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

Gangadhara Reddy Sareddy a, Manas Panigrahi b, Sundaram Challa b, Anita Mahadevana, Phanithi Prakash Babua, *

a Department of Biotechnology and Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India
b Department of Neuro Surgery and Pathology, Nizam Institute of Medical Sciences, Hyderabad, India

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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.

* Corresponding author.
E-mail addresses: ppbul@uohyd.ernet.in, ppphanithi@yahoo.com (P.P. Babu).

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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 remain poor. WHO classified astrocytomas into four clinical grades on the basis of their histology and prognosis. Of these, GBM (glioblastoma multiforme; grade IV) is the most aggressive type. Patients with GBM have a mean survival of 1 year, whereas patients with anaplastic astrocytoma (grade III) have a survival of 2–3 years. Patients with low-grade astrocytoma (Diffuse astrocytoma; grade II) have a better 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 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; Reyna 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 adenomatous 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 histologically 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: II 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% CO2.

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 (radioimmunoprecipitation 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 phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors including 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na3VO4 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 onto 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 Genev 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 BCP-NBT solution (Genev 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 polyclonal 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 GMB 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 deparaffinized in xylene and passed

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Overexpression of DVL-3 in astrocytomas. (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).

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).

2.6. Immunofluorescence

As described in immunohistochemical analysis, after deparaffinization, 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 ground 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-MvLV 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; 80 °C for 1 min; and 80 °C for 1 min.

Fig. 1. Overexpression of DVL-3 in astrocytomas. (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).

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).

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-catenin were used as loading control. (IIp: diffuse astrocytoma peritumor tissue, IIc: diffuse astrocytoma core tumor tissue, IIIp: anaplastic astrocytoma peritumor tissue, IIIc: anaplastic astrocytoma core tumor tissue, IVp: 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 \( \beta \)-catenin levels correlate with histological malignancy

The mRNA expression profile of \( \beta \)-catenin was quantified by using RT-PCR in tumors of different grades. The relative mRNA levels of \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-catenin in grade II (28 ± 1.7, 38.9 ± 1.15, respectively) showed no significant difference with controls.

In addition, we also analyzed the \( \beta \)-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 \( \beta \)-catenin (Fig. 3C). 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 (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. 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 anti-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.](image)

![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.](image)
malignancy (Fig. 10B, C, D and E). Immunostaining of c-Myc, N-Myc 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-25.35, grade III-7.4; control-21.5) and cyclin D1 (grade II-5.3; grade III-6.2; grade IV-6.2; control-5.3) 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 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 childhood 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
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. 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 positive 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.
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 (IIp: diffuse astrocytoma peritumor tissue, IIIp: diffuse astrocytoma core tumor tissue, IIp: anaplastic astrocytoma peritumor tissue, IIIp: anaplastic astrocytoma core tumor tissue, IVp: 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).
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. 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.
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 as 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.

**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.

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**References**


Hong, Y., documentation.


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

Gangadhara Reddy Sareddy · Sundaram Challa · Manas Panigrahi · Phanithi Prakash Babu

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

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, p16INK4A 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 p16INK4A, 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 safely assume that pathological changes are not observed before 90 days of age because ENU itself is rapidly cleared with short half life, which one can safely assume that pathological changes 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 intraperitoneally 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 (radioimmunoprecipitaion 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 14,000 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). Immunoreactivity 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 polyclonal 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% H2O2 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. 2  a Over-expression of \( \beta \)-catenin in ENU-induced gliomas. Western blotting analysis demonstrating \( \beta \)-catenin overexpression in glioma rats, in comparison to saline-treated controls. The protein levels were progressively increased from P90–P180. \( \beta \)-Actin used as a loading control. b Quantitative comparison of \( \beta \)-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 \( \beta \)-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 \( \beta \)-catenin in control (C), P90, P135 and P180 glioma rats. The tumor cells exhibited cytoplasmic and nuclear staining for \( \beta \)-catenin. Original magnification 40×. \((n = 4 \) for each age group)
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 $\beta$-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 $\beta$-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 $\beta$-catenin, immunohistochemical staining was performed. Representative results showed that $\beta$-catenin accumulation in P90, P135 and P180 glioma rats and immunoreactivity observed in cytoplasm and nuclei of tumor cells (Fig. 2c).

Cytoplasmically accumulated $\beta$-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 $\beta$-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/$\beta$-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/$\beta$-catenin pathway components.

$\text{N}$-ethyl-$\text{N}$-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
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 oligidendrogial foci, which

![Image of immunohistochemical analysis](image-url)
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...
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 [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.

![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)](image)
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