Expression of delta-catenin is associated with progression of human astrocytoma

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Abstract

Background: δ-Catenin (CTNND2), which encodes a scaffold protein in humans, has been found in a few malignancies. However, the expression pattern and contribution of δ-catenin to astrocytoma progression are unclear.

Methods: We investigated δ-catenin expression in human astrocytoma samples and its function in astrocytoma cell lines using immunohistochemistry, siRNA knockdown, transfection, MTT, transwell migration and Rac1 pulldown techniques.

Results: δ-Catenin protein expression was detected in cytoplasm of astrocytoma cells by immunohistochemistry. Analysis showed that grade I astrocytoma (0%, 0/11) and glial cells from normal brain tissue exhibited negative staining. δ-Catenin expression was significantly higher in grade III-IV (35%, 29/84) compared to grade II astrocytoma cells (18%, 11/61); *p < 0.01). In addition, CTNND2 overexpression promoted proliferation, invasion and Rac1 activity of U251 astrocytoma cells. Treatment of δ-catenin-transfected cells with a Rac1 inhibitor decreased Rac1 activity and invasion. δ-Catenin knockdown in U87 glioblastoma cell decreased cell proliferation, invasion and Rac1 activity.

Conclusion: The results suggest that δ-catenin expression is associated with the malignant progression of astrocytoma and promotes astrocytoma cell invasion through upregulation of Rac1 activity. δ-Catenin expression levels may serve as a useful marker of the biological behavior of astrocytoma cells.

Background

Astrocytoma arises from neural stem or progenitor cells in the central nervous system. It is the most common primary brain tumor and accounts for approximately 60% of all brain tumors. Despite combined treatment strategies, which include surgery, radiotherapy and chemotherapy, the prognosis for high-grade astrocytoma, especially glioblastoma multiforme, has changed little over the past 10 years, with a median survival of only approximately 1 year [1]. The clinical symptoms and prognosis are closely correlated with tumor location and size and histological grade. Although the histological grade partly reflects the malignant features of astrocytoma, it cannot give an indication of the exact mechanism of tumor invasion and recurrence [2,3]. Thus, it is important to understand the molecular mechanism of astrocytoma cell invasion and identify effective markers in tumorigenesis and progression.

The expression of δ-catenin was initially found to be limited to brain neurons and it can bind to presenilin 1, which is involved in the progression of Alzheimer’s disease [4-6]. δ-Catenin was also implicated in the maintenance of synaptic function and transmission of downstream signals as a scaffold protein [7-11]. It was reported that δ-catenin overexpression changed the morphology of MDCK cells, including the elaboration of lamellipodia [12]. Furthermore, δ-catenin can regulate the activity of small GTPases and therefore induces dendritic protrusions from cells [13]. Small GTPases are critical mediators of cytoskeletal reorganization, signal transduction and gene expression pathways [14,15]. As one of these GTPases, Rac1 is a pleiotropic regulator of many cellular processes, including the cell cycle, cell-cell adhesion, motility (through the actin network) and epithelial differentiation. δ-Catenin also affects invasion and metastasis of lung cancer cells via regulation of small GTPase activity [16]. These studies suggest that δ-catenin plays an important role in many human cancers. Our previous study showed that δ-catenin was overexpressed in non-small-cell lung cancer and...
enhanced cancer cell invasion through small GTPase regulation. However, the expression pattern of δ-catenin in glial cells and human astrocytoma cells is not clear; the relationship between its expression and clinicopathological factors and biological behavior still needs to be addressed.

In the present study, we examined the expression of δ-catenin in 156 astrocytoma tissue specimens and analyzed the correlation between its expression and clinicopathological factors. We also investigated the effect of δ-catenin on Rac1 activity and the biological behavior of astrocytoma cell lines.

Methods

Patients and specimens

The study protocol was approved by the institutional review board of China Medical University. Primary tumor specimens and adjacent normal brain tissue were obtained from 156 patients diagnosed with astrocytoma who underwent resection in the First Affiliated Hospital of China Medical University between 2005 and 2008. None of the patients had received radiotherapy or chemotherapy before surgical resection. There were 95 male (60.9%) and 61 female (39.1%) patients with a median age of 43 years (range 15-67 years). The histological diagnosis and differentiation grade were evaluated for sections stained with hematoxylin and eosin according to the World Health Organization (WHO) classification guidelines. All 156 specimens were re-evaluated with respect to histological subtype and tumor grade. Tumors were graded and classified into grade I (11), grade II (61), grade III (51), and grade IV (33) according to WHO guidelines (2007).

Cell culture and transfection

U251 and U87 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma). Cells were grown on sterilized culture dishes and were passaged every 2 days with 0.25% trypsin (Invitrogen). The pCMV5-FLAG/δ-catenin plasmid was a kind gift from Dr. Shun-ichi Nakamura at Kobe University and was transfected into cells using Attractene Transfection (Qiagen, Hamburg, Germany). A pCMV5 empty vector was used as a negative control. DharmaFECT1 reagent was used for siRNA transfection (Qiagen, Chicago, IL, USA) according to the manufacturer’s instructions. The protein level was assessed 48 h later by western blotting. The δ-catenin siRNA sequences were 5'-CUA CGU UGA CUU CUA CUC AUU-3' and 5'-UGA GUA GAA GUC AAC GUA GUU-3' and were transfected into U87 cell lines, which express relatively high levels of δ-catenin. Nonsilencing siRNA sequences were used as a negative control (5'-UUC UCC GAA CUU GUC ACA UUU-3' and 5'-AUG UGA CAA GUU CGG AGA AUU-3').

Immunohistochemistry

Surgically excised tumor specimens were fixed with 10% neutral formalin and embedded in paraffin, and 4-μm-thick sections were prepared. Normal brain neurons present in the tumor slides were used as positive control. Immunostaining was performed using the avidin-biotin-peroxidase complex method (Ultrasonesitive™, MaiXin, Fuzhou, China). The sections were deparaffinized in xylene, rehydrated with graded alcohol, and then boiled in 0.01 M citrate buffer (pH 6.0) for 2 min in an autoclave. Hydrogen peroxide (0.3%) was applied to block endogenous peroxidase activity and the sections were incubated with normal goat serum to reduce nonspecific binding. Tissue sections were incubated with δ-catenin mouse monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse immunoglobulin (at the same concentration as for the antigen-specific antibody) was used as a negative control. Staining for both antibodies was performed at room temperature for 2 h. Biotinylated goat antimouse serum IgG was used as a secondary antibody. After washing, the sections were incubated with streptavidin-biotin conjugated with horseradish peroxidase, and the peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride. Counterstaining with hematoxylin was performed and the sections were dehydrated in ethanol before mounting.

Two independent blinded investigators examined all tumor slides randomly. Five views were examined per slide, and 100 cells were observed per view at 400× magnification. Immunostaining of δ-catenin was scored on a semiquantitative scale by evaluating in representative tumor areas the intensity and percentage of cells showing significantly higher immunostaining than control cells in normal brain tissues. Cytoplasmic immunostaining in tumor cells was considered positive staining. We counted 400 tumor cells and calculated the percentage of positively stained cells. The proportion of cells exhibiting δ-catenin expression was categorized as follows: 0, absent; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, ≥ 76%. The staining intensity was categorized as follows: 0, negative; 1, moderate; and 2, strong. The proportion and intensity scores were then multiplied to obtain a total score. To obtain final statistical results, a score < 2 was considered negative and scores ≥ 2 were considered positive.

Western blot analysis

Total proteins from tissue and cells were extracted in lysis buffer (Pierce, Rockford, IL) and quantified using the Bradford method. Samples of 50 μg of protein were separated by SDS-PAGE. Samples were transferred to
polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and incubated overnight at 4°C with antibody against δ-catenin (1:1000; Santa Cruz) and a mouse monoclonal antibody against β-actin (1:500; Santa Cruz). After incubation with peroxidase-coupled antimouse IgG (Santa Cruz) at 37°C for 2 h, bound proteins were visualized using ECL (Pierce) and detected using a BioImaging System (UVP Inc., Upland, CA, USA). Relative protein levels were quantified using β-actin as a loading control.

**Figure 1** Expression of δ-catenin in resected astrocytoma tissue. (A) HE staining of normal brain tissue. (B) Immunohistochemical staining of δ-catenin protein in normal brain tissue was negative in glial cells and positive in neurons. (C) HE staining of pilocytic astrocytoma (grade I). (D) Negative δ-catenin staining in pilocytic astrocytoma (grade I). (E) HE staining of grade II astrocytoma. (F) Positive δ-catenin staining (weak staining) in the cytoplasm of grade II glioma. (G) HE staining of grade III astrocytoma. (H) Strong cytoplasmic δ-catenin staining in grade III astrocytoma. (I) HE staining of glioblastoma (grade IV). (J) Strong cytoplasmic δ-catenin staining in glioblastoma (grade IV).
Colony formation and MTT assays

U251 and U87 cells were transfected with siRNA or a δ-catenin plasmid for 48 h and then plated into three 6-cm cell culture dishes (1000 per dish) and incubated for 12 days. Plates were washed with PBS and stained with Giemsa. The number of colonies with more than 50 cells was counted. The colonies were manually counted using a microscope.

Cells were plated in 96-well plates in medium containing 10% FBS at approximately 3000 cells per well 24 h after transfection. For quantitation of cell viability, cultures were stained after 4 days in MTT assays. In brief, 20 μl of 5 mg/ml MTT (thiazolyl blue) solution was added to each well and incubated for 4 h at 37°C. The medium was removed from each well and the resulting MTT formazan was solubilized in 150 μl of DMSO. Each solution was measured spectrophotometrically at 490 nm.

Matrigel invasion assay

Cell invasion assays were performed in 24-well Transwell chambers with a pore size of 8 μm and the inserts were coated with 20 μl of Matrigel (1:3 dilution, BD Bioscience, San Diego, CA, USA). At 48 h after transfection, cells were trypsinized and transferred to the upper Matrigel chamber in 100 μl of serum-free medium containing 3 × 10^5 cells and incubated for 16 h. Medium supplemented with 10% FBS was added to the lower chamber as the chemoattractant. Then non-invading cells on the upper membrane surface were removed with a cotton tip and cells that had passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. The number of invaded cells were counted in 10 randomly selected high-power fields under a microscope. The experiments were performed in triplicate.

Rac1 pulldown assay

Rac1 activity was measured using a Active Rac1 Pull-Down and Detection Kit (Pierce). The Rac1 inhibitor NSC23766 was from Calbiochem (Merck, Whitehouse Station, NJ, USA). Cells were treated for 6 h at a concentration of 50 μM.

Statistical analysis

SPSS version 11.5 for Windows was used for all statistical analyses. A χ² test was used to examine possible correlations between δ-catenin expression and clinicopathologic factors. Student’s t-test was used to compare densitometry data on focus numbers between control and δ-catenin-transfected cells. All p values are based on a two-sided statistical analysis, and p < 0.05 was considered to indicate statistical significance.

Table 1 The relationship between δ-catenin and clinicopathological features

| Clinical parameters | Number | δ-catenin | p value |
|---------------------|--------|-----------|---------|
|                     |        | Negative  | Positive|
| Age                 |        |           |         |
| < 43                | 78     | 61        | 17      | 0.359  |
| ≥ 43                | 78     | 55        | 23      |
| Gender              |        |           |         |
| Male                | 95     | 69        | 26      | 0.578  |
| Female              | 61     | 47        | 14      |
| Grading             |        |           |         |
| Grade I             | 11     | 11        | 0       | 0.009* |
| Grade II            | 61     | 51        | 11      |
| High Grade (II-IV)  | 84     | 55        | 29      |

* p < 0.05

Table 2 Association of δ-catenin expression with tumor location

| Sites       | Total | δ-catenin |
|-------------|-------|-----------|
|             |       | Negative  | Positive|
| Supratentorial |      |           |         |
| Hemisphere  | 124   | 87(70.2%) | 37(29.8%)|
| Diencephalon | 2     | 2(100%)   | 0(0%)   |
| Total       | 126   | 89(70.7)  | 37(29.3%)|
| Infratentorial |     |           |         |
| Cerebellum  | 17    | 13(76.5%) | 4(23.5%)|
| Brain stem  | 5     | 5(100%)   | 0(0%)   |
| Spinal cord | 8     | 8(100%)   | 0(0%)   |
| Total       | 30    | 26(86.7%) | 4(13.3%)*|

* Supratentorial versus Infratentorial, p < 0.05

Results

Expression and localization of δ-catenin in normal brain tissue and astrocytoma cells

In cells from normal brain tissue, only neurons showed strong cytoplasmic (include axon) expression of δ-catenin, whereas glial cells showed no expression (Figure 1a, b). Cytoplasmic staining was present in a subset of astrocytoma cases, while no staining was detected in sections from the same samples subjected to immunohistochemical analysis using mouse immunoglobulin. Of the 156 patients in the study, 40 (25.6%) showed positive δ-catenin expression. δ-Catenin expression predominantly occurs in the cytoplasmic compartment of tumor cells. We analyzed the correlation between δ-catenin protein expression and the histological staging of astrocytoma. As shown in Table 1 Grade I astrocytoma (0%, 0/11) and normal glial cells exhibited negative staining (Figure 1c, d). In grade III-IV astrocytoma (35%, 29/84; Figure 1g-j), δ-catenin expression was significantly higher than in grade II astrocytoma.
(18%, 11/61; Figure 1e, f, p < 0.01), which suggests that δ-catenin expression is associated with astrocytoma progression.

We examined the difference between the expression pattern of δ-catenin according to tumor location. As shown in Table 2 the positive rate of δ-catenin in supratentorial astrocytomas was 29.3%, much higher than the rate in infratentorial astrocytomas (13.3%). (p < 0.05).

**δ-Catenin overexpression promotes astrocytoma cell proliferation and invasion via Rac1**

To determine whether δ-catenin enhances the proliferation and invasion of astrocytoma cells, we carried out δ-catenin transfection in the U251 astrocytoma cell line. siRNA knockdown was performed in the U87 glioblastoma cell line.

As shown in Figure 2, δ-catenin transfection considerably increased protein levels at 48 h after treatment, whereas δ-catenin siRNA knockdown decreased δ-catenin protein in U87 cells. The proliferation rate was determined by MTT assay. A significant increase in proliferation was observed in U251 cells overexpressing δ-catenin compared to cells transfected with the empty vector (Figure 3a). Consistent with the MTT results, colony formation assays showed that transfection of δ-catenin in U251 cells led to an increase in focus numbers (empty vector 109 ± 23 vs δ-catenin 174 ± 26, p < 0.05; Figure 3b). δ-Catenin also promoted tumor cell invasion in a Matrigel assay (empty vector 55 ± 10 vs δ-catenin 73 ± 15, p < 0.05; Figure 4a). In U251 cells overexpressing δ-catenin, immunoblotting showed total Rac1 protein levels were unchanged, but a pulldown assay revealed that Rac1 activity increased significantly (Figure 4b). Conversely, δ-catenin knockdown decreased the proliferation, colony formation ability (control 158 ± 21 vs siRNA δ-catenin 97 ± 17, p < 0.05; Figure 5) and invasiveness of U87 cells (control 59 ± 7 vs siRNA δ-catenin 36 ± 5, p < 0.05). δ-Catenin knockdown also decreased Rac1 activity (Figure 6).
To explore whether the effect of δ-catenin on cell invasion was dependent on Rac1 activity, we measured cell invasion and Rac1 activity after treatment of δ-catenin-transfected cells with the Rac1 inhibitor NSC23766. The inhibitor abolished the effect of δ-catenin on cell invasion (δ-catenin 73 ± 15 vs δ-catenin + Rac1 inhibitor 42 ± 9, \( p < 0.05 \)).

**Discussion**

δ-catenin plays an important role in dendritic morphogenesis, which is associated with changes in the activity of small GTPases [7,13]. The formation and number of dendritic protrusions are indicative of the malignant phenotype of cells, so it is possible that δ-catenin promotes a malignant phenotype in tumor cells. It has been reported that δ-catenin expression was increased in over 80% (55/65) of prostatic adenocarcinomas, and was associated with higher Gleason scores [4]. Some researchers also observed gene amplification of δ-catenin in cervical and bladder carcinoma. δ-catenin mRNA is probably present in esophagus, ovary and breast tumors [17,18]. Our previous study showed that δ-catenin was overexpressed in non-small-cell lung cancer and was correlated with clinicopathological factors [16]. However, the expression pattern and clinical significance of δ-catenin are poorly defined in other human cancers such as astrocytoma.
Analysis of mRNA in primary cultures of neurons and glial cells prepared from fetal rat brain showed that δ-catenin expression levels were higher in neurons than in glial cells [19]. However, its protein expression in neurons and glial cells has not been investigated. In this study, we found strong cytoplasmic δ-catenin expression in neurons but negative staining in human glial cells such as astrocytes, which may due to the low mRNA copy numbers of δ-catenin. Although there was no expression of δ-catenin in 11 grade I astrocytoma cases, positive δ-catenin was found in 18% of grade II and 35% of grade III-IV astrocytoma cases, which suggests that δ-catenin is positively associated with higher grade astrocytoma. Positive rate of δ-catenin in low grade astrocytoma is very low, especially in grade I astrocytoma (Negative expression), and its utilization in the distinguishing grade I astrocytoma from reactive gliosis was limited. The low grade astrocytoma with positive δ-catenin expression may transform into high grade astrocytoma. These findings indicate that δ-catenin might play an important role in astrocytoma progression and could serve as a potential biomarker for the biological behavior of astrocytoma cells.

To explore the relationship between δ-catenin and the biological behavior of astrocytoma cells, we transfected a δ-catenin plasmid into the U251 cell line, which has low δ-catenin expression, and knocked down δ-catenin in the U87MG cell line, which has high δ-catenin expression. We found that δ-catenin overexpression caused a significant increase in the proliferation rate, colony formation and

![Figure 4 δ-Catenin knockdown inhibits proliferation in U87 cells](image-url)
invasion ability of U251 cell lines, with a significant increase in Rac1 activity, which was in accordance with our previous results for lung cancer [16]. On the contrary, δ-catenin knockdown decreased the proliferation rate, colony formation ability and invasiveness of U87 cells, with a decrease in Rac1 activity. We then observed a decrease in invasiveness and Rac1 activity after treatment of δ-catenin-transfected cells with a Rac1 inhibitor, which demonstrates that δ-catenin regulates cell invasion in a Rac1-dependent manner. These results suggest that δ-catenin might enhance the invasive ability of astrocytoma cells via Rac1. However, the exact mechanism of Rac1 regulation by δ-catenin is not clear. In addition, previous studies proved that δ-catenin has an influence in other small GTPases, such as Cdc42 and RhoA, which was not investigated in glioma cell lines [13]. Further studies are required to address these issues.

**Conclusion**

In conclusion, overexpression of δ-catenin in astrocytoma cells was correlated with higher grade and δ-catenin was identified as an important oncoprotein in maintaining the malignant behavior of glioma cells. Overexpression of δ-catenin activated Rac1 and promoted astrocytoma cell proliferation and invasion. Thus, δ-catenin is a candidate target protein for future astrocytoma therapeutics.
Figure 6 δ-Catenin transfection and siRNA knockdown affect Rac1 activity. Overexpression of the δ-catenin gene promoted Rac1 activity in U251 astrocytoma cells. siRNA treatment and a Rac1 inhibitor decreased this activity.

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Authors’ contributions
WM and ZD carried out the immunohistochemistry, cell biological analysis and immunoblot assay. DQ participated in the design of the study and drafted the manuscript. WY participated in its design. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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