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**Original Article**

# Downregulation of WIF-1 by hypermethylation in astrocytomas

Zhuanyi Yang[1†], Ying Wang[2†], Jiasheng Fang[1*], Fenghua Chen[1], Jingfang Liu[1], Jun Wu[1], Yanjin Wang[1], Tao Song[1], Feiyue Zeng[1], and Yihua Rao[1]

[1]Department of Neurosurgery, Xiangya Hospital of Central South University, Changsha 410078, China
[2]Department of Pathology, Basic Medical School of Central South University, Changsha 410078, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-731-84327039; Fax: +86-731-84327436; E-mail: fangjiasheng@yahoo.cn

**Wnt inhibitory factor-1 (WIF-1) acts as a Wnt antagonist and tumor suppressor, but hypermethylation of *WIF-1* gene promoter and low expression of *WIF-1* activate Wnt signaling aberrantly and induce the development of several human tumors. By using RT-PCR, immunohistochemistry and methylation-specific PCR, we analyzed the expression and methylation of WIF-1 in 4 normal brain tissues, 35 freshly resected astrocytoma tissues and 4 glioblastoma-derived cell lines. Significant downregulation of WIF-1 mRNA and protein expression levels was observed in astrocytoma tissues compared with normal brain tissues. Significant association between WIF-1 downregulation and pathological grade of astrocytomas was found. *WIF-1* gene aberrant methylation was observed in 19 of 35 (54.29%) tumor samples. The promoter methylation tumors showed low WIF-1 protein and mRNA expression, whereas the promoter unmethylation tumors displayed high protein and mRNA expression levels. Moreover, complete absence of *WIF-1* mRNA expression was observed in four cell lines, whereas treatment with demethylating agent, 5-aza-2′-deoxycytidine, restored WIF-1 expression. Our results suggested that the *WIF-1* gene is frequently silenced in astrocytoma by aberrant promoter methylation. This may be an important mechanism in astrocytoma carcinogenesis.**

*Keywords* astrocytomas; Wnt inhibitory factor-1 (WIF-1); promoter methylation

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## Introduction

Astrocytomas are the most common primary tumors of the central nervous system. Despite recent advances in diagnosis and therapies such as surgery, radiation, and chemotherapy, the prognosis of high-grade astrocytomas [World Health Organization (WHO) grades III–IV] remains poor and survival time is short. The median survival is only 12–15 months for patients with glioblastomas (WHO grade IV) and 2–5 years for patients with anaplastic astrocytomas (WHO grade III) [1].

The Wnt/β-catenin signaling pathway plays a significant role in various processes of early development and the pathogenesis of human diseases, including human malignancies. Recently, there are several reports, which showed the involvement of Wnt/β-catenin signaling in astrocytoma progression [2–5]. Wnt inhibitory factor-1 (WIF-1) is identified as one of the secreted antagonist that can directly bind Wnt proteins to inhibit Wnt/β-catenin signaling [6]. Downregulation and promoter hypermethylation of *WIF-1* gene have been reported in human hepatocellular, nasopharyngeal, lung, bladder, and gastrointestinal malignancies [7–10]. Yet little is known about the expression and promoter methylation of WIF-1 in astrocytomas.

In this study, we described for the first time that the expression of *WIF-1* was frequently downregulated by its promoter hypermethylation in astrocytomas compared with normal tissue samples, which might contribute to the upregulation of Wnt/β-catenin signaling in astrocytoma carcinogenesis.

## Materials and Methods

### Tissue samples and cell lines

Thirty-five fresh astrocytoma samples (T1–T35) were collected after informed consent from patients who underwent brain operations for astrocytoma at Xiangya Hospital (Changsha, China). Immediately after surgical resection, portions of the tumors were frozen and stored at −80°C for RNA and DNA extraction, and the remainings were fixed in 10% formalin. Tumors were graded and classified according to the WHO (2007) [11], including grade I(1), grade II(14), grade III(6), and grade IV(14). In all cases of astrocytoma, there were 21 (60%) male and 14 (40%) female with the median age of 43 years (5 − 66 years).

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For comparison, four normal human tissues (N1–N4) from patients with contusion and laceration of brain were obtained at the time of decompressive operation (**Table 1**).

Human glioblastoma-derived cell lines U251, SF126, SF767, and T98G were obtained from Xiangya Cancer Research Institute of Central South University (Changsha, China). Cells were maintained in DMEM (Hyclone, Beijing, China) supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured at 37°C in 5% $CO_2$.

**Table 1 Patient's clinical data and results of this study**

| Sample | Sex | Age | WHO grade | IHC scores | mRNA | Methylation status |
|---|---|---|---|---|---|---|
| N1 | F | 60 | 0 | 7 | 0.927 | U |
| N2 | F | 56 | 0 | 7 | 0.907 | U |
| N3 | M | 28 | 0 | 7 | 0.862 | U |
| N4 | M | 56 | 0 | 8 | 0.976 | U |
| T1 | M | 43 | II | 2 | 0.107 | M |
| T2 | F | 50 | III | 0 | 0 | M |
| T3 | F | 38 | II | 5 | 0.653 | U |
| T4 | M | 34 | III | 0 | 0 | M |
| T5 | F | 57 | II | 2 | 0.658 | U |
| T6 | M | 61 | III | 5 | 0.773 | U |
| T7 | M | 54 | IV | 5 | 0.602 | U |
| T8 | M | 66 | IV | 1 | 0 | M |
| T9 | M | 27 | II | 5 | 0.716 | U |
| T10 | M | 55 | III | 1 | 0.134 | M |
| T11 | M | 41 | III | 1 | 0.153 | M |
| T12 | F | 53 | II | 5 | 0.398 | U |
| T13 | F | 45 | IV | 1 | 0.115 | M |
| T14 | M | 29 | IV | 5 | 0.347 | U |
| T15 | F | 40 | II | 2 | 0.151 | M |
| T16 | M | 37 | II | 5 | 0.462 | U |
| T17 | F | 14 | I | 7 | 0.809 | U |
| T18 | F | 55 | IV | 1 | 0.147 | M |
| T19 | M | 58 | IV | 2 | 0.189 | M |
| T20 | F | 39 | III | 6 | 0.897 | U |
| T21 | M | 40 | IV | 0 | 0 | M |
| T22 | M | 43 | IV | 2 | 0.153 | M |
| T23 | F | 39 | IV | 1 | 0.129 | M |
| T24 | F | 33 | II | 5 | 0.704 | U |
| T25 | M | 5 | II | 7 | 0.907 | U |
| T26 | M | 45 | II | 5 | 0.722 | U |
| T27 | M | 66 | II | 5 | 0.478 | U |
| T28 | F | 46 | II | 5 | 0.447 | U |
| T29 | F | 38 | IV | 0 | 0 | M |
| T30 | M | 30 | II | 3 | 0.215 | M |
| T31 | M | 58 | IV | 1 | 0.182 | M |
| T32 | M | 50 | IV | 3 | 0.122 | M |
| T33 | F | 27 | IV | 1 | 0.131 | M |
| T34 | M | 51 | IV | 1 | 0 | M |
| T35 | M | 43 | II | 3 | 0.769 | U |

N, normal brain tissue; T, astrocytoma tissue; M, male; F, female; IHC, immunohistochemistry; U, unmethylation; M, methylation.

### Immunohistochemistry of tissue samples

WIF-1 protein expression was determined by using immunohistochemical staining (IHC) on formalin-fixed paraffin-embedded tissues. Briefly, 5-μm thick sections were deparaffinized, rehydrated using xylene and alcohol, incubated with 0.3% $H_2O_2$ to block endogenous peroxidase activity, and incubated with normal goat serum to block nonspecific antibody binding. Before immunostaining, antigen retrieval was done by immersing sections in 10 mM citrate buffer (pH 6.0) and boiling in a pressure cooker for 2 min. The sections were incubated with 8 μg/ml monoclonal antibody against human WIF-1 protein (R&D, Minneapolis, USA) at 4°C overnight, and then incubated with biotinylated goat anti-mouse IgG antibody (Zymed, San Francisco, USA) for 30 min. The antigen–antibody complexes were visualized using streptavidin-horseradish peroxidase conjugate (Zymed) and diaminobenzidine as a chromogen.

The slides were counterstained with hematoxylin. For WIF-1 protein expression, nuclear staining was considered to be negative, whereas cytoplasmic and membranous expression was analyzed according to the intensity and proportion of positive cells to all cells [9]. IPP6.0 (Media Cybernetics, Bethesda, USA) was applied to semi-quantify immunohistochemical results. Staining was scored for intensity [0 (negative), 1+ (weak), and 2+ (strong)] and percentage of positive staining in malignant cells [0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%)]. The multiplication of intensity and percentage counts were used as the final immunohistochemistry scores [12]. For heterogeneous staining patterns, each component was scored independently and the results were summed. For example, a specimen containing 25% tumor cells with strong intensity ($1 \times 2 + =2$), 25% tumor cells with weak intensity ($1 \times 1 + =1$), and 50% tumor cells without immunoreactivity received a score of $2 + 1 + 0 = 3$. Cytoplasmic and membranous staining in normal brain tissue served as internal positive controls. Negative controls were included in the IHC analyses by omitting the primary antibody.

### RNA extraction and semi-quantitative RT-PCR

Total RNA from tissues and cell lines were isolated using a Trizol procedure (Invitrogen, Carlsbad, USA). An equal amount of RNA from each sample was added to 25 μl of reaction mixture and cDNA was synthesized by First strand cDNA synthesis kit (Fermentas, Burlington, Canada).

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Primers for semi-quantitative RT-PCR were obtained from TaKaRa (Dalian, China). Primer sequences for the human *WIF-1* cDNA were 5′-CCGAAATGGAGGCTTTTGTA-3′ (forward) and 5′-TGGTTGAGCAGTTTGCTTTG-3′ (reverse) [8]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The cycle was defined at 95°C for 5 min, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 40 s. This was followed by the final extension at 72°C for 10 min. The PCR products were electrophoresed in 2% agarose gels. Relative *WIF-1* mRNA levels were evaluated by UVP software (UVP, Upland, USA) and expressed as the fold difference relative to *GAPDH* mRNA levels.

### Genomic DNA extraction and methylation-specific PCR

DNA was extracted from astrocytoma tissues and cell lines by standard proteinase K digestion, phenol chloroform and ethanol precipitation proceeded. Bisulfite modification of genomic DNA was carried out by using an EZ DNA methylation kit (Zymo Research Co, Orange, USA) according to the manufacturer's protocol. *WIF-1* promoter region has been identified and described previously [13]. Bisulfite-treated genomic DNA was amplified using either a methylation-specific (MS) or an unmethylation-specific primer set. GC Rich DNA polymerase (Qiagen, Hilden, Germany) was used in the experiments. Sequences of the MS primers were 5′-GGGCGTTTTATTGGGCGTAT-3′ (forward) and 5′-AAACCAACAATCAACGAAC-3′ (reverse). Sequences of the unmethylation-specific primers were 5′-GGGTGTTTTATTGGGTGTAT-3′ (forward) and 5′-AAACCAACAATCAACAAAAC-3′ (reverse) corresponding to the *WIF-1* promoter region sequences −488 to −468 and −310 to −290, respectively. The PCR was carried out in a Techne TC-412 Thermal Cycler (Keison, Essex, UK) under the following conditions: one cycle of 95°C for 10 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 50 s and extension at 72°C for 50 s. This was followed by the final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 2% agarose gel and samples were evaluated. Normal human lymphocyte DNA was either treated directly with sodium bisulfite or after *in vitro* methylation by SssI methyltransferase (New England Biolabs, Ipswich, USA) to serve as unmethylated or methylated control, respectively.

### *In vitro* treatment of cultured cells with 5-aza-CdR

Cultured cell lines were treated with demethylating agent 5-aza-CdR [5-aza-2′-deoxycytidine (ADC); 10 μM] for 3 days. Fresh drug was added every 24 h. The genomic DNA and total RNA were extracted from the cells before and after 5-aza-CdR treatment and were used for MS-PCR and RT-PCR.

### Immunofluorescence of cultured cells

Cultured cell lines were dissociated into single cell suspension and plated on coverslips with or without 20 μM ADC treatment. Medium was renewed daily. After 3 days, the cells were fixed with 4% paraformaldehyde solution in PBS at room temperature for 30 min, permeabilized with 0.3% Triton X-100 in PBS at room temperature for 5 min, and incubated in 5% bovine serum albumin (BSA)-PBS for 1 h. Cells were incubated at 4°C overnight with 8 μg/ml monoclonal antibody against human WIF-1 protein in 1% BSA-PBS. After washing with PBS, cells were incubated with 1:100 FITC-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, Santa Cruz, USA) diluted in PBS containing 1% BSA. Cells were stained with 4′,6-diamidino-2-phenylindole (300 nM in PBS) to visualize nuclei and photographed with a Nikon Eclipse E800 microscope with Image-Pro Plus software for image analysis.

### Statistical analysis

Statistical analyses were performed using SPSS software version 13.0 (SPSS, Chicago, USA). Data were presented as the mean ± SD. Differences of the variables between groups were tested by Student's *t*-test. $P < 0.05$ was regarded as statistically significant for all the tests.

## Results

### Expression of *WIF-1* mRNA transcript

Semi-quantitative RT-PCR assay was performed to analyze the expression of *WIF-1* at the transcription level. *WIF-1* expression was examined in 4 normal brain tissues as well as in 35 resected astrocytoma tissues (**Table 1** and **Fig. 1**). The results showed that *WIF-1* expression in tumor samples ($0.351 \pm 0.304$) was significantly lower compared with normal brain tissues ($0.918 \pm 0.047$, $P < 0.001$). Significant association was found between *WIF-1* mRNA downregulation and the pathological grade ($P < 0.001$). However, *WIF-1* gene expression was not correlated with age or sex in tumor samples ($P = 0.482$ and 0.713, respectively) (**Table 2**).

### Expression of WIF-1 protein

To further detect the expression level of WIF-1, immunohistochemistry was also performed in 4 normal brain tissues and in 35 astrocytoma tissues (**Table 1** and **Fig. 2**). Reactivity was generally cytoplasmic and membranaceus. The average values of WIF-1 expression were $7.250 \pm 0.500$ and $2.940 \pm 2.182$, respectively, in normal brain tissues and astrocytomas. Statistical analysis indicated significantly lower expression in tumors than in normal brain

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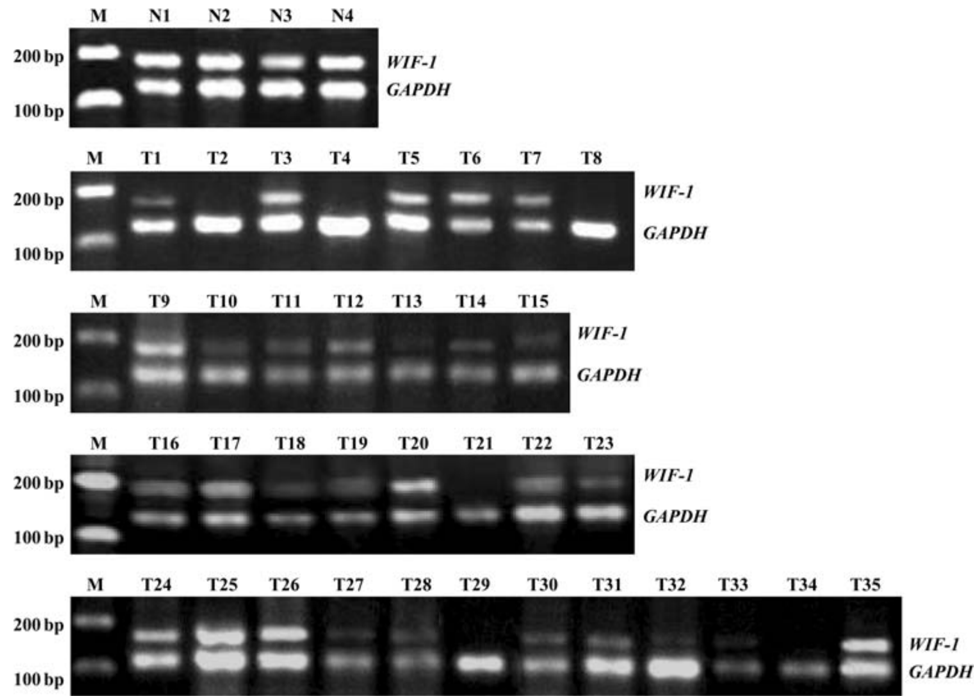


**Figure 1 mRNA expression of *WIF-1* in astrocytoma** RT-PCR results of the *WIF-1* gene in normal brain tissue (N1–N4) and astrocytoma (T1–T35) are shown. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. The fragments of amplified human *WIF-1* and *GAPDH* cDNA are 188 and135 bp, respectively. M, marker; N, normal brain tissue; T, astrocytoma tissue.

**Table 2 The relationship between the expression of WIF-1 and clinicopathological features in 35 cases of astrocytoma**

| Clinical signs | Cases ($n$) | RT-PCR | | IHC | |
|---|---|---|---|---|---|
| | | Mean ± SD[a] | $P$ | Mean ± SD[b] | $P$ |
| Age | | | | | |
| ≤43 | 19 | 0.384 ± 0.333 | 0.482 | 3.160 ± 2.287 | 0.533 |
| >43 | 16 | 0.310 ± 0.270 | | 2.690 ± 1.957 | |
| Sex | | | | | |
| Male | 21 | 0.335 ± 0.302 | 0.713 | 2.950 ± 2.061 | 0.975 |
| Female | 14 | 0.374 ± 0.317 | | 2.930 ± 2.433 | |
| Pathological grading | | | | | |
| Low grade (I–II) | 15 | 0.546 ± 0.248 | 0.000 | 4.400 ± 1.639 | 0.000 |
| High grade (III–IV) | 20 | 0.204 ± 0.259 | | 1.850 ± 1.899 | |

Statistical results showed that protein and mRNA expression of *WIF-1* were not correlated with age or sex, but were decreased as the pathological grade increased in astrocytoma. [a]Relative *WIF-1* mRNA levels were expressed as the fold-difference relative to *GAPDH* mRNA levels; [b]IHC scores of WIF-1. Data were analyzed by Student's *t*-test, $P < 0.05$ was regarded as statistically significant. IHC, immunohistochemistry.

tissues ($P < 0.001$). No significant correlation was found between WIF-1 protein expression and age or sex ($P = 0.533$ and 0.975, respectively). However, WIF-1 protein expression in astrocytomas was decreased as the pathological grade increased ($P < 0.001$) (**Table 2**).

### Relationship between promoter methylation and expression of WIF-1

To examine whether the methylation status of promoter correlates with the expression of WIF-1, MS-PCR was carried out. Unmethylation-specific PCR band might be detected in methylated samples, probably due to unavoidable contamination of nontumor cells, or partial methylation of the gene. No hypermethylation was observed in all normal brain tissues. In contrast, aberrant methylation was observed in 19 of 35 (54.29%) tumor samples. Especially, 16 of 20 (80.0%) high-grade astrocytomas showed promoter hypermethylation (**Table 1** and **Fig. 3**). The promoter methylation tumors showed low *WIF-1* mRNA and protein expression, whereas the promoter



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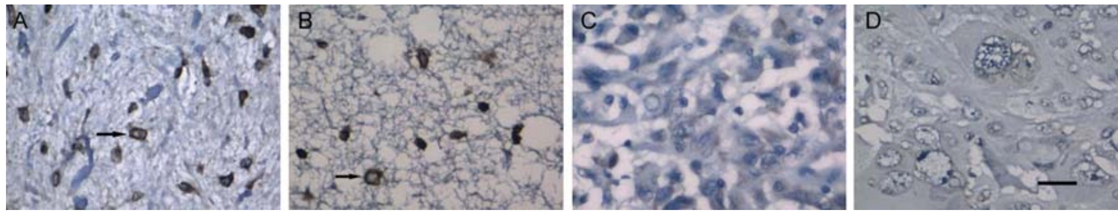


**Figure 2 Immunohistochemical analysis for anti-human WIF-1 antibody** Paraffin-embedded sections of representative astrocytomas and normal brain tissues were stained with the antibody against human WIF-1. The products of WIF-1 expression (brown) located in cytoplasm and membrane (arrow). The photographs in (A) and (B) are normal brain tissues and pilocytic astrocytoma (WHO grade I), which showed strong staining for WIF-1, respectively. In contrast, the anaplastic astrocytoma (WHO grade III) and glioblastomas (WHO grade IV) that have weak or negative expression levels of WIF-1 were shown in (C) and (D), respectively. Pathological malignancy grade of astrocytoma correlated with immunohistochemical score of WIF-1. Scale bar = 100 μm.

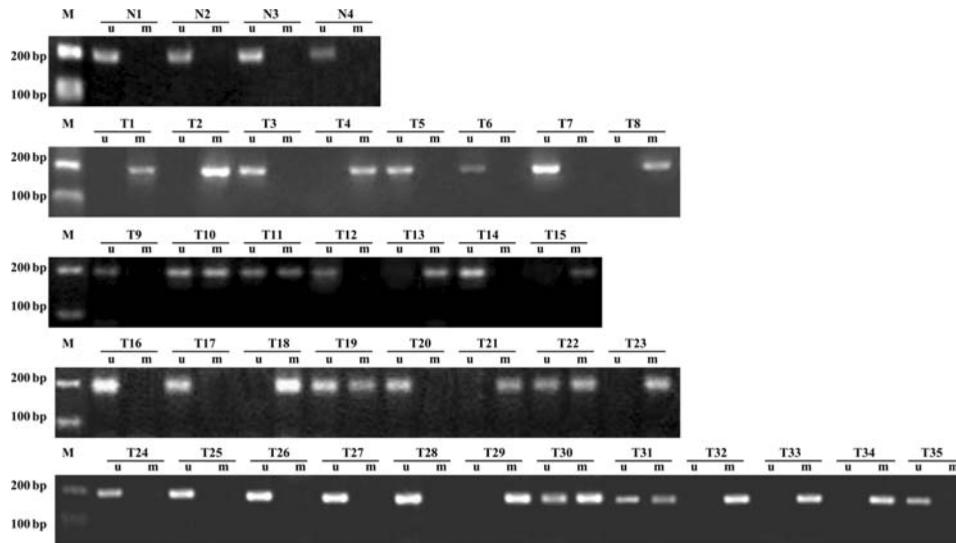


**Figure 3 Promoter methylation of *WIF-1* in astrocytoma** Methylation of the *WIF-1* promoter was analyzed by MS-PCR in normal brain tissue (N1–N4) and astrocytomas (T1–T35). M, marker; N, normal brain tissue; T, astrocytoma tissue; u, unmethylation; m, methylation.

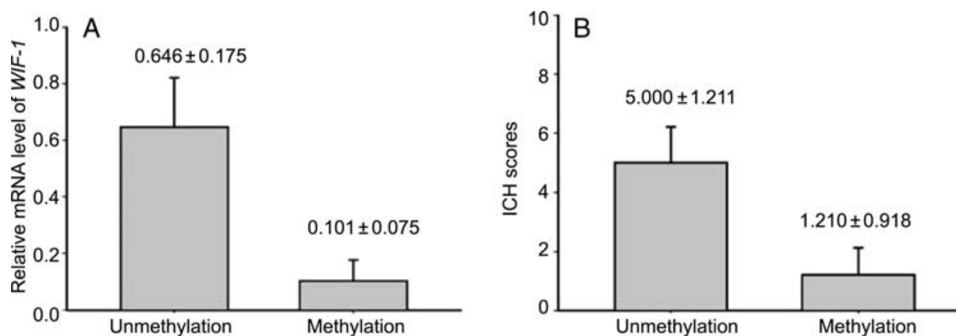


**Figure 4 Correlation between hypermethylation and decreased expression of WIF-1 in astrocytomas** A significant downregulation of the mRNA (A) and protein (B) expression of WIF-1 was observed in astrocytomas with promoter methylation (both $P < 0.001$). The bars in the graph showed the mean ± SD. IHC, immunohistochemistry.

unmethylation tumors displayed high protein and mRNA expression levels. Thus, these data indicated a significant correlation (both $P < 0.001$) between hypermethylation and decreased or weak expression of WIF-1 in astrocytomas (**Fig. 4**).

## Effect of 5-aza-CDR on astrocytoma cell lines

To further estimate if aberrant hypermethylation of the promoter is directly responsible for transcription in astrocytomas, the transcript levels and DNA methylation status of *WIF-1* were analyzed in untreated and ADC-treated human

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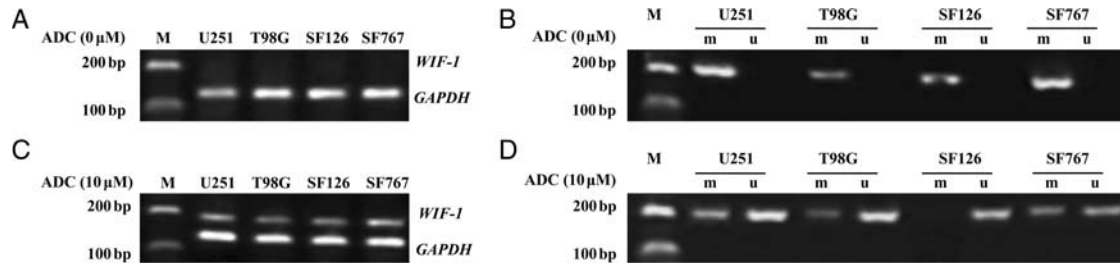


**Figure 5 The transcript levels and DNA methylation status of *WIF-1* by ADC treatment in astrocytoma cell lines** Reactivation of *WIF-1* mRNA expression was observed in all the cell lines after ADC treatment (C) in comparison with untreated controls (A). The unmethylated bands after treatment (D) were observed when compared with untreated controls (B). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. M, marker; N, normal brain tissue; T, astrocytoma tissue; u, unmethylation; m, methylation.

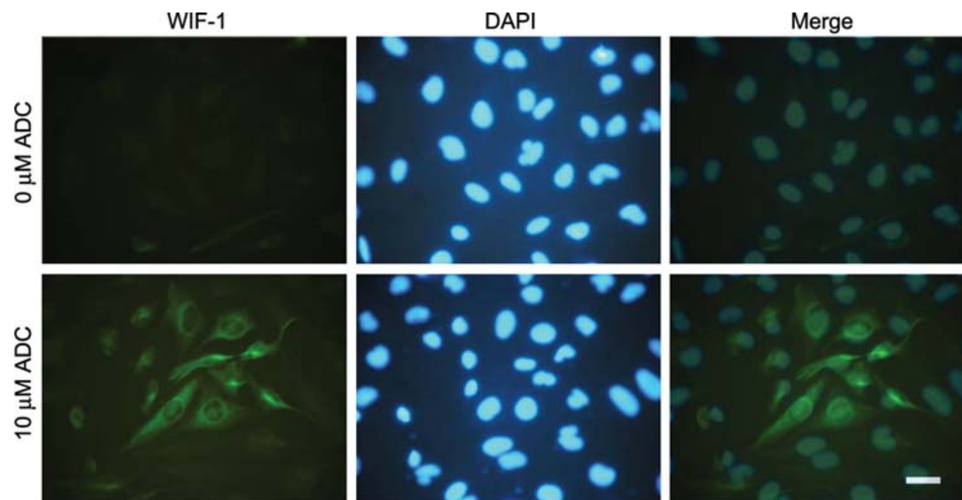


**Figure 6 Immunofluorescence staining of U251 cell lines for the expression of WIF-1** WIF-1 expression was partially restored via demethylation treatment with ADC in U251 cell lines. Nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 100 μm.

glioblastoma-derived cell lines. The RT-PCR analysis showed that *WIF-1* transcription level in the treated cells [**Fig. 5(C)**] was increased compared with that in the untreated cells [**Fig. 5(A)**]. The MS-PCR showed that the unmethylation bands after treatment [**Fig. 5(D)**] were observed in comparison with untreated cells [**Fig. 5(B)**].

To assess whether WIF-1 protein expression is similar to mRNA, immunofluorescence was performed using untreated and ADC-treated cell lines. As shown in **Fig. 6**, WIF-1 were absent in the untreated cells but were observed in cell lines treated with ADC. Taken together, these results suggested that the WIF-1 expression was induced after ADC treatment at the protein level as well at the mRNA level and the examined hypermethylation of the WIF-1 might contribute to reduced expression of WIF-1.

## Discussion

Wnt/β-catenin signaling pathway is important in tumorigenesis and embryogenesis [14,15]. The signaling pathway mediated by Wnt proteins currently includes two classes (canonical and noncanonical) on the basis of the activity of Wnt proteins in cell lines or *in vivo* assays. The canonical pathway, in which β-catenin plays a crucial role, is the most studied Wnt pathway in cancers. The activation of canonical pathway allows β-catenin to accumulate in the cytosol and enter the nucleus and induces expression of Wnt target genes like c-Myc, N-Myc, and cyclin D1 [16–18], many of which have been implicated in human cancers. In astrocytoma, the level of Wnt-2, Wnt-5a, and β-catenin protein is strikingly increased compared with normal brain tissue [2,3,5]. Knockdown of Wnt or its key mediator β-catenin in the canonical Wnt pathway by siRNA in human astrocytoma cells inhibited cell proliferation and invasive ability, induced apoptotic cell death, and reduced tumorigenicity *in vivo*. The above findings suggest Wnt signaling in astrocytoma is constitutively activated and of critical importance in the astrocytoma genesis.

WIF-1 is an endogenous Wnt antagonist. Downregulation of WIF-1 may release the inhibitory effect exerted by WIF-1 on the Wnt/β-catenin signaling [19]. This then enhances the accumulation of β-catenin and

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promotes tumorigenesis. Although it is known that WIF-1 is strongly expressed in embryonic mouse brain [20], its expression in brain tumors has not yet been investigated. In this study, we analyzed the protein and mRNA levels of WIF-1 in astrocytomas using immunohistochemistry and RT-PCR, respectively. *WIF-1* mRNA expression was clearly correlated with protein levels. The level of protein expression in astrocytoma was significantly lower than that in the normal tissues. As the pathological grade increased, the protein and mRNA expression levels of WIF-1 in astrocytoma were decreased. These results indicated that WIF-1 was frequently and significantly downregulated in astrocytomas, especially in high-grade astrocytomas, which might contribute to the upregulation of Wnt/β-catenin signaling in astrocytoma carcinogenesis.

Aberrant methylation of promoter regions that silences transcription of the genes has been recognized as a mechanism for inactivating tumor suppressor genes in human cancer [21,22]. It occurs at cytosine bases located 5′ to a guanosine and so-called CpG dinucleotide short regions of CpG dinucleotides known as CpG islands are found in the proximal promoter region of over half of human genes [22]. The methylation of these gene promoters is generally not detected in normal tissues but in the hypermethylation of CpG islands resulting in a loss of gene function, which is a common feature in many tumor types. Now, many other genes such as *LHX9*, *MGMT*, *CDKN2A*, *PTEN*, and *p15* have been shown to be methylated in astrocytomas [23–27]. WIF-1 silencing may be an early epigenetically carcinogenic event and plays a role in tumor development and progression [28]. In this study, hypermethylation of WIF-1 promoter was not detected in normal brain tissues; however, it was found in 19 of 35 (54.29%) astrocytoma samples. WIF-1 silencing was associated with aberrant methylation of promoter region in tumor tissue samples. In addition, WIF-1 expression could be partially restored via demethylation treatment with ADC in astrocytoma cell lines. Taken together, these findings suggest that promoter methylation is an important mechanism in the inactivation of WIF-1 in astrocytoma.

In summary, we provide evidence that not only WIF-1 is frequently hypermethylated in astrocytomas, but also this epigenetic alteration of the *WIF-1* gene is associated with reduced expression. This study revealed a novel epigenetic event in the pathogenesis of astrocytoma, which may shed light on developing new approaches for this fatal disease. The reversibility of methylation silencing may allow restoration of WIF-1 function and regulation of Wnt signaling. This could be important in the development of new and effective strategy in astrocytoma treatment.

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