs reduced the colon cancer cell line proliferation by inhibiting the β-catenin pathway (Dihlmann et al, 2001; Gardner et al, 2004). In the present study we aimed to check the effects of celecoxib and diclofenac on β-catenin signaling in C6 glioma cell lines.

The activity of Wnt signaling is mediated by the binding of β-catenin/Tcf complex to their specific promoter elements. To investigate whether celecoxib

and diclofenac down-regulated β-catenin/Tcf signaling, we used a set of reporter constructs which contained three copies of the optimal Tcf motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal c-Fos promoter driving luciferase expression (pTOPFLASH and pFOPFLASH respectively). Mechanism of action of these constructs is summarized in figure 4.8.

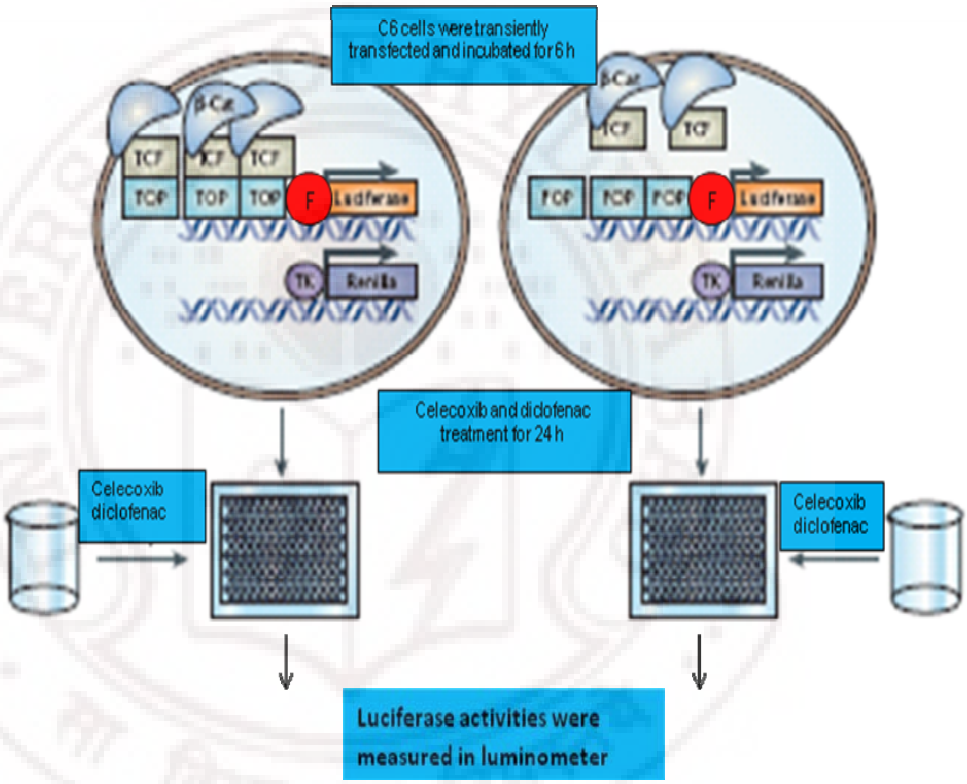


Figure 4.8. Flowchart of the TOPFLASH and FOPFLASH reporter activity assay. A. C6 Cells were transfected with a Tcf reporter gene (TOP-luciferase), which responds to the aberrant Wnt signaling activity by driving high levels of luciferase activity. **B.** C6 cells were transfected with a control plasmid FOP-luciferase, which is unable to respond to active Wnt signaling and consequently drives much lower levels of luciferase activity. This is included to control for non-specific effects on luciferase activity. Both cell lines are also transfected with thymidine kinase–renilla luciferase (pRL-TK), which drives strong Wnt-independent activity of the renilla gene and serves as a measure of cell viability. The reporter cell lines are independently plated out into culture plates and treated with celecoxib and diclofenac. After an incubation time of 48 h, cells are lysed and luciferase and renilla activities measured using a luminometer. (Part of the figure reproduced from Barker et al, 2006)

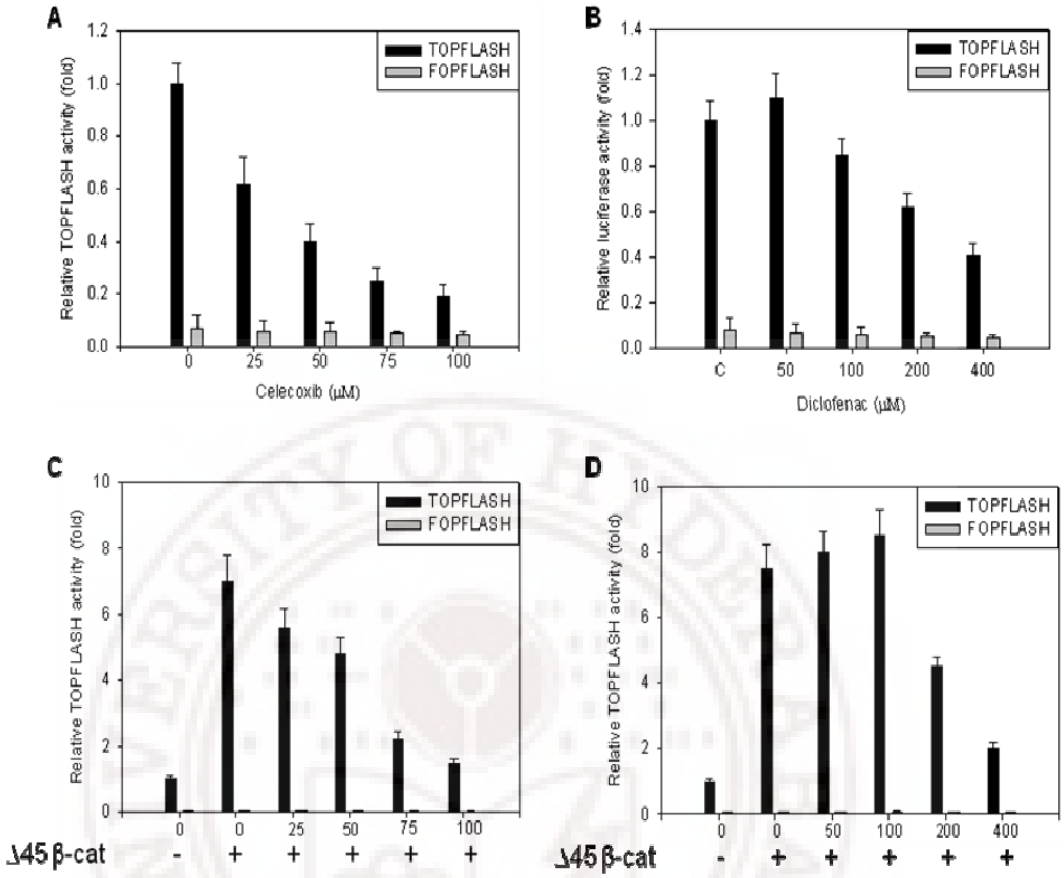


Figure 4.9. Effect of celecoxib and diclofenac on Tcf reporter activity. **(A, B)** C6 cells were transfected with TOPFLASH or FOPFLASH reporter plasmids together with pRL-TK plasmid. **(C, D)** C6 cells were transfected with reporter plasmids TOPFLASH, FOPFLASH, pRL-TK together with mutant β-catenin vector (pCMVΔ45). Following 3 h transfection transfected cells were treated for 48 h with different concentrations of celecoxib and diclofenac. TOPFLASH and FOPFLASH activities were corrected for transfection efficiency using the pRL-TK activity. Celecoxib and diclofenac dose dependently decreased the Tcf reporter activity in both the cases. All data presented are the mean + SD and are representative of three independent experiments. C denotes carrier control.

C6 cells were transiently transfected with the pTOPFLASH and pFOPFLASH reporter plasmids together with pRL-TK plasmid. Reporter assays revealed that celecoxib and diclofenac down-regulated the β-catenin/Tcf signaling in a dose dependent manner (figure 4.9A, B). In order to exclude nonspecific effects of celecoxib and diclofenac on gene expression,

pTOPFLASH activity is corrected by the effect of drug on pFOPFLASH activity which contains mutant inactive Tcf binding sites. This was confirmed by the fact that pFOPFLASH was not influenced by celecoxib and diclofenac.

Interaction with β-catenin is very important in Tcf activation. To check whether celecoxib and diclofenac inhibits upstream or downstream of β-catenin signaling, C6 cells were transiently transfected with constitutively active mutant β-catenin (deletion of serine 45) together with the reporter constructs. Overexpression of mutated β-catenin (pCMVΔ45) significantly increased the Tcf activity in C6 glioma cell lines. When treated with celecoxib and diclofenac the increased Tcf activity is reduced to almost basal level in a dose dependent manner (figure 4.9C, D).

The above results clearly suggested that celecoxib and diclofenac inhibited β-catenin signaling activity in a dose dependent manner.

4.3.4. Celecoxib and diclofenac does not affect the levels of β-catenin but repress its target gene cyclin D1

When Wnt signaling is active β-catenin is accumulated in cytosol and further translocated to nucleus. Since Tcf activation is largely dependent on the level of β-catenin, we analyzed the total amount of β-catenin in response to celecoxib and diclofenac treatment. The total levels of β-catenin were not altered following celecoxib (figure 4.10.A, B) and diclofenac (figure 4.10.C, D) treatments. To further confirm the effect of celecoxib and diclofenac on β-catenin levels, C6 cells were transfected with constitutively active mutant β-catenin plasmid pCMVΔ45 and analyzed the β-catenin levels. Following transfection, β-catenin levels were enhanced compared to control C6 cells.

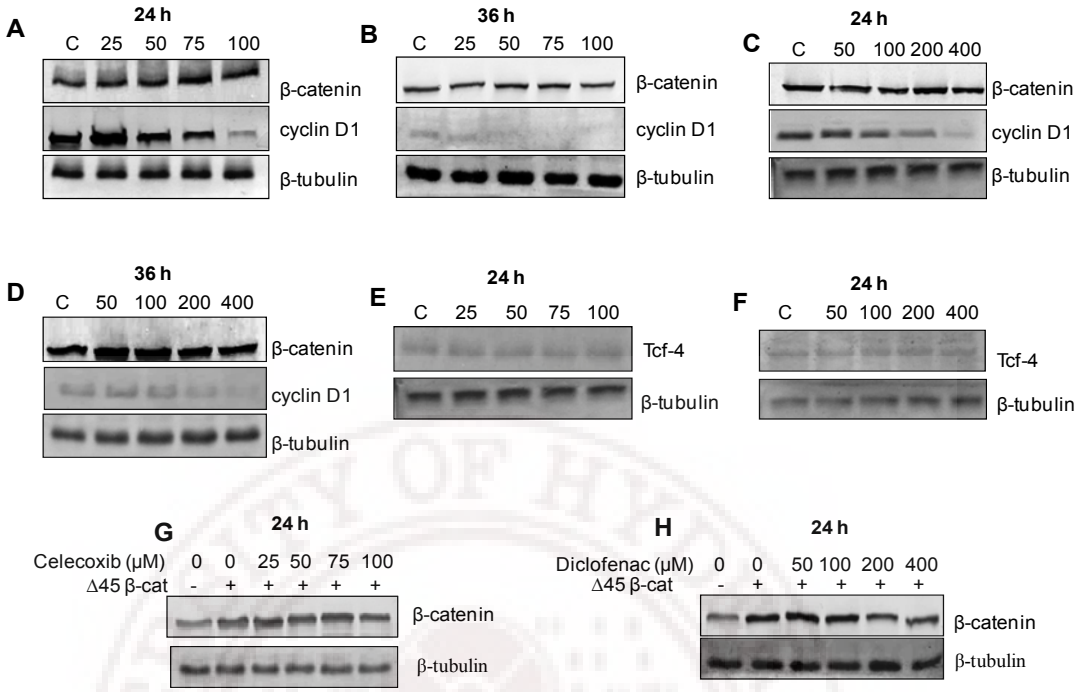


Figure 4.10. Effect of celecoxib and diclofenac on β-catenin, cyclin D1 protein levels. Cell lysates of C6 glioma cells treated with different concentrations (μM) of celecoxib **(A, B)** and diclofenac **(C, D)** were subjected to western blot analysis against β-catenin and cyclin D1. Celecoxib and diclofenac does not alter the levels of b-catenin but decrease the levels of cyclin D1 in a dose dependent manner.

Effect of celecoxib and diclofenac on Tcf4 protein levels. Cell lysates of C6 glioma cells treated with different concentrations (μM) of celecoxib **(E)** and diclofenac **(F)** were subjected to western blot analysis against Tcf4. Tcf levels were also not altered following celecoxib and diclofenac treatment.

Effect of celecoxib and diclofenac on β-catenin levels in constitutively active mutant β-catenin expressing cells. (G, H) Cell lysates of the mutant β-catenin (pCMVΔ45) transfected cells were subjected to western blot analysis against β-catenin. C denotes the carrier control and β-tubulin used as loading control. In transfected cells the levels of b-catenin were enhanced compared to nontransfected cells and these increased levels are not affected by celecoxib and diclofenac treatment.

But these enhanced levels were not affected following celecoxib and diclofenac treatments (figure 4.10G, H).

The above results suggested that celecoxib and diclofenac suppressed the β-catenin pathway activity by a mechanism independent from the β-catenin levels. To further confirm the β-catenin response transcription (CRT) inhibition data obtained from luciferase assays we checked whether celecoxib and diclofenac affects the expression of β-catenin target gene cyclin D1, a known target of β-catenin signaling. Western blot analysis showed that both celecoxib and diclofenac significantly decreased the protein levels of cyclin D1 in a dose dependent manner in C6 glioma cells (figure 4.10A, B, C, D). In addition we also checked the protein levels of Tcf4 following celecoxib and diclofenac treatments. Both celecoxib and diclofenac did not affect the protein levels of Tcf4 (figure 4.10E, F). This result implies that celecoxib and diclofenac reduced β-catenin signaling without altering the levels of the transcription factor Tcf4.

4.3.5. Celecoxib and diclofenac alter the sub-cellular localization of β-catenin

β-Catenin accumulation is essential for modulating the Tcf4 activity and further pathway activation. To confirm whether celecoxib and diclofenac inhibited β-catenin signaling by inhibiting nuclear localization of β-catenin, immunofluorescence analysis was performed. Celecoxib and diclofenac altered β-catenin distribution by retaining the β-catenin in the cytoplasm (figure 4.11A, B, C). This result clearly manifested that celecoxib and diclofenac down-regulated the β-catenin signaling by inhibiting the nuclear translocation of β-catenin.

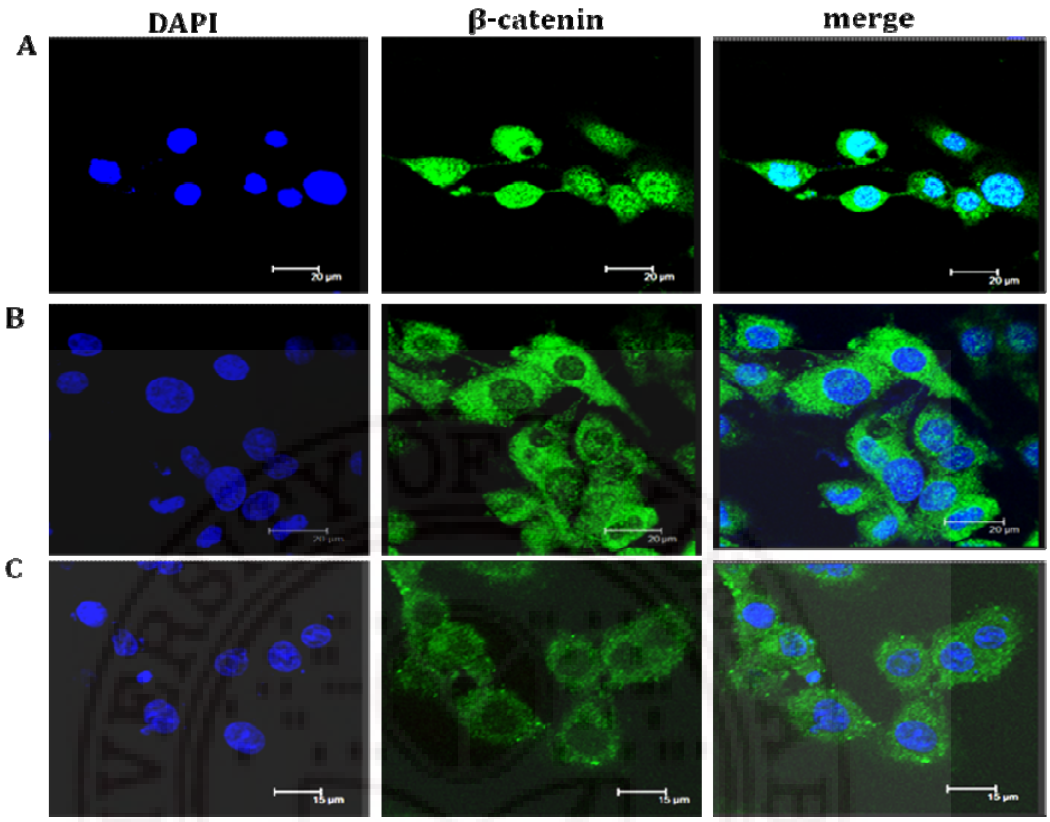


Figure 4.11. Effect of celecoxib and diclofenac on β-catenin localization. C6 cells were treated with vehicle (0.1%) **(A)** or celecoxib (100μM) **(B)** and diclofenac (400μM) **(C)** for 48 h and fixed with paraformaldehyde and incubated with β-catenin primary antibody overnight at 4°C and anti-TRITC secondary antibodies for 1-2 h at room temperature. Fluorescence was captured under Leica confocal microscope. In untreated C6 cells, b-catenin was localized in cytosol and nucleus, whereas in celecoxib and diclofenac treated cells most of the β-catenin was localized to cytosol.

4.3.6. Celecoxib and diclofenac reduced the pAkt levels

Akt participated in Wnt signaling by inactivating GSK3β, resulting in the stabilization of β-catenin. Akt activity is increased in response to Wnt or Dishevelled (Dvl) over expression. Activated Akt bound to the Axin-GSK3β complex in the presence of Dvl, phosphorylated GSK3β and increased free β-catenin (Fukumoto et al, 2001).

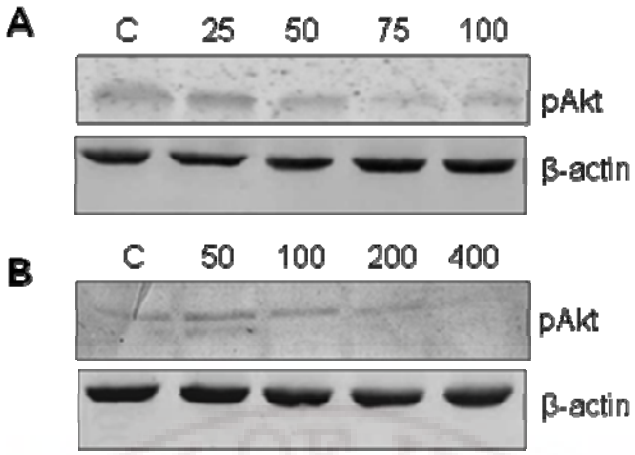


Figure 4.12. Effect of celecoxib and diclofenac on pAkt protein levels. Cell lysates of C6 glioma cells treated with different concentrations of celecoxib **(A)** and diclofenac **(B)** were subjected to western blot analysis against p-Akt. C denotes the carrier control and β-actin used as loading control. Both celecoxib and diclofenac dose dependently reduced the levels of pAkt.

To check whether celecoxib and diclofenac affect pAkt we examined the pAkt levels in response to celecoxib and diclofenac treatments. Both celecoxib and diclofenac significantly decreased the levels of pAkt in a dose dependent manner, suggesting that celecoxib and diclofenac attenuated the Wnt signaling by acting on pAkt (figure 4.12A, B).

4.3.7. Celecoxib and diclofenac inhibits the TNFα-induced nuclear translocation of p65

To characterize the effect of celecoxib and diclofenac on the TNFα-induced nuclear translocation of NF-κB, U373 cells were treated with celecoxib and diclofenac and analyzed the nuclear levels of p65. The nuclear p65 levels were increased following TNFα stimulation compared to unstimulated cells and further treatment with celecoxib and diclofenac significantly reduced the p65 levels in a dose dependent manner (figure 4.13).

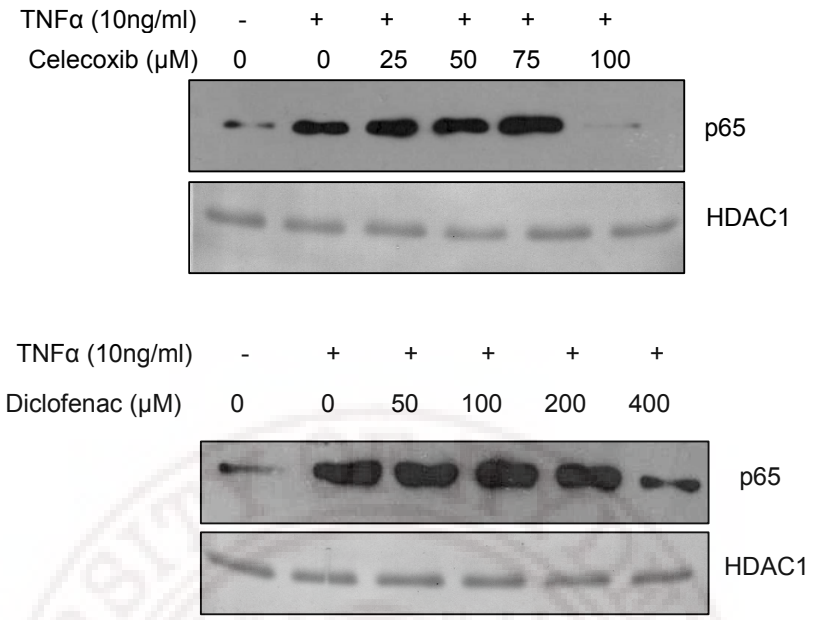


Figure 4.13. Effect of celecoxib and diclofenac on TNFα induced nuclear p65 levels. U373 cells were seeded in 100-mm dishes and treated with varying concentrations of celecoxib and diclofenac for 48 h followed by TNFα stimulation for 30 min. Nuclear extracts were prepared and subjected to western blotting for p65. Celecocib and diclofenac decreased the TNFα induced nuclear p65 levels in a concentration dependent manner. HDAC-1 used as loading control and the blots were representatives of three independent experiments. 'C' denotes the carrier control.

To confirm further the inhibition of nuclear translocation of p65, U373 cells were treated with celecoxib and diclofenac, stimulated with TNFα and subjected to immunofluorescence analysis. We observed the nuclear localization of p65 following TNFα stimulation in U373 cells (figure 4.14A), where as in celecoxib and diclofenac treated cells p65 was localized in cytosol (figure 4.14B, C). These findings clearly demonstrated that celecoxib and diclofenac significantly inhibits the nuclear translocation of p65.

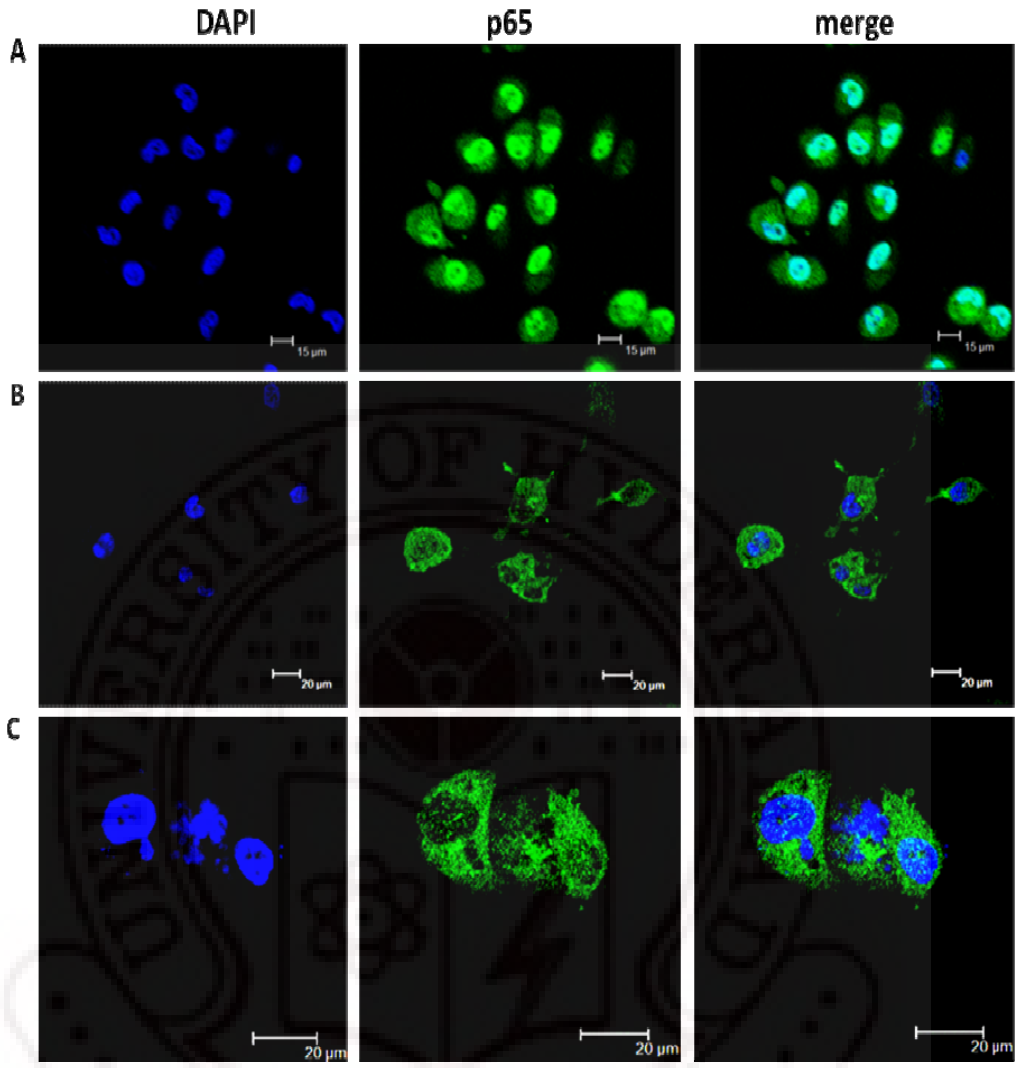


Figure 4.14. Effect of celecoxib and diclofenac on TNFα induced nuclear translocation of p65. U373 cells were seeded on to coverslips in 24 well plates and pretreated with vehicle **(A)** celecoxib (100 µM) **(B)** and diclofenac (400 µM) **(C)** for 48 h, followed by stimulation with TNFα (10 ng/ml) for 30 min. Cells were then fixed in 4% paraformaldehyde and incubated with p65 primary antibody and anti-FITC secondary antibodies for 1 h at room temperature. Fluorescence was captured under Leica confocal microscope. DAPI was used to visualize the nuclei. Upon stimulation with TNFα P65 was translocated to nucleus. Celecoxib and diclofenac treatment resulted in the inhibition of nuclear translocation of p65 following TNFα stimulation and most of the p65 is localized in the cytosol.

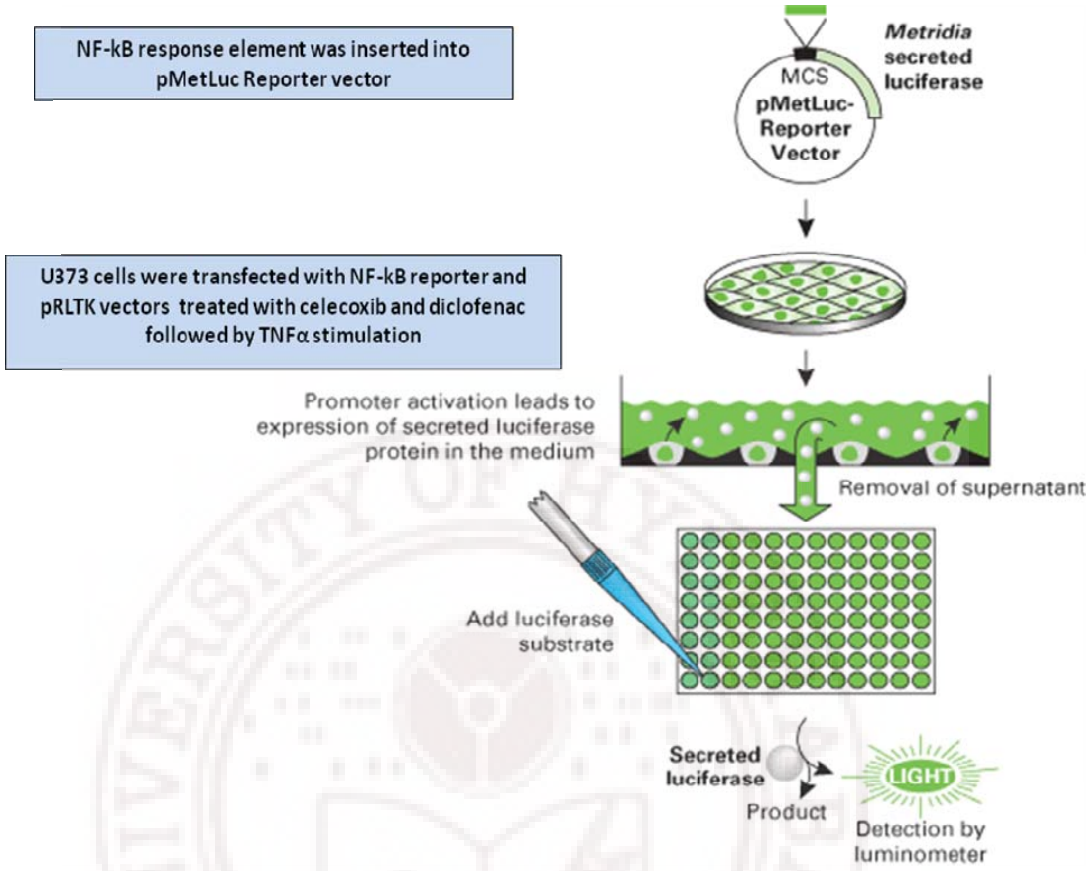


Figure 4.15. Flowchart of the Ready-To-Glow NF-κB Secreted Luciferase Reporter Assay. The Ready-To-Glow Secreted Luciferase Reporter System combines the advantages of a live cell assay with the sensitivity of an enzyme-based system—all in a "one-step" reaction which detects the activity of the secreted reporter enzyme in the supernatant of transfected cells without the need for cell lysis.

4.3.8. Celecoxib and diclofenac down-regulates the NF-κB reporter gene expression

Once IκB inhibitory effects are relieved p50/65 complex is translocated to nucleus and binds to and activate expression of their target genes. In order to examine the effect of celecoxib and diclofenac on NF-κB activation U373 cells were transiently transfected with pNF-κB-MetLuc2-Reporter, and pRL-TK (for normalization of transfection efficiency) plasmids, and treated with celecoxib and diclofenac followed by TNFα stimulation.

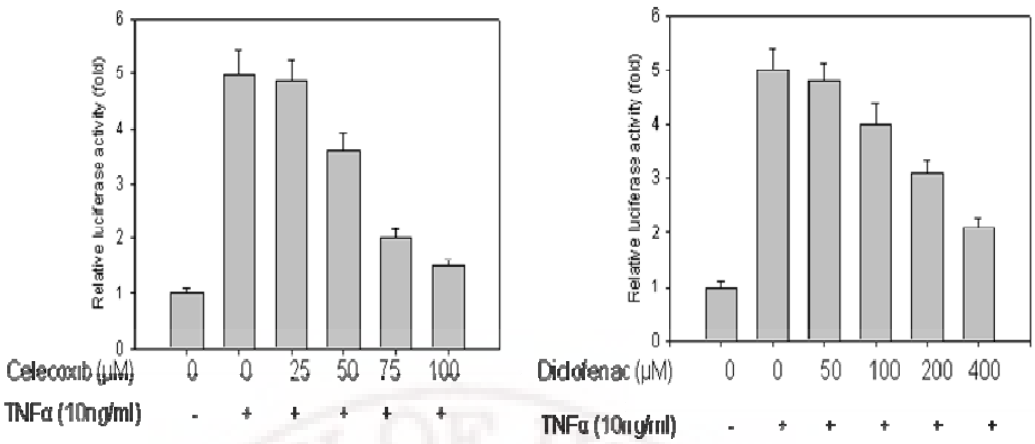


Figure 4.16. Effect of celecoxib and diclofenac on NF-κB dependent reporter gene expression. U373 cells were seeded in 24 well plates and transiently transfected with pNF-κB-MetLuc2-Reporter and pRL-TK (for normalization of transfection efficiency) plasmids using lipofectamine 2000 as described in materials and methods. After 6 h cells were treated with indicated concentrations of celecoxib and diclofenac for 48 h followed by TNFα stimulation for 24 h. Culture medium was then harvested and analyzed for metridia luciferase activity and cells were lysed in passive lysis buffer and analyzed for renilla luciferase activity. The metridia luciferase activities were normalized against renilla luciferase activities. Both celecoxib and diclofenac reduced the NF-kB dependent reporter activity in a dose dependent manner. Data expressed as mean + SD and are representative of three independent experiments. 'C' denotes the carrier control.

Mode of action of pNF-κB-MetLuc2-Reporter plasmid and measurement of luciferase activities were summarized in figure 4.15.

NF-κB reporter gene activity is enhanced greatly following TNF-α stimulation compared to unstimulated cells and this enhanced reporter activity is potentially inhibited by celecoxib and diclofenac in a concentration dependent manner (figure 4.16). These results clearly manifested that celecoxib and diclofenac down-regulated the NF-κB dependent reporter gene expression.

4.3.9. Celecoxib and diclofenac reduce the pERK1/2 and pJNK levels

Mitogen activated protein kinases play critical roles in cell proliferation, differentiation and control of cellular responses to various growth factors and stress condition (Pearson et al, 2001). In addition, they play a critical role in the modulation of NF-κB activity (Vanden Berghe et al, 1998). In order to

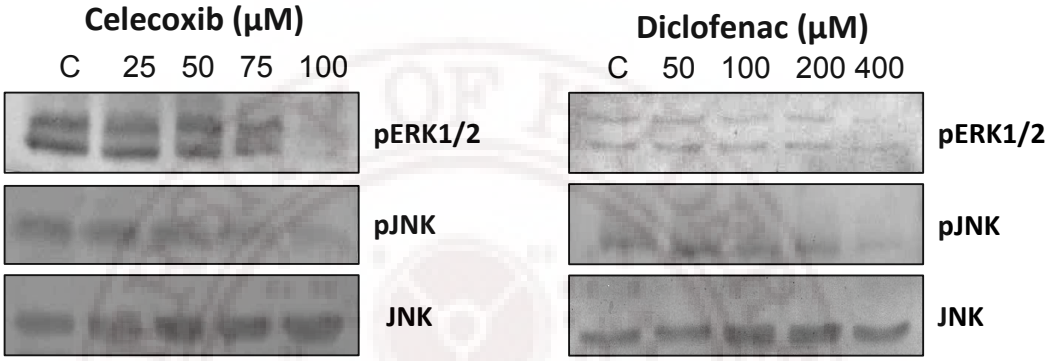


Figure 4.17. Effect of celecoxib and diclofenac on pERK1/2 and pJNK levels. Cell lysates were prepared from U373 cells treated with vehicle (0.1% DMSO) or different concentrations of celecoxib and diclofenac for 48 h and subjected to western blot analysis against pERK1/2 and pJNK. JNK used as loading control and the blots were representatives of three independent experiments. 'C' denotes the carrier control. Both celecoxib and diclofenac dose dependently reduced the levels of pERK1/2 and pJNK.

know the effect of celecoxib and diclofenac on MAP kinases, U373 cells were treated with different concentrations of celecoxib and diclofenac for 48 h and subjected to western blot analysis. The levels of pERK1/2 and pJNK were significantly reduced following celecoxib and diclofenac treatment in a dose dependent manner (figure 4.17), suggesting that celecoxib and diclofenac suppress the growth of GBM cells by inhibiting the MAP kinase levels.

4.4. DISCUSSION

NSAIDs are used extensively for the relief of pain, treatment of minor injuries and headaches, degenerative joint diseases such as rheumatoid arthritis, and prophylaxis against cardiovascular disease. The well known molecular targets for NSAIDs function are cyclooxygenase enzymes. However, accumulating evidences suggesting that NSAIDs have wide range of COX independent targets including β-catenin, NF-κB, PPAR delta, NAG-1, bcl-2 suggesting that distinct molecular pathways plays a role in the anti-neoplastic activity of these drugs. Understanding the COX independent molecular targets is important for chemo preventive effects of NSAIDs.

It is well known that NSAIDs inhibited cell proliferation and induce apoptosis in several colon cancer cells (Smith et al, 2000). The effect of NSAIDs on GBM cell lines is not completely understood. However, recently it was identified that NSAIDs flurbiprofen and indomethacin inhibited the proliferation of glioma cells. Most importantly, celecoxib entered into phase I and Phase II clinical trials for the treatment of GBM. Recent findings reported that anti-tumor activity is enhanced by low-dose combination of the recombinant urokinase kringle domain and celecoxib in glioma models (Kim et al, 2009) and Phase II study of temozolomide, thalidomide, and celecoxib for newly diagnosed glioblastoma in adults (Kesari et al, 2008) have stimulated the use of NSAIDs and provides a desirable strategy for anti-glioma therapy. These findings tempted us to investigate the effects of NSAIDs celecoxib and diclofenac on human glioblastoma cells.

In this study first we set out to investigate whether celecoxib and diclofenac have significant anti-proliferative effect on glioblastoma cell lines.

MTT assay, cell viability and clonogenic assays revealed that celecoxib and diclofenac have significant anti-proliferative effects on various glioblastoma cell lines. In addition, cell cycle analysis showed that celecoxib and diclofenac arrest the cell cycle at G2-M phase, and increases the apoptotic sub G1 fraction. Moreover, celecoxib and diclofenac significantly increased the Caspase-3 and -8 activities and induced the PARP cleavage.

The oncogenic activation of β-catenin pathway resulted from several consequences like loss of APC function due to mutations or oncogenic mutations in phospho-acceptor sites of β-catenin or inhibition of GSK3β activity by Wnt secreted proteins or by PI3K/Akt signaling or loss of regulation of Wnt suppressors like Dkk-1 and secreted frizzled receptors (Barker et al, 2006). Most of the colon cancer cells having highly constitutive β-catenin due to mutations in APC or β-catenin itself. In contrast glioma cells did not harbor such kind of mutations in APC or β-catenin. To date no prominent mutations were observed in β-catenin but it was reported that β-catenin is mutated at S33 as sporadic event in a brain metastasis (Lee et al, 2009). Further, this mutated β-catenin enhances the Tcf dependent promoter activity. In the previous chapters we showed that Wnt signaling components were up regulated in human astrocytomas and ENU-induced rat gliomas and showed their positive correlation with histological malignancy. Several other reports supported the functional role of Wnt signaling in gliomas. Inhibition of Wnt2 and β-catenin by anti-sense oligonucleotides block the tumor cell growth in both in vivo and in vitro (Pu et al, 2009). Over-expression of negative regulators of Wnt signaling like Secreted frizzled related proteins (sFRPs) (Roth et al, 2000) and Dkk-1 inhibited the motility and sensitized the glioma

cells to apoptosis (Shou et al, 2002). Therefore, it seems that Wnt/β-catenin pathway activation in gliomas may be result from over-expression of β-catenin upstream elements like Wnt 5a, Wnt10b, Wnt13 ligands (Yu et al, 2007), frizzled receptors (Zhang et al., 2006) dishevelled (Sareddy et al, 2009a) and loss of negative regulators of Wnt signaling.

Several reports demonstrated that there exists a link between the effects of chemo preventive agents and β-catenin/Tcf4 activity (Smith et al, 2000; Dihlmann et al, 2001). We therefore focused on β-catenin itself as a major regulator of Tcf-responsive transcription for being a target of celecoxib and diclofenac action. We first used a Tcf reporter assay to assess the effects of celecoxib and diclofenac on the activity of the pathway as a whole and then used western blotting to detect the changes in the levels of β-catenin, Tcf4 and its target cyclin D1. We found reduction of β-catenin/Tcf signaling and expression of β-catenin/Tcf target cyclin D1 in a concentration dependent manner. But the levels of total β-catenin and Tcf4 were not altered. But with immunofluorescence studies we found that β-catenin is relocated to cytosol following celecoxib and diclofenac treatments.

Though the transcriptional activity of the β-catenin/Tcf4 complex is down-regulated by celecoxib and diclofenac we did not detect alterations in total β-catenin. Therefore we suspect that celecoxib and diclofenac use alternate mechanisms such as post translational modifications like the phosphorylation status of β-catenin and/or Tcf4, or inactivation of co-activators or activation of co-repressors of β-catenin/Tcf complex. In addition to Ser/Thr-phosphorylation sites which are involved in degradation of the protein, β-catenin harbors Tyr-phosphorylation sites, which connect β-catenin

pathway with other signaling pathways (Hoschuetzky et al, 1994). In normal conditions, β-catenin signaling is repressed by Groucho (Grg/TLE) family of transcriptional co-repressors (Brantjes et al, 2001). When β-catenin is available in the nucleus, it recruits an array of co-activator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless, Mediator and Hyrax (Hecht et al, 2000; Hoffmans et al, 2005; Barker et al, 2006).

NF-κB is a family of dimeric transcription factors that play vital roles in diverse physiological processes and various human malignancies. NF-κB family includes RelA/p65, RelB, c-Rel, p50/p105 and p52/p100 which possess a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD) containing the dimerization, nuclear localization, and DNA-binding domains. Among these RelA, c-Rel, and RelB, have a transactivation domain at the C-terminus. In unstimulated cells, NF-κB/Rel dimers are bound to IκB and retained in cytoplasm. In response to stimulus such as TNFα, LPS the IκB proteins are phosphorylated by an activated IκB kinase (IKK) complex at positions 32 and 36 followed by polyubiquitination and degradation by 26S proteasome releasing free NF-κB dimers. Then the p50/65 complex is translocated to nucleus and binds to their target gene promoters and drives their transcription (Karin et al, 2002; Ghosh et al, 2002; Nishikori et al, 2005; Karin et al, 2000).

Glial tumors develop as a result of occurrence of genetic alterations in low grade lesions, which further acquire additional mutations and progress to more malignant tumors. Mutation of the p53, RB and PTEN, deletion of p16INK4A, activation of the Ras and Akt pathways, and amplification of CDK4 and EGFR contribute to the development of astrocytomas (Ohgaki and

Kleihues, 2007; Louis, 1997; Hayashi et al, 1997). Like wise, NF-κB signaling pathway is constitutively activated in gliomas and associated with enhanced growth, cell cycle progression, and inducible chemo resistance. NF-κB is one of the COX independent targets of NSAIDs, through which NSAIDs exert anti-tumor activities. Because inhibition of NF-κB activation has been linked with anti-tumor activities, we hypothesize that celecoxib and diclofenac mediates their effect at least partly through inhibition of NF-κB activation. In this study we checked the inhibitory effects of celecoxib and diclofenac on nuclear translocation of p65. Our results showed that TNFα-induced nuclear translocation and NF-κB dependent reporter gene expression were inhibited following celecoxib and diclofenac treatment. Moreover, celecoxib and diclofenac decreased the levels of pERK1/2 and pJNK in a dose dependent manner.

In summary, our results demonstrated that the NSAIDs celecoxib and diclofenac reduced the proliferation of GBM cells by suppressing the Wnt signaling pathway and partly by NF-κB activation and MAP kinase phosphorylation. These findings might help in providing the rationale to initiating in vivo studies to examine the efficacy of NSAIDs as chemo preventive agents against gliomas and explain some features of NSAIDs-mediated chemoprevention.

Summary

The current understanding of glioma biology reveals effective targets for anti-invasive therapy which include manipulations of extra cellular matrix and receptors, growth factors and cytokines, proteases, cytoskeletal components, oncogenes and tumor suppressor genes. The treatment of patients with glioblastoma multiforme (GBM) is conventionally considered to be a palliative venture with no hope of cure. Traditionally, patients are treated with maximal surgical resection, although surgery is not a curative procedure, a major resection provides for a longer survival and better quality of life. Radiotherapy increases the duration of survival, but again is not a curative intervention. The role of chemotherapy, specifically focusing on a foundation of chloroethylating agents such as carmustine (BCNU) or lomustine (CCNU), has been controversial with an equal number of clinicians arguing in favor of or against this treatment.

Despite tremendous improvement in these treatment strategies, gliomas in particular GBM still carry a bleak prognosis. In the recent past, there has been an intensive effort shedding on gliomas to gain an understanding of the cellular and molecular mechanisms that contribute to the pathogenesis of gliomas as a first step toward the development of better treatments for the deadliest tumors. Understanding of such molecular features can help predict not only the natural history of glioma but also the likelihood that it will respond to conventional chemotherapeutic agents and novel molecular targeted therapy. Genetic modeling of gliomas in mice suggests that neural precursor cells represent preferred cellular substrates of gliomas or that whether glial cell or precursor cells constitute potential cells-of-origin of gliomas. During normal brain development, neural precursor cells including

stem cells acts as a source of glial cells. As the mechanism/molecular pathways that control gliogenesis during normal brain development become better understood, it will be important to determine if deregulation of these mechanisms might contribute to the pathogenesis of astrocytomas.

Wnt signaling pathway a major developmental pathway critically regulates the processes of self-renewal and dedifferentiation of neural precursor cells. In addition, Wnt signaling pathway is required at several stages of CNS development during embryogenesis and adult life. Aberrant activation of this pathway has been implicated in variety of human cancers including colon, breast, prostate, ovary, and CNS malignancies including medulloblastoma and subependymal giant cell astrocytomas. The present study is therefore designed to unravel the relation between Wnt signaling with respect to glioma progression in humans as well as in ENU-induced glioma rat model. As, oncogenic role of Wnt signaling is evident in several cancers its specific inhibition by chemical agents may have an advantage in chemotherapy of tumors. Chemo preventive effects of NSAIDs are mainly mediated through COX-2 inhibition. However, recently several evidences suggesting that alternative targets are also implicated in the tumor suppressive effects of NSAIDs which include Wnt and NF-κB signaling pathways. In the present study we set out to investigate whether celecoxib and diclofenac have anti-proliferative effect on glioma cells and on status of Wnt/β-catenin/Tcf and NF-κB signaling pathways.

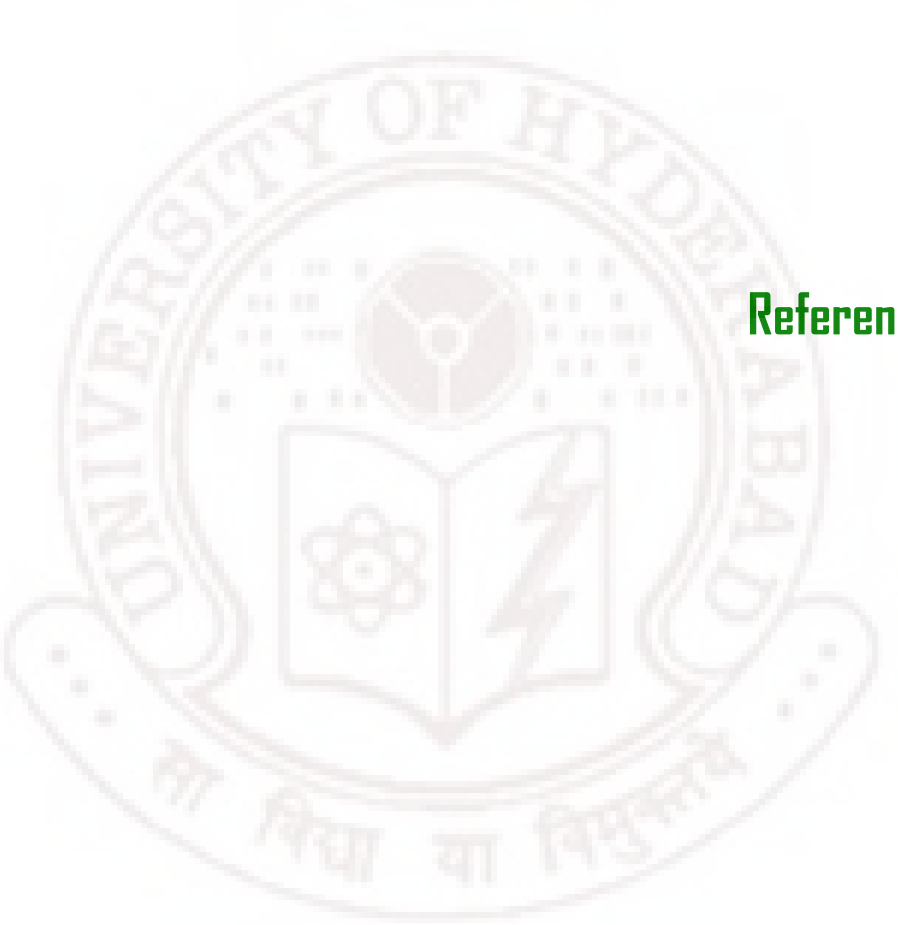
In this thesis, we have addressed the possible role of Wnt/β-catenin/Tcf signaling in human and ENU-induced rat gliomas with respect to histological malignancy and its therapeutic significance in inhibiting glioma progression.

Salient findings:

- ✓ Overexpression of *Dvl-3* through RT-PCR analysis and its significant positive correlation with histological malignancy may indicate its possible role associated with astrocytoma malignancy.
- ✓ The elevated levels of β-catenin and its transcriptional partners Lef1 and Tcf4 in human and rat glioma tumors and their significant positive correlations with degree of malignancy may demonstrate their possible role in glioma progression.
- ✓ Overexpression of Wnt target genes c-Myc, N-Myc, c-jun and cyclin D1 in human and rat glioma samples may indicate that β-catenin exert neoplastic activities by binding to the promoters and activating the expression of c-Myc, N-Myc, c-jun and cyclin D1. Further their significant positive correlations with histological malignancy may suggest that aberrant expression of Wnt/β-catenin/Tcf signaling pathway components leads to the tumor formation and progression.
- ✓ Reduced cell proliferation, clonogenic efficiency and increased Caspase-3, Caspase-8 activities, PARP cleavage and percent of subG1 cell population (apoptotic cells) following NSAIDs (celecoxib and diclofenac) treatment suggest that these drugs have significant antiproliferative and/or proapoptotic effects on glioma cell lines.
- ✓ Wnt/β-catenin/Tcf signaling activity and cyclin D1 protein levels were down-regulated following celecoxib and diclofenac treatment but total β-catenin and Tcf4 protein levels were not altered. Further, TNF-α induced nuclear NF-κB translocation and its reporter gene expression,

MAP kinases and pAkt levels were significantly inhibited following celecoxib and diclofenac treatment in a dose dependent manner.

In summary, the present study demonstrates the possible role of Wnt/β-catenin/Tcf signaling pathway in human and ENU-induced rat gliomas and its therapeutic significance in inhibiting glioma progression.



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Activation of Wnt/β-catenin/Tcf signaling pathway in human astrocytomas

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ABSTRACT

Astrocytomas are the most common form of primary brain tumors. Understanding the molecular basis of development and progression of astrocytomas is required to develop more effective therapies. Although, over activation of Wnt/β-catenin/Tcf pathway is a hallmark of several forms of cancer, little is known about its role in human astrocytomas. Here, we report the evidence that Wnt/β-catenin/Tcf signaling pathway is constitutively activated in astrocytic tumors. In the present study, human astrocytic tumors with different clinical grades were analyzed for mRNA expression of *Dvl-1*, *Dvl-2*, *Dvl-3*, β-*catenin*, *c-myc* and *cyclin D1* and protein levels of β-catenin, Lef1, Tcf4, c-Myc, N-Myc, c-jun and cyclin D1. RT-PCR analysis demonstrated the overexpression of *Dvl-3*, β-*catenin*, *c-myc* and *cyclin D1* in astrocytomas. Western blotting revealed upregulation of β-catenin, Lef1, Tcf4 and their target proteins in the core tumor tissues in comparison to peritumor and normal brain tissues. The protein and mRNA levels were positively correlated with the histological malignancy. Cytoplasmic and nuclear accumulation of β-catenin, nuclear localization of Lef1, Tcf4, c-Myc, N-Myc, c-jun and cyclin D1 were demonstrated by immunohistochemical staining. Our studies tend to suggest that Wnt/β-catenin/Tcf signaling pathway is implicated in malignancy of astrocytomas.

1. Introduction

Astrocytomas are the most common and deadliest form of primary brain tumors. Despite recent advances in diagnosis and therapies such as surgery, radiation, and chemotherapy, the prognosis and survival times remains poor. WHO classified astrocytomas into four clinical grades on the basis of their histology and prognosis. Of these, GBM (glioblastoma multifrome; grade IV) is most aggressive and highly invasive type. Patients with GBM have a mean survival of 1 year, whereas patients with anaplastic astrocytoma (grade III) have the life expectancy of 2–3 years. Patients with low-grade astrocytoma (Diffuse astrocytoma; grade II) have favorable outcome and can survive for as long as 10–15 years (Kleihues and Cavenee, 2000; Lacroix et al., 2001; Simmons et al., 2001; Holland, 2001). GBM include two subtypes, those, which develop *de novo*, are primary GBM and secondary GBM develop through progression from low grade diffuse or anaplastic astrocytomas (Kleihues and Ohgaki, 1999,2007). Astrocytic tumors develop as a result of stepwise accumulation of genetic alterations, which results in the activation of oncogenes and inactivation of tumor suppressor genes (Cavenee, 1992). These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. Mutation of the p53, RB and PTEN, deletion of p16INK4A, activation of the Ras and Akt pathways, and amplification of CDK4 and EGFR contribute to the development of astrocytomas (Kleihues and Ohgaki, 2007; Louis, 1997; Hayashi et al., 1997).

Wnt signaling pathway plays crucial roles in normal embryonic development and abnormal pathological processes in vertebrates (Moon et al., 1997; Robb and Tam, 2004; Zorn et al., 1999). Aberrant activation of Wnt signaling pathway has been implicated in variety of human cancers including colon, breast, prostate, and ovary. The Wnt pathway activation in oncogenesis and its consequences has been extensively reviewed (Logan and Nusse, 2004; Moon et al., 2004; Polakis, 1999; Reya and Clevers, 2005). Activation of canonical Wnt pathway requires the binding of Wnt ligands to frizzled (FZD) receptors together with the co-receptors LRP5 or LRP6 (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Binding of Wnt proteins to FZD–LRP complex results in the activation and membrane recruitment of the phospho protein Dishevelled (Dvl). Dvl recruits Axin to the plasma membrane, where it binds directly to the cytoplasmic tail of LRP5-6, results in the inactivation of destruction complex (Cliffe et al., 2003; Moon et al., 2004). This allows the accumulation and translocation of unphosphorylated free β-catenin to the nucleus, where it interacts

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with members of the Tcf/Lef transcription factors to induce expression of Wnt target genes like c-Myc, N-Myc, c-jun and cyclin D1, which instruct the cell to actively proliferate and remain in an undifferentiated state (He et al., 1998; Shiina et al., 2003; Shtutman et al., 1999). β-Catenin is the central hub of the Wnt signaling pathway. In the absence of Wnt signal, β-catenin is captured by adenomatus polyposis coli (APC) and Axin within the cytoplasmic destruction complex, which allows its phosphorylation by kinases CK1α and GSK3β, facilitating its degradation via the ubiquitin–proteosome pathway (Brown and Moon, 1998; Wodarz and Nusse, 1998).

The aim of this study is to investigate whether Wnt/β-catenin/Tcf signaling pathway plays a role in the malignant progression of human astrocytic tumors. Here we found the expression profile of β-catenin, its transcriptional counter parts Lef1, Tcf4 and its potential target genes c-Myc, N-Myc, c-jun and cyclin D1 were up regulated in astrocytomas and positively correlated with histological malignancy. This may suggest the role of Wnt/β-catenin/Tcf pathway in the pathology of astrocytoma.

2. Materials and methods

2.1. Sample collection

Human astrocytic tumor samples were collected from patients who underwent surgical resection at Nizam Institute of Medical Sciences (Hyderabad, India). The tumors were classified histopathologically according to the WHO classification: 8 diffuse astrocytomas (grade II), 7 anaplastic astrocytomas (grade III), and 17 glioblastoma multiforme (grade IV) (Table 1). Peritumor tissues (1 cm beyond the tumor margin) were also collected from the patients during the surgical resection. Two brain samples consisting of periventricular region were obtained from patients with epilepsy. These tissues were apparently normal histologically. All samples were obtained with informed consent. A part of the surgically removed samples were immediately snap frozen in liquid nitrogen and then stored at −80 °C until analysis. The remaining samples were fixed with formalin and embedded in paraffin for histological studies. Human glioblastoma derived cell lines A127, T98G (gifted by Dr. Ellora Sen, National Brain Research Center, India), GO-G-CCM, U373, and U87 and rat C6 glioma cell line were obtained from National Center for Cell science (Pune, India) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Biowhittaker, Lonza Pri Ltd, Mumbai, India), 10 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C and 5%CO_2.

Table 1
Clinical characteristics in astrocytoma patients. Abbreviations: F = female; M = male; II = diffuse astrocytoma; III = anaplastic astrocytomas; IV = glioblastoma multiforme (GBM).

No.	Age	Sex	WHO grade	Location	Survival days	Outcome
1	34	F	II	Fronto-temporal	1988	Alive
2	35	M	II	Frontal	663	Alive
3	27	M	II	Fronto-temporal	1225	Alive
4	30	M	II	Frontal	1269	Alive
5	16	M	II	Frontal	1442	Alive
6	60	M	II	Parietal	412	Dead
7	37	F	II	Tempero-frontal	389	Alive
8	40	M	II	Frontal	869	Alive
9	29	M	III	Frontal	345	Dead
10	62	F	III	Parietal	432	Alive
11	35	M	III	Frontal	326	Dead
12	37	M	III	Frontal	363	Alive
13	30	F	III	Frontal	419	Dead
14	45	F	III	Fronto-temporal	986	Dead
15	43	M	III	Frontal	1021	Dead
16	45	M	IV	Parietal	431	Alive
17	36	F	IV	Frontal	446	Dead
18	16	M	IV	Frontal	321	Dead
19	52	M	IV	Frontal	496	Dead
20	20	M	IV	Frontal	462	Dead
21	19	M	IV	Tempero-parietal	56	Dead
22	27	M	IV	Frontal	120	Dead
23	48	M	IV	Temporal	175	Dead
24	15	M	IV	Frontal	32	Dead
25	58	F	IV	Fronto-tempero-parietal	93	Dead
26	61	M	IV	Frontal	18	Dead
27	50	M	IV	Corpus callosal	39	Dead
28	55	M	IV	Frontal	172	Dead
29	5	M	IV	Multiple intra cranial	113	Dead
30	30	M	IV	Frontal	269	Alive
31	40	M	IV	Parietal	59	Dead
32	34	M	IV	Temporal	213	Dead

2.2. Preparation of soluble cell lysates

Deep frozen samples were thawed gradually and further homogenized by using dounce homogenizer in 5 volumes of RIPA (radioimmunoprecipitaiton assay) buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.4% deoxycholate, 1% NP-40 containing protease inhibitors including 1 mM phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitors including 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na_3Vo_4 and 0.3 mM aprotinin. The lysates were sonicated for 2 min and then centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant (whole tissue lysate) was collected and frozen at −80 °C before use. Protein concentrations were determined by Lowry method.

2.3. Western blotting

Seventy-five micrograms of cellular protein from human tissues and cell lines were mixed with SDS sample buffer, boiled for 5 min and then subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred on to nitrocellulose papers. After blocking the nitro cellulose paper in non-fat dry milk (5%) in Tris Buffered Saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature, the membranes were incubated for 12–24 h in primary antibodies at 4 °C. Blots were again incubated with secondary antibodies conjugated to alkaline phosphatase (ALP) (anti-rabbit and anti-mouse IgG conjugated to ALP obtained from Genei Pvt Ltd, Bangalore, India), for 1–2 h at room temperature. Before and after incubation of blots with secondary antibodies, blots were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivty was visualized by incubating the blots with BCIP-NBT solution (Genei Pvt Limited, Bangalore, India). Blots were analyzed quantitatively using scion image software (NIH).

The primary antibodies used in these experiments included rabbit polyclonal antibody against β-catenin, rabbit monoclonal antibody against Lef1, Tcf4, c-jun and cyclin D1 (obtained from Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal antibody against N-Myc (gifted by Dr. Robert Eisenman, Fred Hutchinson Cancer Research Center, Washington, USA), mouse monoclonal antibody against c-Myc (gifted by Naidu, Manipal Institute of Medical Sciences, India), and mouse polyconal β-actin (gifted by Prof. K. Anand Kumar, University of Hyderabad, India).

2.4. Co-immunoprecipitation

Co-immunoprecipitation was performed with anti-β-catenin antibody in 200 μg of nuclear protein from human GBM tissue and T98G cell lysates. The samples were pre-cleared using protein A-agarose (Sigma Chemicals, USA) and incubated with anti-β-catenin antibody over night at 4 °C. After the addition of protein A-agarose, samples were further incubated for 2–3 h at room temperature. Then washed thrice with phosphate buffered saline containing 0.1% NP-40 and the beads were resuspended in SDS sample buffer and subjected to western blot analysis.

2.5. Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded specimens. Briefly, sections were deparafinized in xylene and passed

Table 2
Sequences of specific primers: F = forward primer; R = reverse primer.

Gene	Primer sequence	Amplicon size (bp)
Dvl-1 F	5′-GTCAGGGCTACCCCTACCAG-3′	480
Dvl-1 R	5′-TGATGTCCACGAAGAACTCG-3′	
Dvl-2 F	5′-GCACCATTACATCTGGATCGT-3′	190
Dvl-2 R	5′-GGTAGAGCCAGTCAACCACAT-3′	
Dvl-3 F	5′-CACGTGGTTGCTTCACATTGC-3′	198
Dvl-3 R	5′-GACAAGTGGAAGTCGTCTAGG-3′	
β-catenin F	5′-CACAAGCAGAGTGCTGAAGGTGC-3′	189
β-catenin R	5′-AAGGAGGCCTTCCATCCCTTC-3′	
c-myc F	5′-CTGGAAGAAATTCGAGCTGA-3′	220
c-myc R	5′-ACATACAGTCCTGGATGATGA-3′	
cyclin D1 F	5′-GTGGCCTCTAAGATGAAGGAG-3′	281
cyclin D1 R	5′-GAACTTCACATCTGTGGCACAG-3′	
GAPDH F	5′-ACCACAGTCCATGCCATCAC-3′	186
GAPDH R	5′-ACACGGAAGGCCATGCCAGTG-3′	

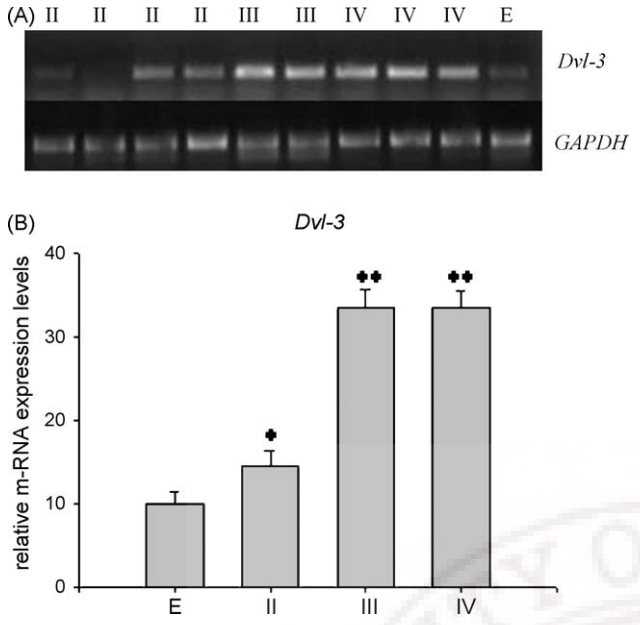

Fig. 1. Overexpression of *DVL-3* in astrocytic tumors. (A) Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *Dvl-3* specific primers. The house keeping gene *GAPDH* was used as an internal quantitative control (II: diffuse astrocytoma; III: anaplastic astrocytoma; IV: glioblastoma multiforme; E: control brain). (B) Densitometric analysis of *Dvl-3* expression revealed the mRNA levels of *Dvl-3* were correlated with the histological grading of astrocytomas. Data are represented as mean ± standard error from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.001$ indicate significant difference relative to the corresponding control).

through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by micro waving the sections for 10–14 min in 10 mM citrate buffer (pH 6.0). Sections were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase followed by incubation with serum for 1 h at room temperature in a humid chamber. The sections were then incubated overnight at 4 °C with primary antibodies against β-catenin, Lef1, Tcf4, c-jun, c-Myc, N-Myc and cyclin D1 diluted as per data sheet in blocking solution. Peroxidase conjugated

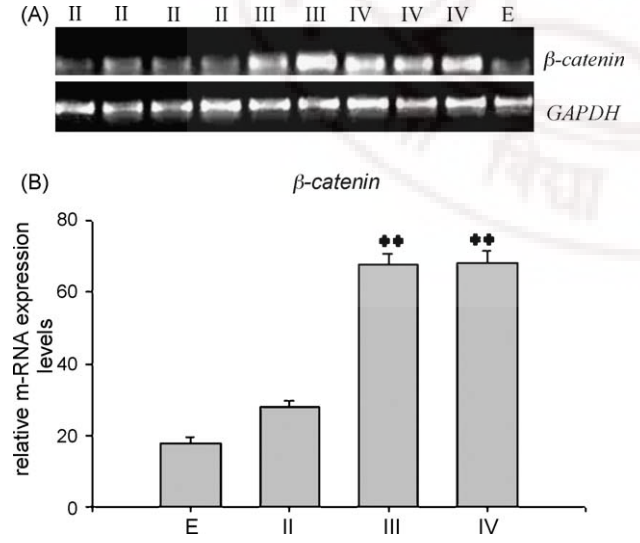

Fig. 2. Overexpression of *β-catenin* mRNA in astrocytomas. (A) Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *β-catenin* specific primers. The house keeping gene *GAPDH* was used as an internal quantitative control (II: diffuse astrocytoma; III: anaplastic astrocytoma; IV: glioblastoma multiforme; E: control brain). (B) Quantitative densitometric analysis showing the expression levels of *β-catenin* were correlated with the histological malignancy. Data are represented as mean ± standard error from three independent experiments ($^{**}p < 0.001$ indicate significant difference relative to the corresponding control).

secondary antibody was used for 1 h incubation time at room temperature followed by TBS washes (3× 5 min each). Diaminobenzidine (DAB) in buffer was used till sections develop color. Then sections were counterstained using haematoxylin. Sections were washed with distilled water followed by dehydration in graded alcohols, xylene and mounted with DPX (kit obtained from Biogenex Pvt Limited, India). In each experiment, a negative control was included in which the primary antibody step was skipped and replaced by non-immune serum.

2.6. Immunofluorescence

As described in immunohistochemical analysis, after deparafinization, rehydration and cooking, tissue sections were blocked with 5% serum for 1 h at room temperature in a humid chamber. Then incubated the sections in a cocktail of two primary antibodies (mouse polyclonal GFAP obtained from Sigma Chemicals, USA, and rabbit polyclonal β-catenin antibodies) overnight at 4 °C. The sections were subsequently washed and incubated in a mixture consisting FITC and TRITC secondary antibodies (Genei Pvt Limited, Bangalore, India) at 1:250 dilution for 1 h at room temperature. The sections were washed and mounted using 90% glycerol and visualized using a Leica confocal microscope.

2.7. RNA isolation and semi quantitative RT-PCR

Total RNA was isolated from astrocytic tumor tissues (grade II-4, grade III-2 and grade IV-3) using TRI reagent (Sigma Chemicals, USA). Briefly, frozen tissues (50–100 mg) were grounded in liquid nitrogen and homogenized in 1 ml of TRI reagent for RNA isolation following manufacturer's instructions. Purified total RNA was dissolved in DEPC treated water and stored at −80 °C before use. Five micrograms of RNA was treated with DNase I and reverse transcribed to cDNA using oligo $(dT)_{18}$ primer and M-mLV reverse transcriptase enzyme (Invitrogen, Sandiego, CA, USA) as per the protocol recommended by manufacturer. The amplification conditions were initial denaturatuion at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min;

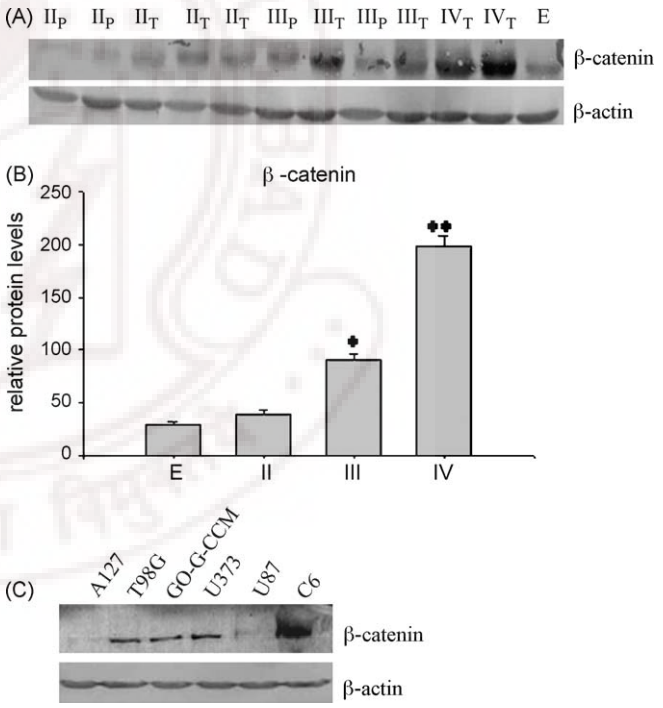

Fig. 3. Upregulation of β-catenin in astrocytomas. (A) Whole cell lysates extracted from human astrocytic samples separated on 10% SDS gels and transferred on to nitrocellulose membranes. Membranes were subjected to immunoblot analysis with a β-catenin specific primary antibody. The expression levels of β-actin were used as loading control. (II_P: diffuse astrocytoma peritumor tissue, II_T: diffuse astrocytoma core tumor tissue, III_P: anaplastic astrocytoma peritumor tissue, III_T: anaplastic astrocytoma core tumor tissue, IV_T: GBM core tumor tissue, E: normal control brain). (B) Densitometric analysis showing the β-catenin levels were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.001$ indicate significant difference relative to the corresponding control). (C) Proteins were extracted from different GBM cell lines, separated on 10% SDS gel and transferred on to nitrocellulose membranes and immunoblots were detected with β-catenin specific primary antibody. T98G, GO-G-CCM, U373 and C6 cell lines bears more β-catenin expression, while A127 and U87 cell lines bears low β-catenin expression. β-actin used as a loading control.

58 °C for 45 s; 72 °C for 45 s. PCR amplification was conducted using the described primer sets (Table 2). The house keeping gene *GAPDH* was used as an internal quantitative control.

2.8. Statistics

Statistical analysis was performed using sigmastat version 3.1. All data were represented as mean ± S.D. obtained from individual patients of each grade and were compared with control. The significant differences of the data were determined using one-way ANOVA. The correlation of the data was determined by Pearson's test. Differences between tumor samples and controls were compared by paired Student's *t*-test. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Overexpression of Dvl-3 in astrocytic tumors

In order to examine *Dvl* expression in astrocytomas, we analyzed tumor tissues for variation in expression levels of *Dvl-1*, *Dvl-2* and *Dvl-3*, using total RNA for semi quantitative RT-PCR experiments. The mRNA expression levels of *Dvl-3* in astrocytic tumors were increased significantly in comparison to the controls (Fig. 1A). Data represents mean expression levels of *Dvl-3* in grade II (14.5 ± 1.8), grade III (33.5 ± 2.1) and grade IV (33.4 ± 2.12) were statistically significant ($p < 0.05$) when compared to control brain (10 ± 1.5) and positively correlated with the degrees of histological grading (Fig. 1B). In contrast, the expression of *Dvl-1* and *Dvl-2* were not found in astrocytomas.

3.2. Increased β-catenin levels correlate with histological malignancy

The mRNA expression profile of *β-catenin* was quantified by using RT-PCR in tumors of different grades. The relative mRNA levels of *β-catenin* were significantly high in higher grade astrocytomas (III, IV), moderate in low grade astrocytomas (grade II), in comparison to control (Fig. 2A). The mean mRNA expression levels of *β*-catenin in grade III (67.5 ± 3.2) and grade IV (68 ± 3.4) were statistically significant ($p < 0.05$) when compared to control (18 ± 1.6) and correlated with histological malignancy (Fig. 2B). Western blotting analysis demonstrated the relative protein levels of β-catenin were progressively increased from low grade (II) to higher grade (III, IV) astrocytomas. Moreover, the protein levels were higher in the core tumor tissues in comparison to peritumor and normal brain tissues (Fig. 3A). The mean protein expression levels of β-catenin in grade III (91.3 ± 1.45) and grade IV (199 ± .6.06) were statistically significant ($p < 0.05$) when compared with that of control (28.6 ± 1.45) and showed positive correlation with histological grading (Fig. 3B). But the mean mRNA and protein expression levels of β-catenin in grade II (28 ± 1.7, 38.9 ± 1.15, respectively) showed no significant difference with controls.

In addition, we also analyzed the β-catenin expression in different glioblastoma cell lines (A127, T98G, GO-G-CCM, U87, U373 and C6). Representative western blotting results showed that T98G, GO-G-CCM, U373 and C6 cell lines were overexpressing β-catenin (Fig. 3C). Moreover, the growth inhibition of different GBM

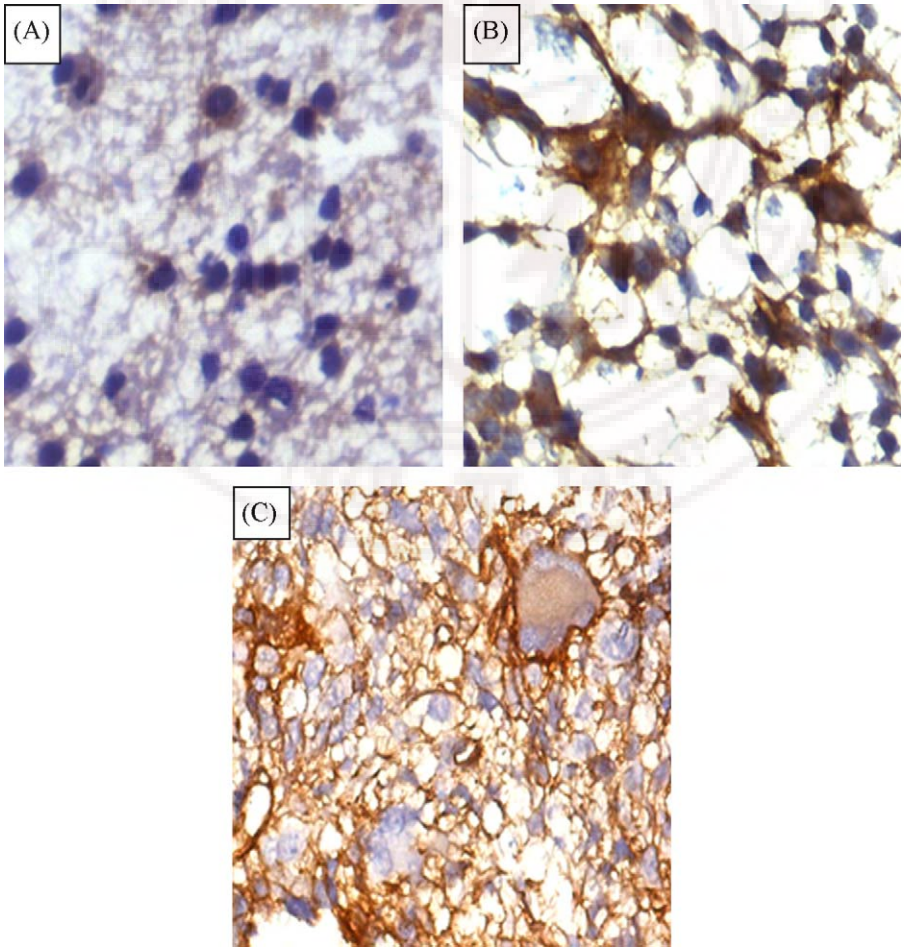

Fig. 4. Cytoplasmic and nuclear accumulation of β-catenin. Paraffin-embedded sections were prepared from astrocytoma specimens. Sections were incubated with β-catenin specific primary antibody (1:100 dilution) overnight at 4 °C and peroxidase conjugated secondary antibody was used for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Representative results showed cytosolic and nuclear positivity for β-catenin in tumor cells (A: diffuse astrocytoma; B: anaplastic astrocytoma; C: glioblastoma multiforme).

cell lines was demonstrated using non-steroidal anti-inflammatory drugs (NSAIDS) like diclofenac and celecoxib, which are the specific inhibitors of the Wnt pathway. Diclofenac and celecoxib suppressed the growth of GBM cell lines in culture and reduced the β-catenin dependent expression (data not shown).

In order to confirm the cell type and sub-cellular distribution for the expression of β-catenin, immunohistochemistry and immunofluorescence experiments were performed. Immunohistochemical analysis showed a strong β-catenin expression in astrocytic tumors (DA, AA and GBM). The cytosolic accumulation and nuclear translocation of β-catenin which is the hallmark of Wnt pathway activity was evidenced by the strong positive staining in the cytoplasm and nuclei of tumor cells (Fig. 4). Double immunofluorescence analysis also showed the cytoplasmic and nuclear localization of β-catenin in GFAP expressing tumor cells (Fig. 5).

3.3. Constitutive activation of Tcf4 and Lef1

Cytoplasmically accumulated β-catenin translocates to nucleus and activates the Tcf4 and Lef1 transcriptional factors. In order to know whether β-catenin interacted with Tcf4, we performed co-immunoprecipitation experiments. The corresponding results showed that β-catenin formed a complex with Tcf4 in human GBM tissue (Fig. 6A) and cultured T98G cell lysates (Fig. 6B). Further, to check the activation of Tcf4 and Lef1 transcriptional factors, we performed western blotting and immunohistochemical staining. Our results showed that the relative protein levels of Tcf4 and Lef1 were significantly higher in core tumor samples than in peritumor and control brain samples (Fig. 7A). The mean expression levels of Lef1 in grade II (52.6 ± 1.45), grade III (61.3 ± 2.02), grade IV (84.2 ± 3.47) and Tcf4 in grade II (22.6 ± 1.51), grade III (29.5 ± 0.86), grade IV (40.52 ± 2.59) were statistically significant ($p < 0.05$) when compared with that of controls (11 ± 1.15, 12.3 ± 1.76, respectively) and showed significant positive correlation with the degrees of histological malignancy (Fig. 7B and C). Further, immunohistochemical analyses of Lef1 and

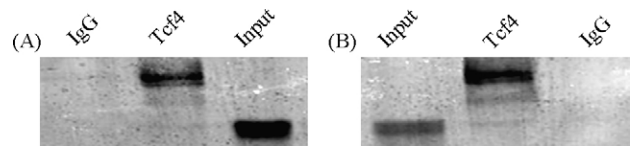

Fig. 6. Binding of β-catenin with Tcf4. Nuclear extracts from human GBM tissue (A) and T98G cells (B) overexpressing β-catenin and Tcf4 were immunoprecipitated with anti-β-catenin antibody (normal IgG as a negative control). Proteins in the β-catenin complex were analyzed by western blotting with anti-Tcf4 antibodies.

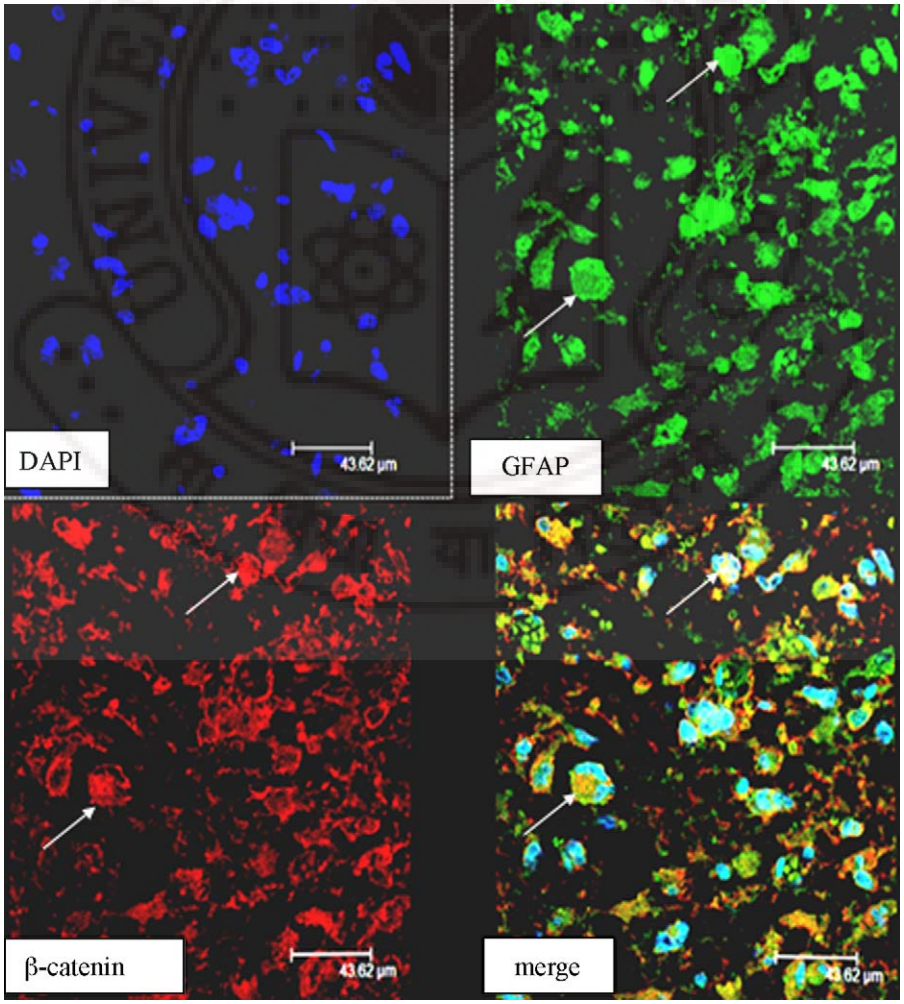

Fig. 5. Colocalization of β-catenin and GFAP. Paraffin-embedded astrocytoma sections were prepared from astrocytoma specimens. Sections were incubated with anti-rabbit-β-catenin and anti-mouse-GFAP specific primary antibodies (1:100 dilution) overnight at 4 °C and ant-rabbit-TRITC and anti-mouse-FITC secondary antibodies were used for 1 h at room temperature. DAPI was used for the detection of nuclei and fluorescence was captured under Leica confocal microscope. Representative figure showed the cytosolic and nuclear localization of β-catenin in GFAP positive tumor cells.

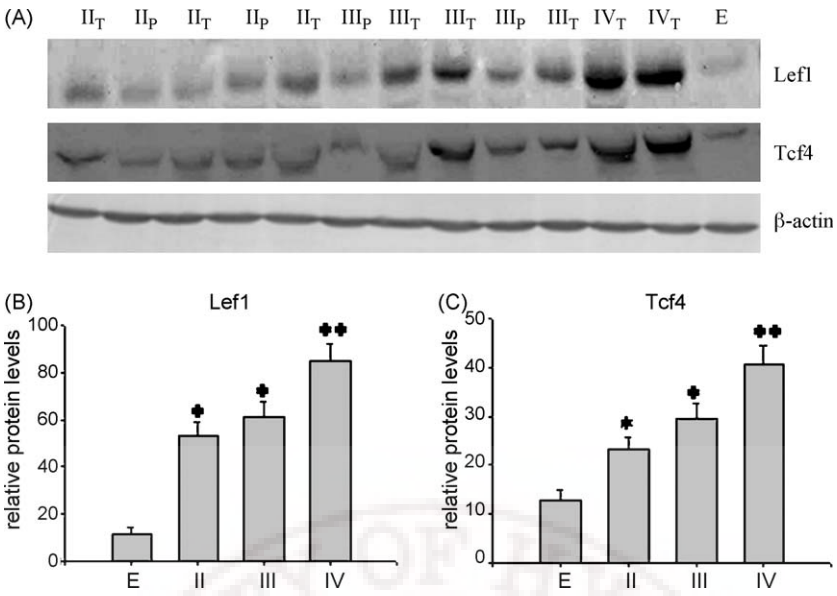

Fig. 7. Overexpression of Lef1 and Tcf4. (A) Whole cell protein extracted from human astrocytic tumor samples were separated on 10% SDS gels and transferred on to nitrocellulose membranes. Immunoblots were detected with Lef1 and Tcf4 specific primary antibodies. The expression levels of β-actin were used as loading controls (II_P: diffuse astrocytoma peritumor tissue, II_T: diffuse astrocytoma core tumor tissue, III_P: anaplastic astrocytoma peritumor tissue, III_T: anaplastic astrocytoma core tumor tissue, IV_T: GBM core tumor tissue, E: normal control brain). Densitometric analysis showing the Lef1 (B) and Tcf4 (C) levels were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments ($^*p < 0.05$ and $^{**}p < 0.001$ indicate significant difference relative to the corresponding control).

Tcf4 in DA, AA and GBM showed strong nuclear positivity in tumor cells (Fig. 8).

3.4. Upregulation of Wnt target genes: c-Myc, N-Myc, c-jun and cyclin D1

To assess the gene expression of c-Myc, N-Myc, c-jun and cyclin D1 in tumors as well as in controls we performed RT-PCR, western blotting and immunohistochemistry. RT-PCR analysis in astrocytic tumors showed the upregulation of *c-myc*, and *cyclin D1* in tumor samples than in controls (Fig. 9A). Quantitative densitometric analysis showed the expression levels of *c-myc* in grade II (18 ± 1.43), grade III (44 ± 2.14) and grade IV (45 ± 2.4) and *cyclin D1* in grade II (25 ± 1.16), grade III (50 ± 2.24) and grade IV (58 ± 2.7) were statistically significant ($p < 0.05$) when compared to controls (6 ± 1.0, 11 ± 1.1, respectively) and positively correlated with histological malignancy (Fig. 9B and C). Western blotting results showed the elevated protein levels of c-Myc, N-Myc, c-jun and cyclin D1 in core tumor tissues than in peritumor and control brain tissues (Fig. 10A). The mean protein expression levels of c-Myc (grade II-61.6 ± 2.61; grade III-68.7 ± 4.2; grade IV-114.25 ± .7.4; control-23.7 ± 5.9) N-Myc (grade II-63 ± 5.3; grade III-70 ± 6.2; grade IV-103.5 ± 8.0; control-21.5 ± 3.6) c-jun (grade II-62.1 ± 5.2; grade III-69.5 ± 6.3; grade IV-90.5 ± .7.3; control-32.25 ± 1.5) and cyclin D1 (grade II-171.3 ± 15.4; grade III-213.25 ± 18.8; grade IV-291.8 ± .19.54; control-128.6 ± 3.2) were statistically significant when compared with that of controls ($p < 0.05$) and showed significant positive correlation with the progression of histological malignancy (Fig. 10B, C, D and E). Immunostaining of c-Myc, N-Myc (Fig. 11), c-jun and cyclin D1 (Fig. 12) showed strong nuclear positivity in tumor cells of DA, AA and GBM.

3.5. Correlations among Dvl-3, β-catenin, Lef1 and Tcf4 in astrocytomas

The expression levels of every two genes were compared. The mRNA levels of β-*catenin* and *Dvl-3* showed strong positive correlation ($n = 10$, $r = 0.924$, $p < 0.001$) and the protein and mRNA levels of β-catenin showed highly significant positive correlation ($n = 10$, $r = 0.887$, $p < 0.001$). Also, the protein levels of β-catenin and Lef1 ($n = 32$, $r = 0.875$, $p < 0.001$), β-catenin and Tcf4 ($n = 32$, $r = 0.893$, $p < 0.001$), Lef1 and Tcf4 ($n = 32$, $r = 0.931$, $p < 0.001$) exhibited a highly significant positive correlation.

4. Discussion

Cancer may arise because the developmental pathways that create the dramatic alterations in form and structure in embryonic development are potentially interrupted. The cells in our body retain memories of these pathways and cancer can occur later in life if imperfections occur in the fidelity of these pathways. Wnt signaling pathway is a major developmental pathway, which regulates CNS development during embryogenesis and also later in adult life (Fogarty et al., 2005). Activation of this pathway appears to play a critical role in carcinogenesis (Morin, 1999; Polakis, 2000). Its role as a critical mediator in carcinogenesis was evident in many cancers (Giles et al., 2003), including CNS malignancies like medulloblastoma (Huang et al., 2000), a major child hood brain tumor, and subependymal giant cell astrocytomas (Jozwiak et al., 2007). Our earlier studies reported the Wnt/β-catenin/Tcf signaling pathway activation in progression of ENU induced rat gliomas (Sareddy et al., in press). Expression of Wnt signaling cascade genes in human astrocytic tumors has not been investigated so much, only few reports showed that Wnt5a, Wnt10b and Wnt13 ligands (Yu et al., 2007; Howng et al., 2002), and frizzled 9 receptor were upregulated in GBM (Zhang et al., 2006) and also little has been studied on correlation between the histological malignancy of human astrocytic tumors and expression profile of the Wnt pathway components. To our knowledge, this is the first report showing the upregulation of Wnt signaling cascade genes in the malignant progression of astrocytomas.

Dvl genes are upstream key mediators of β-catenin, activated in response to Wnt binding to frizzled receptor complex. Dvl activates β-catenin by suppressing the inhibitory activities of GSK3β and relocating Axin to plasma membrane. Upregulation of different *Dvl* homologs *Dvl-1* and *Dvl-3* was evident in many cancers (Okino

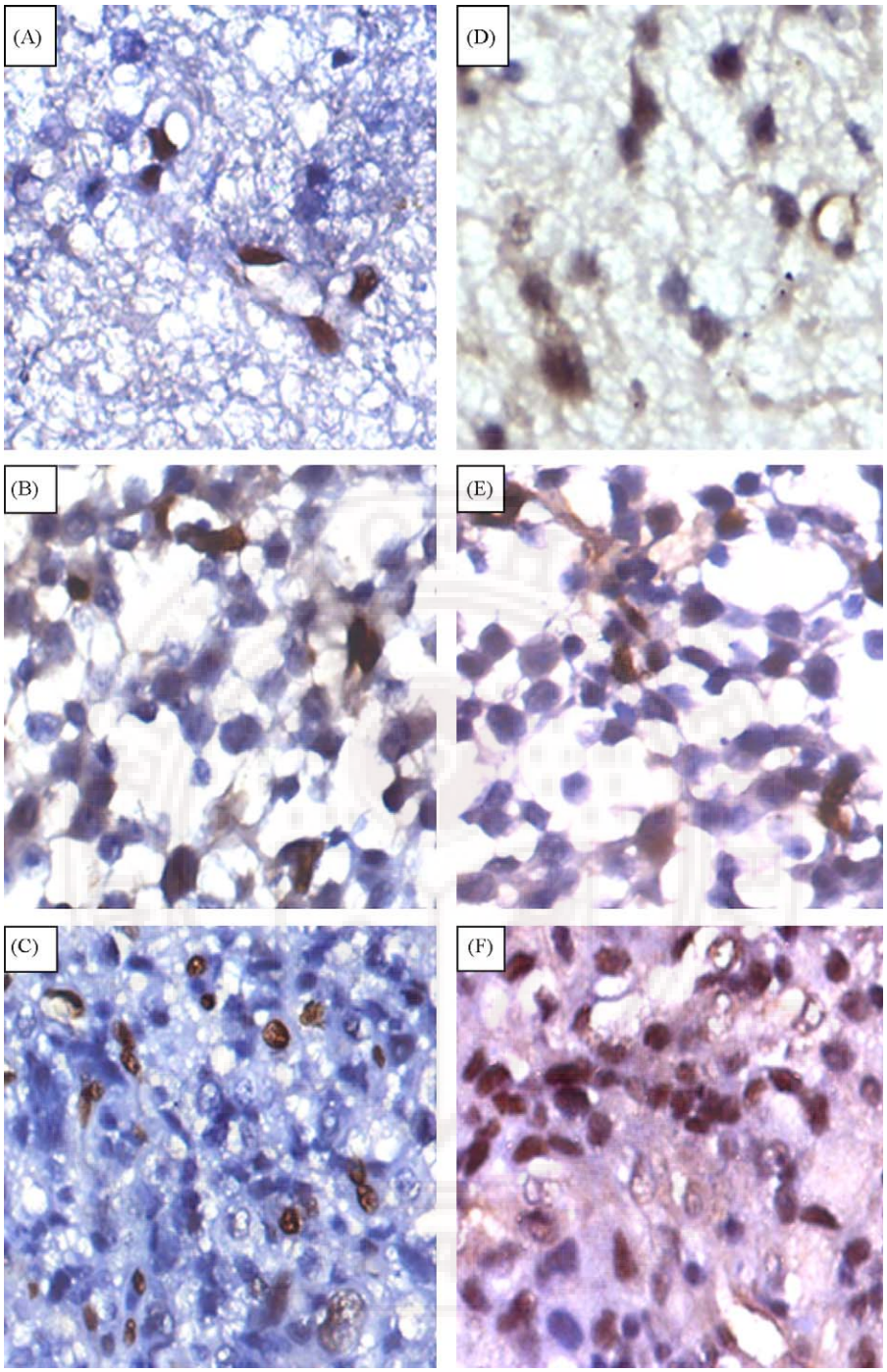

Fig. 8. Immunohistochemical staining of Lef1 and Tcf4 in astrocytomas. Paraffin-embedded astrocytoma sections prepared from astrocytoma specimens. Sections were incubated with Lef1 and Tcf4 specific primary antibodies (1:200 dilutions) overnight at 4 °C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positivite nuclear staining for Lef1 (A, B, C) and Tcf4 (D, E, F). A and D: diffuse astrocytoma; B and E: anaplastic astrocytoma; C and F: GBM.

et al., 2003; Nagahata et al., 2003; Uematsu et al., 2003). The present study has shown an enhanced expression of *Dvl-3* mRNA in astrocytic tumors and its positive correlation with astrocytoma malignancy. However *Dvl-1* and *Dvl-2* were not expressed in astrocytomas. Activated Dvl-3 transduces signals to β-catenin in response to the binding of Wnt to Frizzled and LRP. In this study we observed the relative β-catenin protein and mRNA levels were moderate in low-grade tumors, higher in high-grade tumors, compared to control brain samples. The mRNA and protein levels of β-catenin showed positive correlation with histological malignancy. The evidence from immunohistochemistry and immunofluorescence studies suggested nuclear and cytoplasmic accumulation of β-catenin in astrocytomas which is the hallmark of active β-catenin/Tcf signaling. In the absence of β-catenin, Tcf/Lef factors suppress the Wnt target gene expression by binding with members of the Groucho (Grg/TLE) family of transcriptional co-repressors (Brantjes et al., 2001). Translocation of β-catenin converts Tcf family proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of co-activator proteins including CBP, TBP, BRG1, BCL9/PYG, Legless,

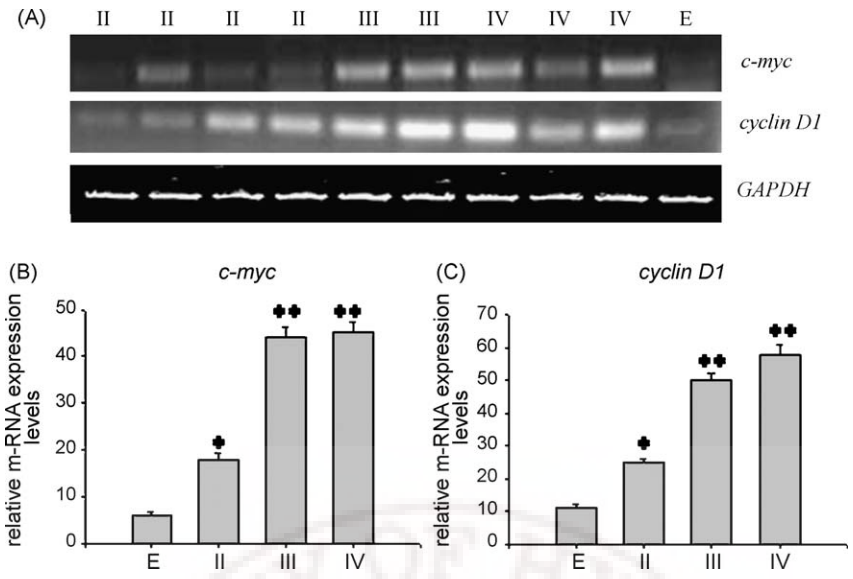

Fig. 9. Overexpression of *c-myc* and *cyclin D1* mRNA in astrocytic tumors. (A) Total RNA was isolated from astrocytoma samples and reverse transcribed to cDNA. PCR amplification was conducted using *c-myc* and *cyclin D1* specific primers. Amplification of *GAPDH* served as an internal control (II: diffuse astrocytoma; III: anaplastic astrocytoma; IV: glioblastoma multiforme; E: control brain). Densitometric analysis showing the expression levels of *c-myc* (B) and *cyclin D1* (C) were positively correlated with the histological grading of astrocytomas. Data are represented as mean ± standard error from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.001$ indicate significant difference relative to the corresponding control).

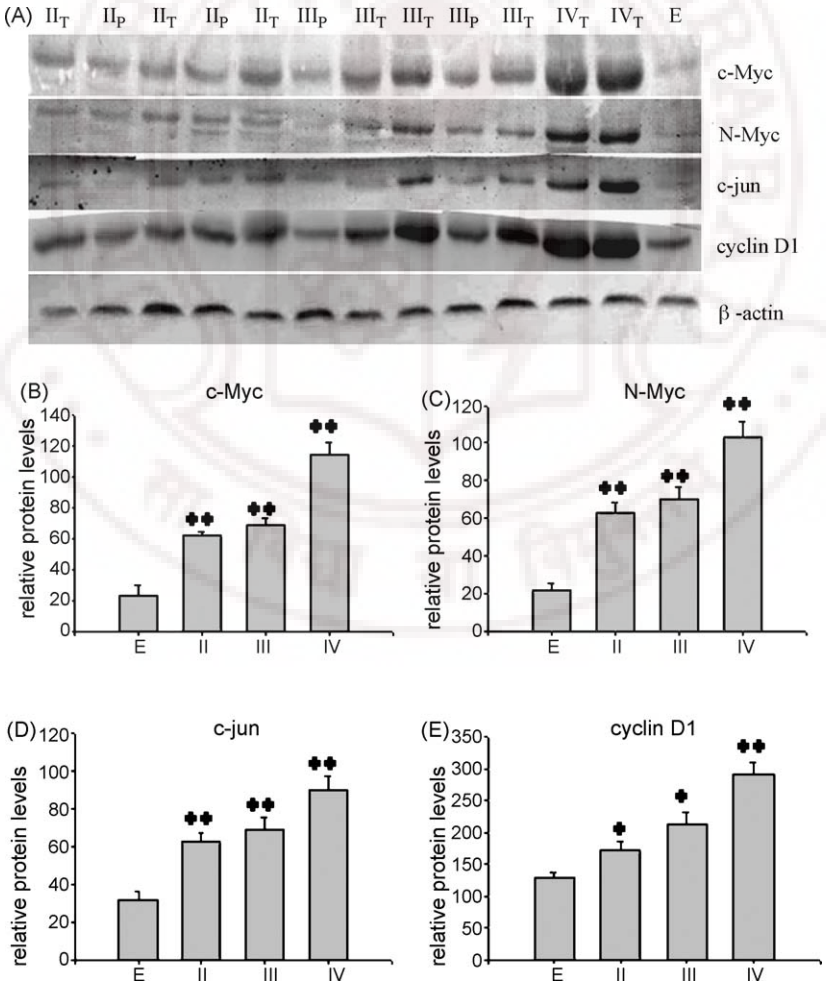

Fig. 10. Upregulation of β-catenin target genes c-Myc, N-Myc, c-jun and cyclin D1. (A) Whole cell protein extracted from human astrocytic samples separated on 10% SDS gels and transferred on to nitrocellulose membranes. Immunoblots were detected with c-Myc, N-Myc, c-jun and cyclin D1 specific primary antibodies. The expression levels of β-actin were used as loading controls (II_P: diffuse astrocytoma peritumor tissue, II_T: diffuse astrocytoma core tumor tissue, III_P: anaplastic astrocytoma peritumor tissue, III_T: anaplastic astrocytoma core tumor tissue, IV_T: GBM core tumor tissue, E: normal control brain). Densitometric analysis showing the protein levels of c-Myc (B), N-Myc (C), c-jun (D) and cyclin D1 (E) were positively correlated with histological grading of tumors. Data are represented as mean ± standard error from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.001$ indicate significant difference relative to the corresponding control).

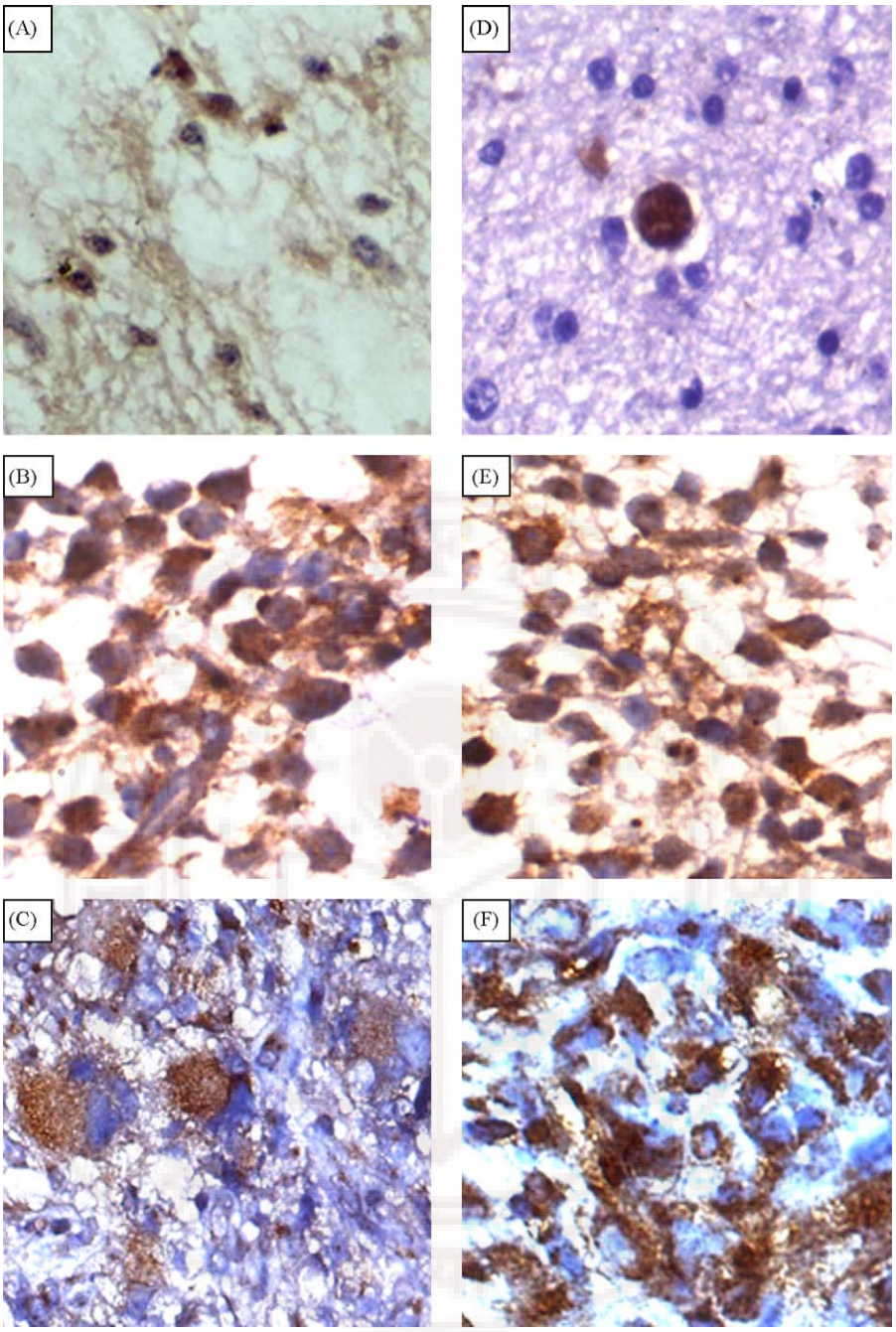

Fig. 11. Paraffin-embedded astrocytoma sections were prepared from astrocytoma specimens. Sections were incubated with c-Myc and N-Myc (1:100 dilutions) specific primary antibodies overnight at 4 °C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positive nuclear staining for c-Myc (A, B, C) and N-Myc (D, E, F). A and D: diffuse astrocytoma; B and E: anaplastic astrocytoma; C and F: GBM.

Mediator and Hyrax (Hecht et al., 2000; Takemaru and Moon, 2000; Hoffmans et al., 2005; Kramps et al., 2002; Barker and Clevers, 2006). β-catenin does not have a DNA binding domain, but it has a potent transcription activation domain. In general, Lef/Tcf transcription factors do not have a strong transcription activation domain, but they have a good DNA binding/bending domain (Waterman, 2004). Thus, when β-catenin binds to a Lef/Tcf protein, a potent transcription regulatory complex is formed. The present study has shown elevated protein levels of Tcf4 and Lef1 in astrocytomas and their positive correlation with histological grading. Immunohistochemical staining showed Tcf4 and Lef1 were prominent in nuclei of tumor cells. With co-immunoprecipitation experiment it has been demonstrated that β-catenin interacted with Tcf4 in the nuclear fractions of GBM tissue and T98G cell line. The effects of Wnt signaling are mediated by the expression of their targets like c-jun, c-Myc, N-Myc and cyclin D1. We observed over expression of *c-myc* and *cyclin D1* mRNA in cancerous tissues than controls. In addition, protein levels of c-Myc, N-Myc, c-jun, and cyclin D1 were up regulated in astrocytic tumors and correlated with histological malignancy as well.

Astrocytic tumors develop and progress as a result of occurrence of genetic alterations in low grade lesions, which further acquire additional mutations or genetic alterations when progress towards more malignant lesion. These genetic alterations results in the activation of oncogenes and inactivation of tumor suppressor genes. These genetic alterations disrupt the cell cycle

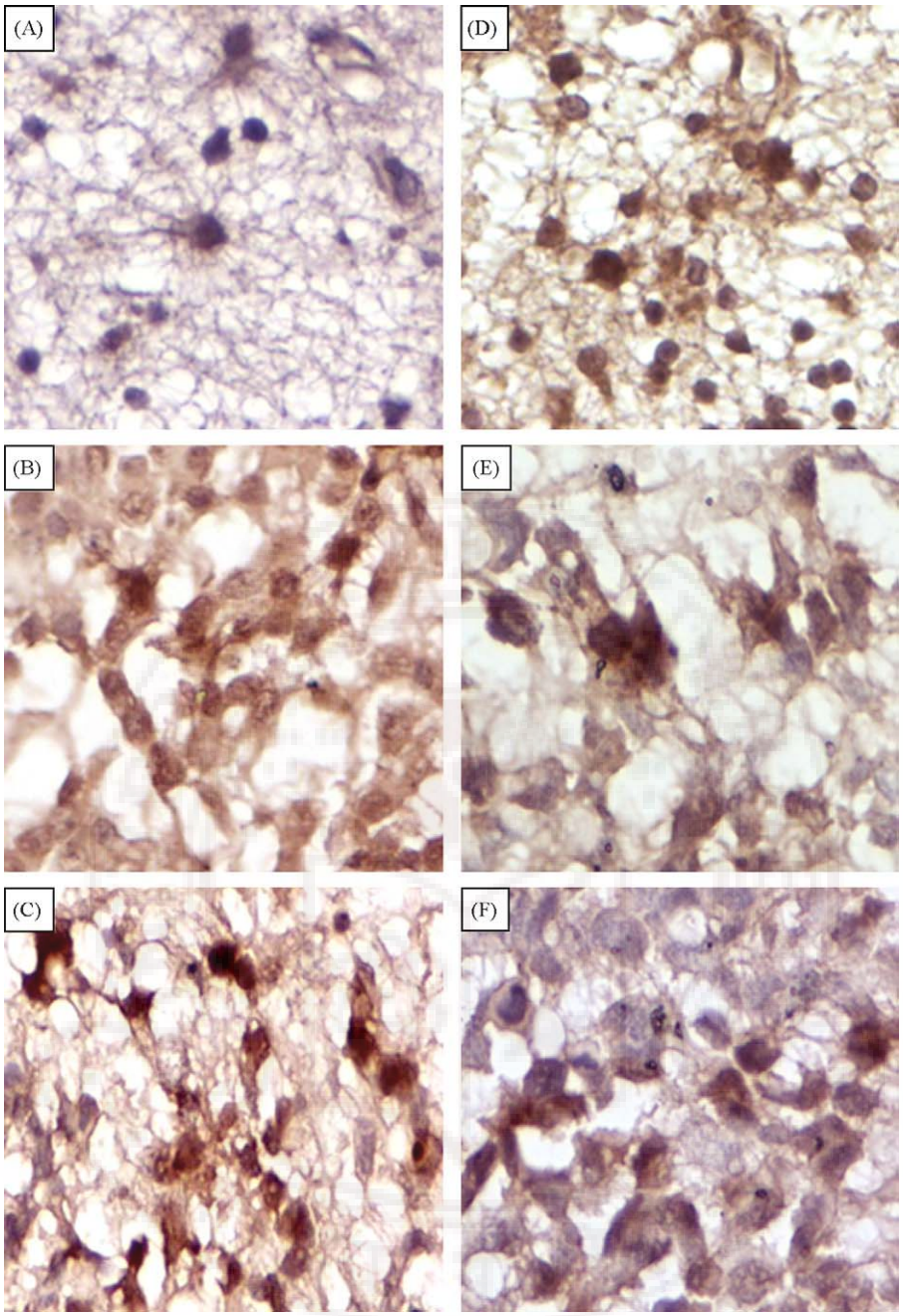

Fig. 12. Paraffin-embedded astrocytoma sections prepared from astrocytoma specimens. Sections were incubated with c-jun and cyclin D1 (1:50 dilutions) specific primary antibodies for overnight at 4 °C and peroxidase conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by diaminobenzidine and sections were counterstained using haematoxylin. Tumor cells exhibited positive nuclear staining for c-jun (A, B, C) and cyclin D1 (D, E, F). A and D: diffuse astrocytoma; B and E: anaplastic astrocytoma; C and F: GBM.

arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. In the present study, we observed the elevated protein and mRNA profile of Wnt signaling pathway components in tumors of low grade as well in high grades and their expression patterns significantly correlated with histological malignancy of astrocytomas. This clearly indicated that the Wnt/β-catenin/Tcf signaling activation is implicated the development and malignant progression of human astrocytomas. Future studies utilizing conditional expression of β-catenin and/or dnTcf4 *in vivo* at different stages of carcinogenesis will be required to corroborate our current data suggesting the involvement of the β-catenin/Tcf signaling pathway in astrocytoma development.

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ORIGINAL PAPER

Wnt/β-catenin/Tcf Signaling Pathway Activation in Malignant Progression of Rat Gliomas Induced by Transplacental *N*-Ethyl-*N*-Nitrosourea Exposure

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Abstract Although Wnt/β-catenin/Tcf signaling pathway has been shown to be a crucial factor in the development of many cancers, little is known about its role in glioma malignancy. In the present study, we report the first evidence that Wnt/β-catenin/Tcf signaling pathway is constitutively activated in experimental gliomas induced by single transplacental dose of *N*-ethyl-*N*-nitrosourea (ENU). In the present study we analyzed ENU induced rat gliomas of different stages (P90, P135 and P180) for the expression of β-catenin, Lef1, Tcf4 and their targets c-Myc, N-Myc and cyclin D1. Western blot analysis revealed upregulation of β-catenin, Lef1, Tcf4, c-Myc, N-Myc and cyclin D1 in gliomas compared to controls and their levels were progressively increased from initial stage (P90) to progression stage (P180). In consistent with this, immunohistochemistry revealed the cytoplasmic and nuclear accumulation of β-catenin, and nuclear positivity was evident for Lef1, Tcf4, c-Myc, N-Myc and cyclin D1. Based on these results, we conclude that Wnt/β-catenin pathway may play a major role in the tumorigenesis and tumor progression in ENU induced rat gliomas.

Keywords Glioma · β-catenin · Tcf4 · Lef1 · c-Myc · N-Myc · cyclin D1 · Tumor

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Introduction

Malignant gliomas are the most common primary tumors of the central nervous system. Despite advances in surgical and clinical neuro-oncology, their prognosis remains poor. Glioma includes the tumors derived from astrocytes, oligodendrocytes and ependymal cells. WHO classified gliomas into four clinical grades on the basis of histology and prognosis. Glioblastoma multiforme (GBM-grade IV) is the most malignant and highly invasive type with worst prognosis. Patients with GBM have the mean survival of 1 year, whereas patients with anaplastic glioma (grade III) can survive for 2–3 years, and patients with low grade (grade II) gliomas have favorable outcome and can survive for as long as 10–15 years. Pilocytic astrocytomas (grade I), major childhood brain tumors, are curable by surgery and might represent a separate disease from the gliomas of other grades [1–5]. GBM includes two subtypes: primary GBM, which arise de novo, and secondary GBM, which develop from low grade gliomas. Majority of cases (90%) are primary GBMs, which develop without clinical or histological evidence of a less malignant precursor lesion and affect mainly elderly patients and are genetically characterized by the loss of heterozygosity 10q, epidermal growth factor receptor (EGFR) amplification, $p16^{INK4A}$ deletion, and phosphatase and tensin homolog (PTEN) mutations. Secondary glioblastomas develop through progression from low grade gliomas and manifest in younger patients, bearing the mutations in p53 and are the most frequent and earliest detectable genetic lesions [6]. Glial tumors develop as a result of stepwise accumulation of genetic alterations, which results in the activation of oncogenes and inactivation of tumor suppressor genes [7]. These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways

that are activated by receptor tyrosine kinases. Mutation of the p53, retinoblastoma (RB) and PTEN, deletion of $p16^{INK4A}$, activation of the Ras and Akt pathways, and amplification of CDK4 and EGFR contribute to the development of gliomas [8, 9].

Wnt proteins are a large group of secreted glycoproteins, which play a critical role in regulating cell fate, differentiation, proliferation and potentially tumor formation [10, 11]. Aberrant activation of Wnt signaling pathway has been implicated in a variety of human cancers including colon, breast, prostate, and ovary. The Wnt pathway activation in oncogenesis and its consequences has been extensively reviewed [12–15]. Activation of canonical Wnt signaling pathway requires the binding of Wnt ligands to frizzled (FZD) receptors together with the co-receptors LDL receptor-related protein 5 (LRP5) or LRP6 [16–18]. β-catenin is the central hub of the Wnt signaling pathway. It is well documented that in the absence of Wnt signaling, β-catenin is captured in the destruction complex consists of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by GSK3β and CK1α results in its ubiquitination and subsequent proteosomal degradation [19, 20]. Eventually, the resulting β-catenin drought ensures that the Tcf/Lef transcription factors (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoter and enhancers. Binding of Wnt ligands to their cognate receptors FZD and to co-receptors LRP at the membrane results in the formation of Dishevelled (Dvl)–Fzd complex and relocation of Axin from the destruction complex to the cell membrane [13, 21]. This allows β-catenin to accumulate in the cytosol and enter the nucleus, where it interacts with members of the Tcf/Lef transcription factors to induce expression of Wnt target genes like c-Myc, N-Myc, and cyclin D1, which instruct the cell to actively proliferate and remain in an undifferentiated state [22–24].

Though there are several reports which evident the involvement of Wnt/β-catenin signaling in several malignancies, little is known about the role of Wnt/β-catenin signaling pathway in glioma development. To begin addressing this question, therefore, we have been using a rat model of neurocarcinogenesis in which gliomas invariably develop several months after a single prenatal exposure to *N*-ethyl-*N*-nitrosourea (ENU) [25–27]. ENU-induced glioma rat model is a well established animal model by which one can better study the pathological changes and various signal transduction pathways that may play a crucial role in tumorigenesis and progression in a way similar to the progression of low grade gliomas to GBM in humans. Its ability to induce neuroectodermal tumors in 100% of the offspring [28, 29] following a single transplacental exposure has stimulated its use in experimental neuro-oncology, and ENU-induced neoplasms in rats have morphological and biological similarities to naturally occurring glial tumors in humans [30, 31]. Detailed understanding of the tumor biology during promotion and progression stage may be highly significant for therapeutic targeting of human malignant GBMs.

This model is uniquely suited to address this question because ENU itself is rapidly cleared with short half life, after which tumors are not observed before 90 days of age in the offspring. This creates a large temporal window in which one can safely assume that pathological changes are because of developing tumor and not from continued exposure to mutagens.

Materials and Methods

Tumor Induction

The animal model used in the present study is approved by institutional ethics committee. ENU (Sigma Aldrich, St Louis, MO, USA) was freshly prepared by dissolving 10 mg/ml in 0.9% saline and adjusted pH to 4.5 with crystalline ascorbic acid. A single transplacental dose of (75 mg/kg body weight) of ENU was administered intraperitonially to pregnant Wistar rats ($n = 4$) on 18th day of gestational period and control rats received saline. All the ENU treated pregnant rats were normal. 34 (out of 38) offspring rats with glioma symptoms (corneal erosion observed from birth, limb paresis, shaggy, loss of fur along with discoloration of skin, depressive or aggressiveness, loss of weight and other neurological symptoms) were used for experimentation [32]. Ten rats at postnatal day (P) 90, 12 rats at P135 and 12 rats at P180 were used for western blot and immunohistochemistry analyses.

Western Blotting

Rat brain tissues were homogenized in 5 volumes of 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, 0.3 mM Na_3Vo_4 and 0.3 mM aprotinin. The lysate was sonicated for 2 min and centrifuged at 14,000 g for 15 min at 4°C. The supernatant was collected as whole tissue lysate and frozen at −80°C before use. Protein concentrations were determined by Lowry method. 75 µg of cellular protein were mixed with SDS sample buffer, boiled for 5 min and subjected to

electrophoresis on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking the nitrocellulose membranes in non-fat dry milk (5%) in tris buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature, they were incubated for 12–24 h with primary antibodies. Then membranes were incubated with secondary antibodies conjugated to alkaline phosphatase (ALP) (anti-rabbit and anti-mouse IgG conjugated to ALP obtained from Genei Pvt Ltd, Bangalore, India), for 1–2 h at room temperature. Before and after incubation with secondary antibodies, membranes were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivty was visualized by incubating the membranes with BCIP-NBT solution (Genei Pvt Ltd, Bangalore, India). Membranes were analyzed quantitatively using ImageJ software (NIH).

The primary antibodies used in these experiments included rabbit polyclonal antibody against β-catenin, rabbit monoclonal antibody against Lef1, Tcf4, and cyclin D1 (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal antibody against N-Myc (gift of Dr. Robert Eisenman, Fred Hutchinson Cancer Research Center, Washington, USA), mouse monoclonal antibody against c-Myc (gift of Naidu, Manipal Institute of Medical Sciences, India), and mouse polyconal β-actin (gift of Prof. K. Anand Kumar, University of Hyderabad, India).

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded specimens. Briefly, sections were deparafinized in xylene and passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by microwaving the sections for 10–14 min in 10 mM citrate buffer (pH 6.0). Sections were then treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase followed by blocking with serum for 1 h at room temperature in a humid chamber. The sections were then incubated overnight at 4°C with primary antibodies against β-catenin, Lef1, Tcf4, c-Myc, N-Myc and cyclin D1 diluted as per data sheet in blocking solution. Peroxidase conjugated secondary antibody was used for 1 h incubation at room temperature followed by TBS washes (3 × 5 min each). Diaminobenzidine (DAB) in buffer was used till sections develop color. Sections were counter-stained using hematoxylin. Sections were washed with distilled water followed by dehydration in graded alcohols, xylene and then finally mounted by using DPX (kit obtained from Biogenex Pvt Ltd, India). In each experiment, a negative control was included in which the primary antibody step was skipped and replaced by non-immune serum.

Statistics

Densitometric data from quantitative western blotting was expressed as means ± SD. Differences between tumors and controls were compared by paired Student's *t*-test ($n = 34$). Values of $P < 0.05$ were considered statistically significant.

Results

Hematoxylin and Eosin staining of tissue sections of P90 glioma rats have shown the presence of early neoplastic proliferation (ENP) centers in the form of nodules. These were observed in the periventricular white matter of the

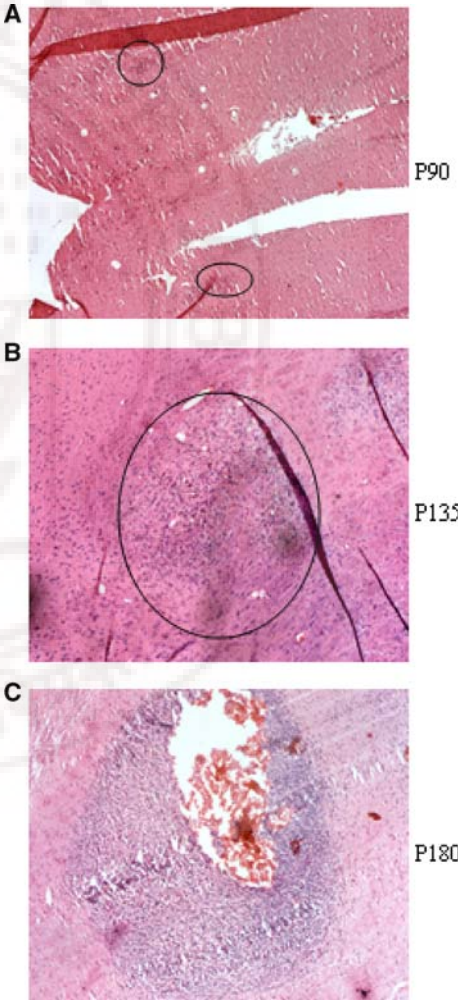

Fig. 1 H&E staining of brain sections of ENU-induced glioma rats. **a** P90 (initiation stage) glioma rat showing the presence of ENP centers in the form of nodules. **b** P135 (promotion stage) glioma rat showing the presence of increased tumor mass with increased cellularity. **c** P180 (progression stage) glioma rat showing the profound increase in tumor mass, angiogenesis, and cellularity. Original magnification 10×. "P" indicates postnatal in days

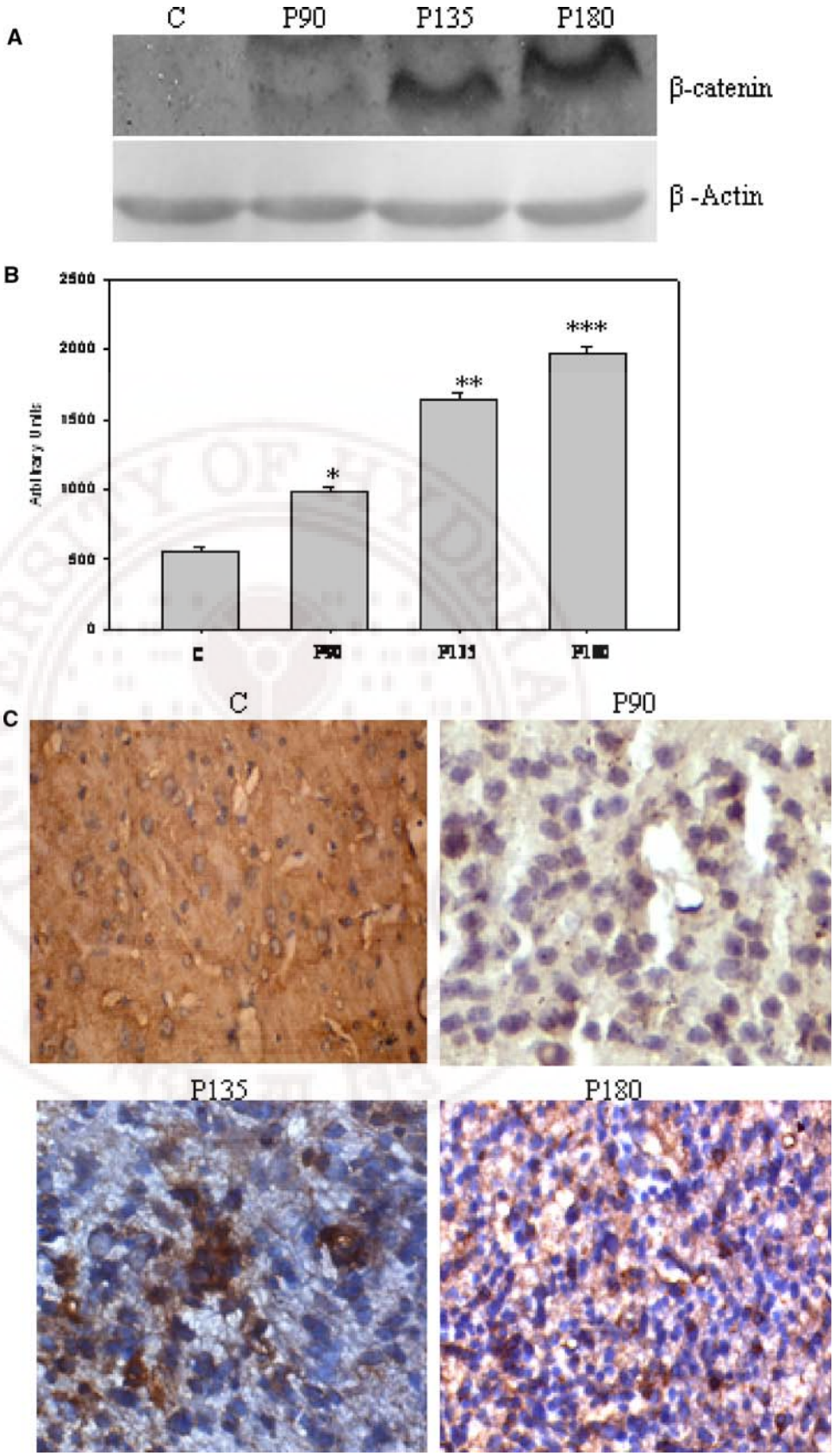

Fig. 2 **a** Over-expression of β-catenin in ENU-induced gliomas. Western blotting analysis demonstrating β-catenin overexpression in glioma rats, in comparison to saline-treated controls. The protein levels were progressively increased from P90–P180. β-Actin used as a loading control. **b** Quantitative comparison of β-catenin protein levels between glioma and control rats. The protein levels, determined by western blotting analysis, were analyzed by densitometry and normalized with expression levels of β-actin. The data were presented as mean $\pm$ SD of relative protein expression levels in tumors and control. Statistical significance is calculated using paired t-test between control ($n = 4$) and glioma rats of P90 ($n = 6$) (* represents $P < 0.001$), P135 ($n = 8$) (** represents $P < 0.0002$) and P180 ($n = 8$) (*** represents $P < 0.0001$). **c** Representative immunohistochemical staining for β-catenin in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited cytoplasmic and nuclear staining for β-catenin. Original magnification 40×. ($n = 4$ for each age group)

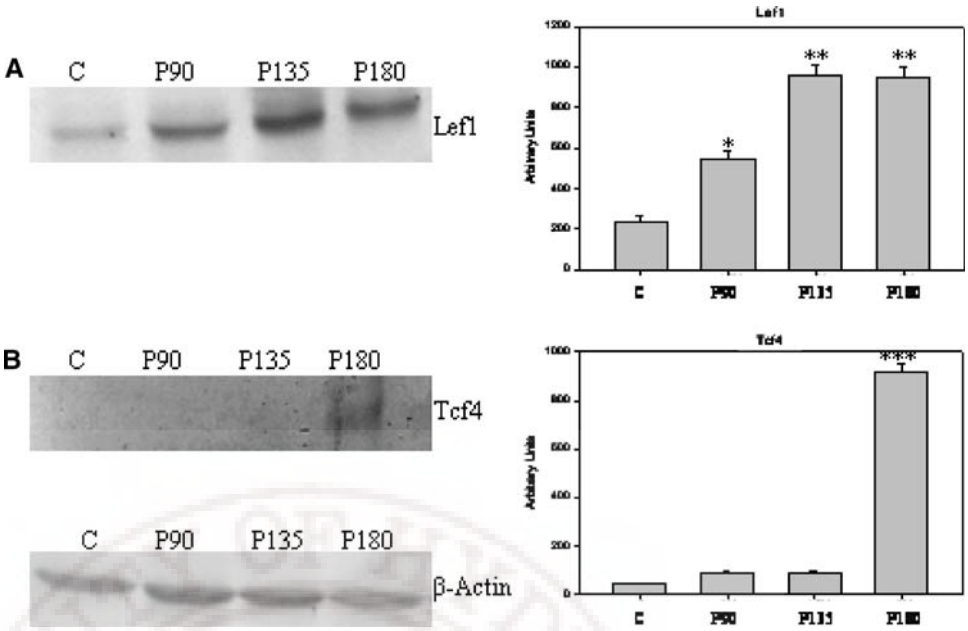

Fig. 3 Expression levels of Lef1 (**a**) and Tcf4 (**b**) in glioma and control rats determined by western immunoblotting analysis. Lef1 expression levels progressively increased from P90–P180 glioma rats, while Tcf4 expression was evident only in P180 rats. Expression levels of β-actin used as loading controls. Data are shown as the means ± SD of independent densitometric measurements among different glioma rats. Statistical significance is calculated using paired *t*-test between control ($n = 4$) and glioma rats of P90 ($n = 6$) (* denotes $P < 0.001$), P135 ($n = 8$) (** denotes $P < 0.0006$) and P180 ($n = 8$) (** denotes $P < 0.0007$, *** indicates $P < 0.00001$)

cerebral hemispheres (Fig. 1a). P135 glioma rats showed increased cellularity, signs of angiogenesis and mitotic figures (Fig. 1b), and these features were markedly increased to the progression stage (P180) (Fig. 1c).

In order to examine whether aberrant activation and accumulation of β-catenin in ENU-induced gliomas, P90, P135 and P180 rats were examined using western blot analysis. Saline-treated rats were used as controls. Glioma rats showed a significant increase in β-catenin levels in comparison to control (Fig. 2a), and expression levels were progressively increased from P135 to P180 as evident from densitometric analysis (Fig. 2b). In order to confirm the cell type and sub-cellular distribution for the expression of β-catenin, immunohistochemical staining was performed. Representative results showed that β-catenin accumulation in P90, P135 and P180 glioma rats and immunoreactivity observed in cytoplasm and nuclei of tumor cells (Fig. 2c). Cytopslasmically accumulated β-catenin that is translocated to the nucleus there it activates the Tcf4 and Lef1 transcriptional factors. So, in order to reveal the activation of Tcf4 and Lef1 transcriptional factors, we performed western blotting and immunohistochemical staining. Representative results showed the relative protein levels of Tcf4 and Lef1 were significantly higher in tumors than control. We observed a significant increase in Tcf4 expression at P180 only (Fig. 3b). Lef1 expression was observed in all glioma rats and relative protein levels were significantly increased from P90 to P180, while very low expression was observed in controls (Fig. 3a). Further, immunohistochemistry of Lef1 and Tcf4 showed their nuclear positivity in P90, P135 and P180 glioma rats (Fig. 4).

We assess the gene expression of c-Myc, N-Myc, and cyclin D1 that are activated in response to constitutive activation of Tcf4, Lef1 and β-catenin using western blot and immunohistochemistry. Western blot results showed significantly elevated levels of c-Myc (Fig. 5a), N-Myc (Fig. 5b) and cyclin D1 (Fig 5c) in gliomas when compared to controls from P90 to P180. Further immunohistochemical analysis demonstrated the nuclear immunoreactivity for c-Myc, N-Myc (Fig. 6) and cyclin D1 (Fig. 7) in tumors of P90, P135 and P180 glioma rats.

Discussion

Cancer may arise because the developmental signaling pathways that regulate embryonic development in form and structure are potentially interrupted. The cells in our body retain memories of these signaling pathways and cancer can occur later in life if the signaling pathways are interrupted [33]. Wnt signaling pathway is one of the major developmental pathway, which regulates CNS development during embryogenesis and also later in adult life [34]. Activation of this pathway appears to play a critical role in carcinogenesis [35, 36], and its role as a critical mediator for carcinogenesis was evident in many cancers [37]. Activation of Wnt/β-catenin signaling cascade genes in glial tumors has hardly been investigated, only a few reports showed that Wnt5a, Wnt10b and Wnt13 ligands [38, 39], and FZD 9 receptor were upregulated in GBM [40] and also little has been studied on the association between malignant progression and expression profile of the Wnt/β-catenin pathway components.

N-ethyl-*N*-nitrosourea is a potent resorptive neuro-carcinogen that has been found to be mutagenic in a wide variety of mutagenicity test systems and carcinogenic in

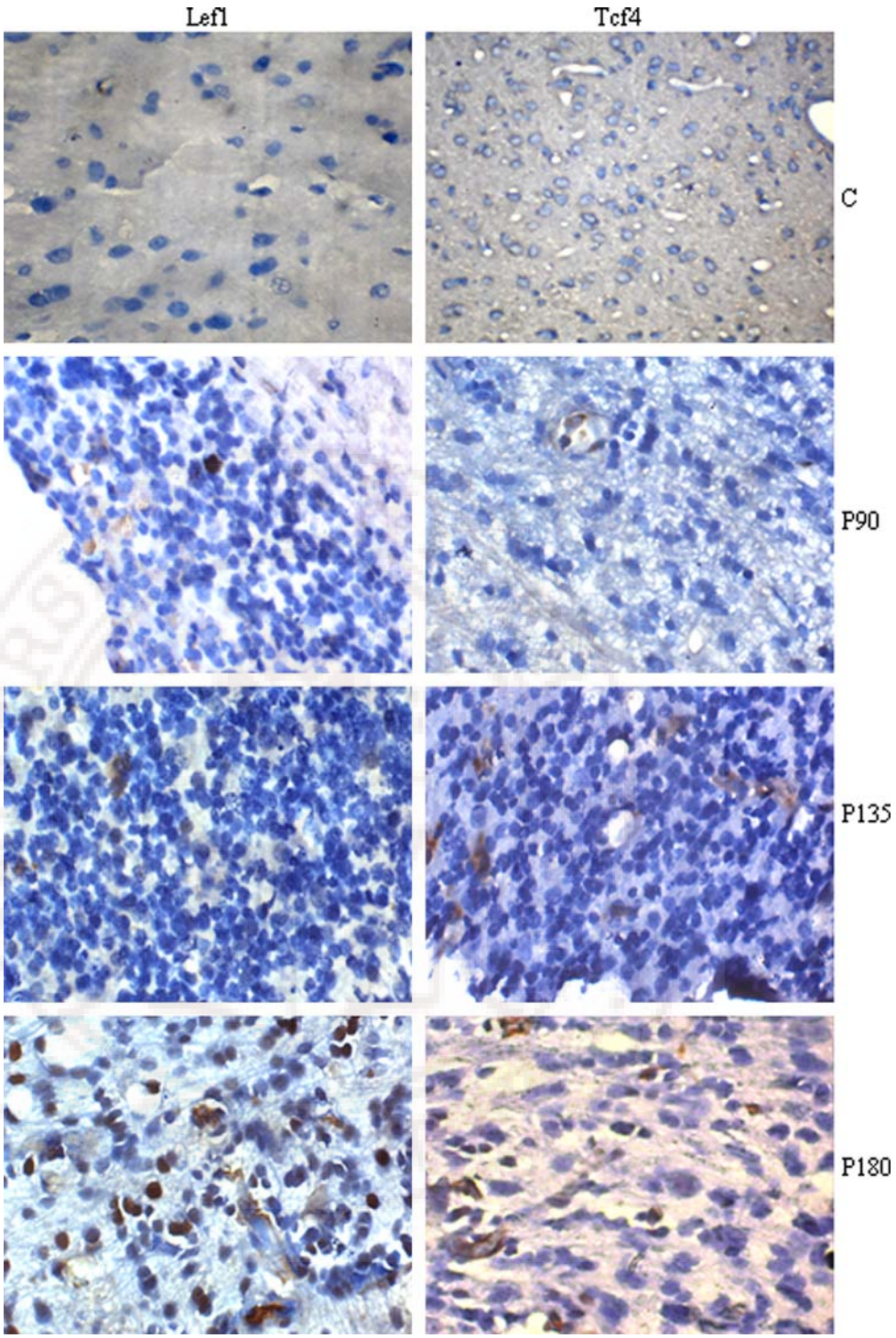

Fig. 4 Immunohistochemical analysis of Lef1 and Tcf4 in control (C), P90, P135 and P180 glioma rats demonstrated their nuclear positivity in tumor cells. In control rats no immunoreactivity was observed. Original magnification 40×. ($n = 4$ for each age group)

various organs of mammals [27]. ENU has referential transplacental and neonatal action, a single intraperitoneal injection induced almost 100% incidence of neural neoplasms and the incidence is higher in prenates and neonates than adults [31]. Multistage nature of carcinogenesis through initiation, promotion and progression stage in tumor development was reported earlier [41]. Characterization of different phases may be significant in the identification of effective molecular targets. This ENU-induced glioma rat model is a suitable model to study stage specific alterations during the tumor progression [42]. In the present study, we studied the stage specific alterations of β-catenin/Tcf signaling at initiation (P90), promotion (P135) and progression stages (P180).

Tumors in ENU glioma rats were reported to appear first as an ENP centers, or oligidendroglial foci, which

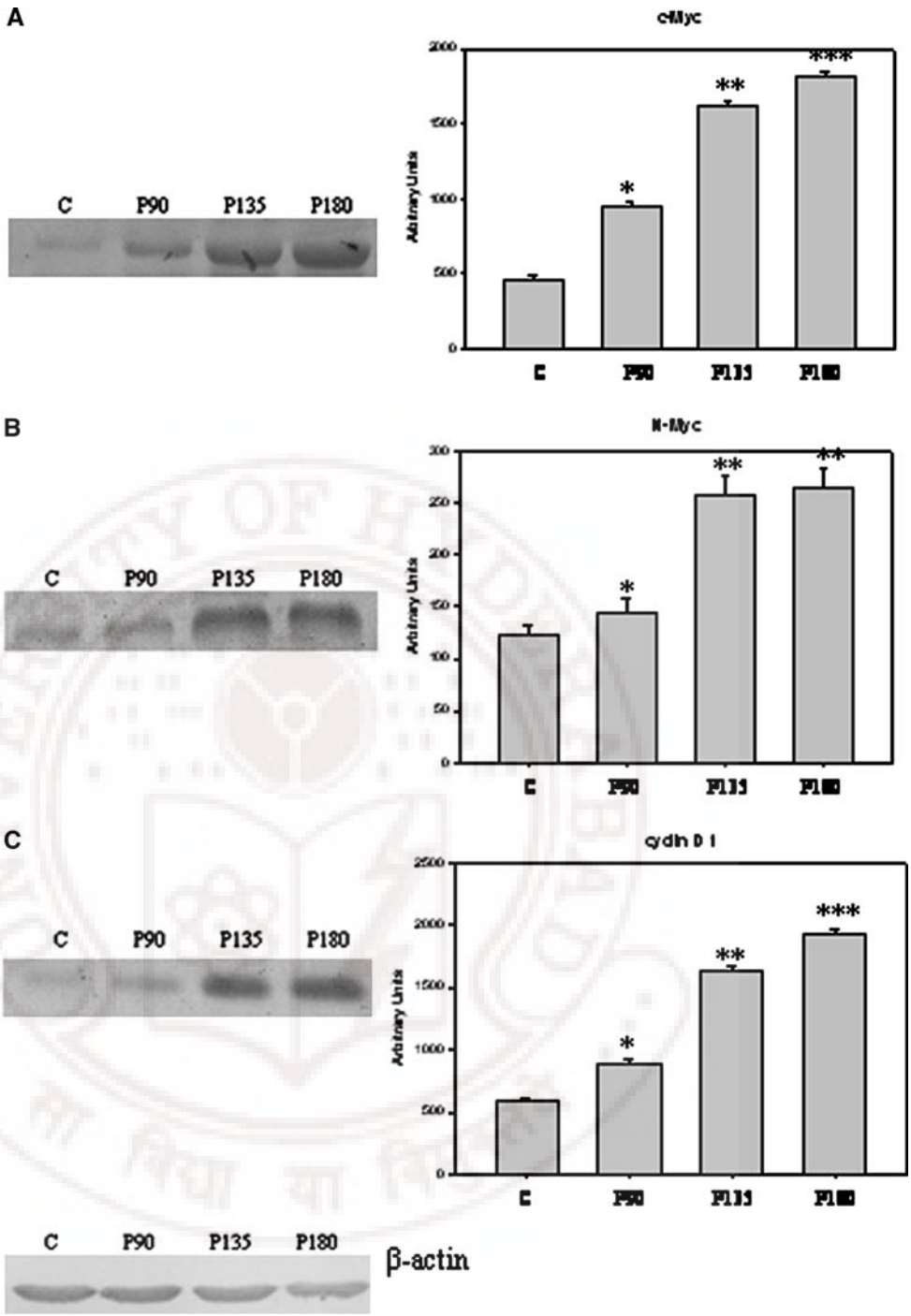

Fig. 5 Upregulation of β-catenin target genes c-Myc (**a**), N-Myc (**b**), and cyclin D1 (**c**). Western immunoblotting demonstrating elevated protein levels of c-Myc, N-Myc, and cyclin D1 in glioma rats and protein levels were significantly increased from P90–P180. The expression levels of β-actin were used as loading controls. The data are presented as mean $\pm$ SD of relative protein expression levels in tumors and control. Statistical significance is calculated using paired t-test between control ($n = 4$) and glioma rats of P90 ($n = 6$), P135 ($n = 8$) and P180 ($n = 8$). In c-Myc histogram *, ** and *** indicates $P < 0.0001$, 0.0002 and 0.00006 respectively. For N-Myc * and ** indicates $P < 0.005$ and 0.001 respectively. For cyclin D1 *, ** and *** represents $P < 0.0005$, 0.0001 and 0.00002 respectively

subsequently progress to "microtumors" and then "tumors" at different stages of tumor development as described by Koestner et al. [43]. ENPs appear at the end of postnatal 2 months and continue to appear till 3.5 months. Variable nestin expression was observed in ENU-induced glioma rats between 30 and 90 days and nestin expressing cells of the nodules was reported to represent the early stage of the neoplastic process [44, 45]. In contrast to sections of untreated rats, H&E staining sections of ENU-induced glioma rats shows cells with apoptotic features and marked cellularity including mitotic figures and abnormal pleomorphic cells [46].

As a first step to analyze the activation of β-catenin/Tcf signaling during the development of gliomas, we studied the expression profile of β-catenin. The relative protein levels of β-catenin were higher in glioma rats compared to controls and significantly increased from 3 to 6 months. Consistent with this, nuclear and cytoplasmic accumulation

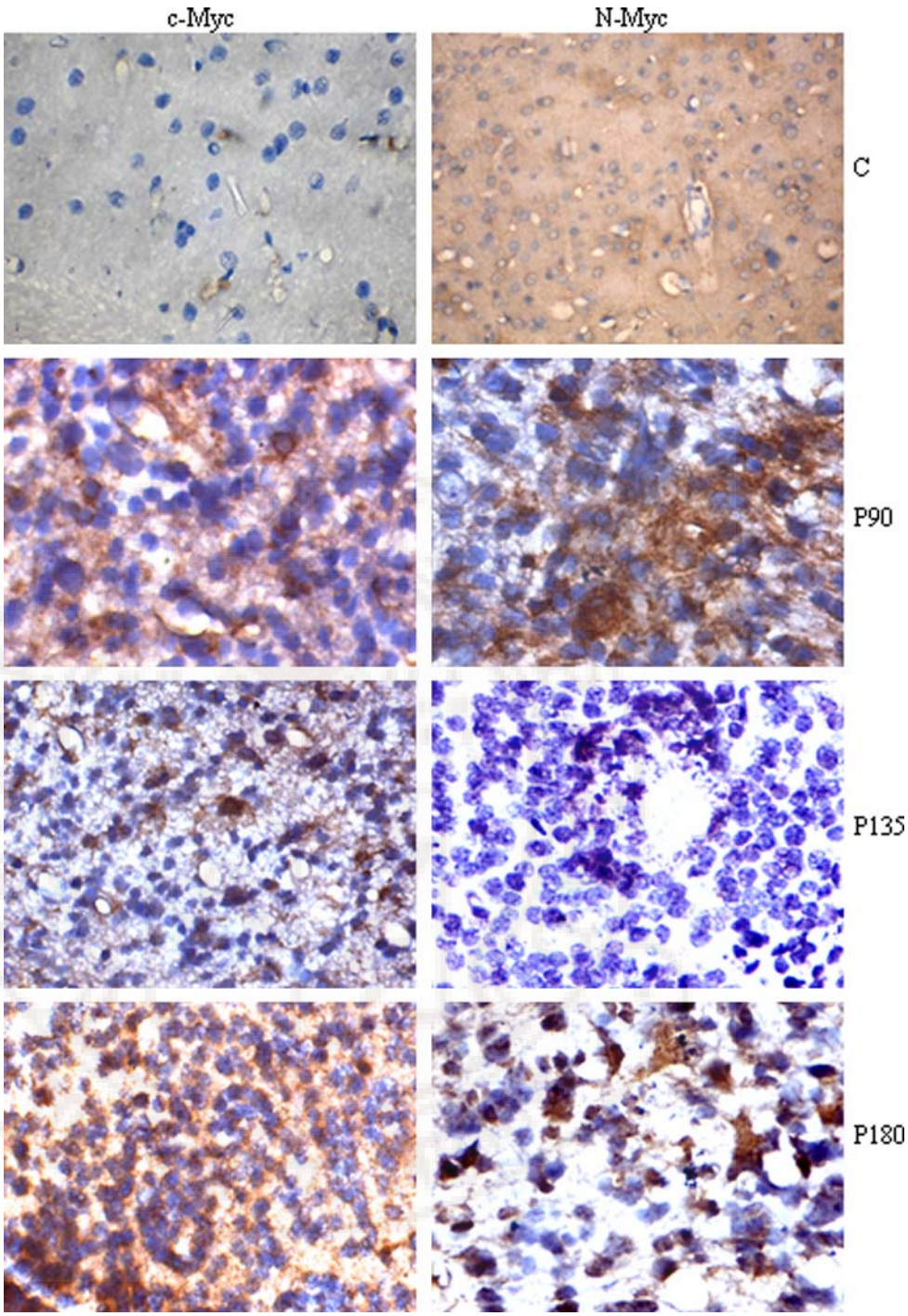

Fig. 6 Representative immunohistochemical staining for c-Myc and N-Myc in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited nuclear staining for c-Myc, N-Myc and cyclin D1. Original magnification 40×. ($n = 4$ for each age group)

of β-catenin was observed in sections of gliomas, which is the hallmark of active β-catenin/Tcf signaling. Another illustration of β-catenin/Tcf activation during glial carcinogenesis is the overexpression of β-catenin counterparts Tcf4 and Lef1. We observed the elevated protein levels of Tcf4 and Lef1 in gliomas and their levels were significantly higher in 6 months glioma rats. Immunohistochemical analysis has shown Tcf4 and Lef1 immunoreactivity in nuclei of tumor cells. Activated β-catenin and its transcriptional counter parts Lef1 and Tcf4 mediate Wnt pathway activity by enhancing the expression of their targets like, c-Myc, N-Myc and cyclin D1. We observed the protein levels of c-Myc, N-Myc, and cyclin D1 were upregulated in gliomas and increased from initiation stage (P90) to progression stage (P180) as well.

β-catenin levels are regulated by the ubiquitin-dependent proteolysis system and β-catenin ubiquitination is preceded by phosphorylation of its N-terminal region by

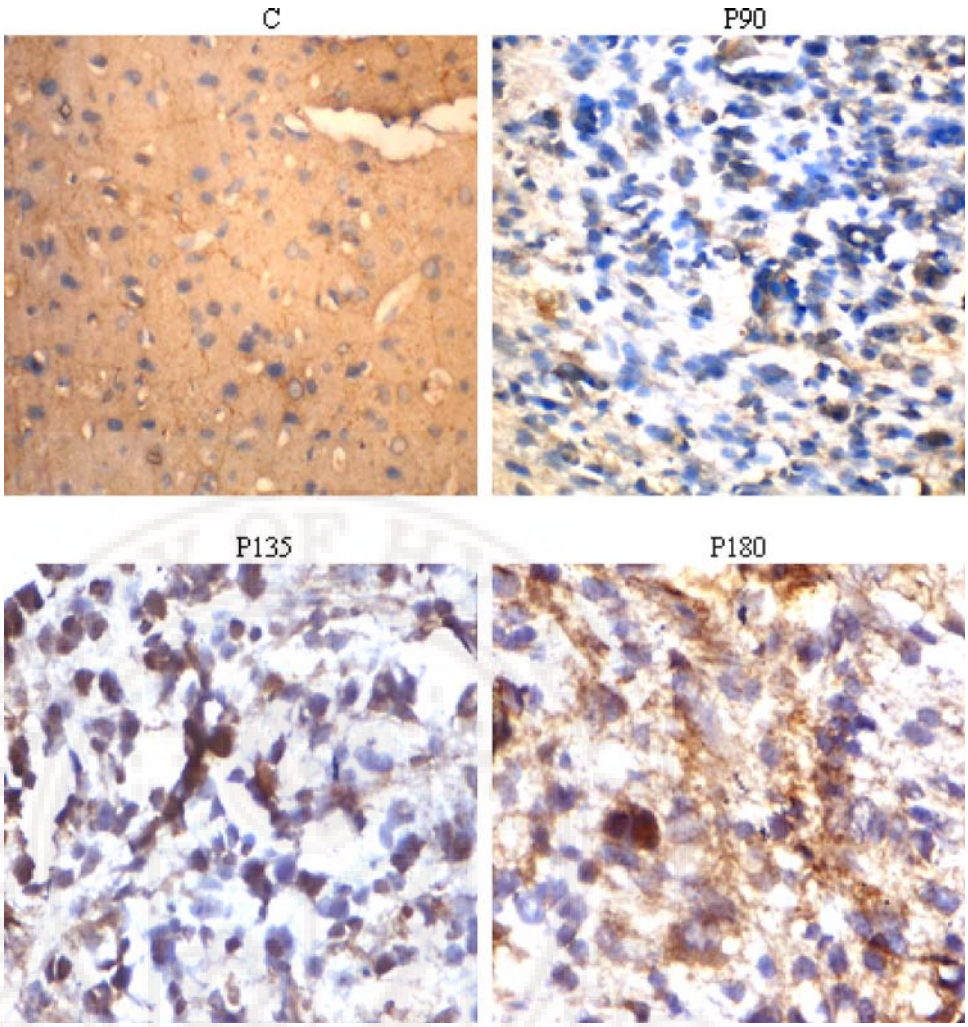

Fig. 7 Immunohistochemical analysis of cyclin D1 in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited nuclear staining for cyclin D1. Original magnification 40×. ($n = 4$ for each age group)

the GSK-3β/Axin/APC complex. In the absence of the Wnt signal, GSK-3β constitutively phosphorylates β-catenin, leading to low expression levels as a result of ubiquitin-mediated proteolysis of β-catenin. Mutations of the adenomatous polyposis coli (APC) tumor suppressor gene are the most common genetic events in colorectal cancers [47]. A recent study showed that β-catenin is a target for the ubiquitin–proteasome pathway and that phosphorylation of serine/threonine residues at positions 29, 33, 37, 41 and 45 by GSK-3β appears to be a prerequisite for ubiquitination [48]. Abnormal accumulation of β-catenin resulting from deregulation of the proteolytic machinery via phosphorylation/ubiquitination is the most likely cause of tumorigenesis of several cancers.

Gliomas develop and progress as a result of occurrence of genetic alterations in low grade gliomas, which further acquire additional mutations or genetic alterations when progress towards more malignant tumors. Secondary GBM develop through progression from low grade glioma or anaplastic glioma, while primary GBM develop de novo with no signs of precursor lesion. These genetic alterations disrupt the cell cycle arrest pathways, or activate various signal transduction pathways that are activated by receptor tyrosine kinases. The understanding of the signal transduction pathways associated with cell proliferation and cell death have great importance in finding new drug targets against glial tumors.

In the present study, we observed the upregulation of β-catenin/Tcf signaling in ENU-induced rat gliomas compared to controls. Further, the relative expression of β-catenin/Tcf signaling pathway components was significantly increased from initiation phase to progression phase and correlating with glioma malignancy. This clearly indicates that the Wnt/β-catenin/Tcf signaling pathway may play a significant role in gliomagenesis and its malignant progression to higher grades. Further studies require analyzing the Wnt pathway activity with respect to tumor behavior, and inhibition of this pathway at early stage may promise a reliable therapy for gliomas. Together, our findings suggest that Wnt/β-catenin/Tcf signaling is significantly activated in the development of glial tumors.

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