**ARID1A is a tumour suppressor and inhibits glioma cell proliferation via the PI3K pathway**

Y Zeng¹, Z Liu¹, J Yang², Y Liu¹, L Huo¹, Z Li¹, S Lan¹, J Wu¹, X Chen¹, K Yang¹, C Li¹, M Li²*, J Liu¹*

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**Abstract**

The AT-rich interactive domain 1A gene (ARID1A) plays an important role in malignant tumorigenesis, but its role in gliomas remains unclear. This study aims to identify a possible biomarker that could be used in the diagnosis and tumour grade assessment of gliomas. Additionally, the biological role of ARID1A was further characterized in glioma cells. Data were collected from sporadic glioma specimens (n = 55) and normal brain tissues (n = 5), and ARID1A expression was examined by quantitative RT-PCR and Western blot. We verified the differential expression of ARID1A and evaluated the associations of ARID1A expression with the pathological characteristics of gliomas. An ARID1A overexpression plasmid was constructed and transfected into the human glioblastoma cell line U87, and cell proliferation and apoptosis were examined. Our results showed that the ARID1A mRNA in gliomas was significantly down-regulated compared with that in normal brain tissues. As the pathological grade (World Health Organization classification 2007) increased, the expression of ARID1A decreased. Overexpression of ARID1A was able to inhibit cell proliferation and arrest cell cycle progression in the G1/S phase as well as induce cell apoptosis in glioma cells. Furthermore, ARID1A overexpression was accompanied by the suppression of glioma cell proliferation via the phosphatidylinositol 3-kinase pathway and decreased expression of pAKT and pS6K. Therefore, ARID1A may be a useful target for the diagnosis and therapy of gliomas.

**Introduction**

Glioma is the most common primary central nervous system tumour and often results in the death of patients within 1–2 years. The aggressive nature of glioma is characterized by intense cell proliferation, diffuse infiltration and high resistance to apoptosis. With few advances in early detection, most patients are at advanced stages at the time of diagnosis, which decreases the chances for successful treatment. To date, no tumour marker has been found to detect glioma at a potentially curative stage, and the factors that mediate glioma proliferation and recurrence are still poorly understood. Consequently, there is a meaningful urgency to identify critical carcinogenic pathways and discover potential genes as diagnostic and therapeutic targets.

The AT-rich interactive domain 1A gene (ARID1A), which encodes one of the subunits in the switch/sucrose non-fermentable (SWI/SNF) chromatin remodelling complex, has been shown to function as a tumour suppressor in various cancer types. Recent studies found that ARID1A was mutated in 46% of ovarian clear cell carcinomas, 30% of endometrioid carcinomas, 13% of hepatitis B virus-associated hepatocellular carcinomas and 10% of gastrointestinal neoplasms. Mutations were also identified in 2–8% of pancreatic, breast, brain (medulloblastomas), prostate and lung tumours. These data suggest that the loss of ARID1A may play an important role in the process of carcinogenesis. Therefore, ARID1A is a candidate for targeted therapy for various cancers. Restoring ARID1A expression significantly inhibited cell proliferation and colony formation in gastric and ovarian cancer cells. Moreover, it appears to be a good biomarker for the early diagnosis of cancer. Some researchers found ARID1A mutation or loss to be an early event in the carcinogenesis of endometrioid uterine carcinomas. Also, the loss of ARID1A was associated with an advanced invasion depth of endometrioid uterine carcinomas and gastric cancer.

Unfortunately, the expression of ARID1A and its role in gliomas remain largely unknown. In this current study, we used quantitative real-time polymerase chain reaction (RT-PCR) and Western blot analysis to investigate the correlation between ARID1A expression and glioma grade. To explore its associated molecular mechanisms in glioma cells, we examined the effect of ARID1A gene over-expression on cell proliferation and apoptosis in vitro. These findings will be useful in identifying potential candidates for the diagnosis and targeted therapy of glioma.

**Materials and methods**

**Patients and tissue samples**

A total of 55 glioma specimens were collected in this study. These specimens were obtained from patients who underwent either explorative or radical surgery at the Xiangya Hospital of Central South University (Hunan, China) from February 2011 to May 2012 after informed consent was obtained. The gliomas were histopathologically and clinically diagnosed and classified according to World Health Organization (WHO) classification of tumours of the central nervous system (2000). The gliomas included grade II astrocytomas (n = 13), grade II oligodendrogliomas (n = 1), grade II oligoastrocytomas (n = 1), grade III anaplastic astrocytomas (n = 4), grade III oligoastrocytomas (n = 2), grade IV glioblastomas (n = 28), and grade IV gliosarcomas (n = 2). The normal brain tissue samples were provided by the Department of Neurosurgery, Xiangya Hospital of Central South University (n = 3).

**ARID1A expression**

ARID1A mRNA expression in glioma tissues was determined by quantitative RT-PCR analysis. Briefly, total RNA was extracted from the glioma tissues using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using the SuperScript II RNase H-Reverse Transcriptase System (Invitrogen) and then amplified using the specific primer sets. The primers (Invitrogen) used are as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ - 3’)</th>
<th>Product size (bp)</th>
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<tr>
<td>ARID1A/ACTB</td>
<td>forward: GCTTATCCAGATGGCGACCGC</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>reverse: GATTTAGGTCAACGTTCGAGG</td>
<td></td>
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</tbody>
</table>

**Western blot analysis**

Total protein was extracted from the glioma and normal brain tissues using a lysis buffer (Santa Cruz Biotechnology, USA) containing 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM potassium phosphate (pH 7.4), 100 mM NaF, 1 mM Na3VO4, 10 mM EDTA, 1 mM dithiothreitol, 4 mM phenylmethylsulphonyl fluoride, 3 mM leupeptin, and 5 µg/ml aprotinin. Proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween 20 (TBST) and incubated with antibodies (1:1000) against ARID1A, pAKT (Ser 473) (1:1000), pS6K (Ser 235/236) (1:1000). Blots were incubated with the appropriate secondary antibody (1:5000) and visualized with an enhanced chemiluminescence system (GE Healthcare) according to the manufacturer’s instructions.

**RT-PCR**

ARID1A expression in the glioma cell line U87 was evaluated by quantitative real-time polymerase chain reaction (RT-PCR) analysis. Briefly, the total RNA was extracted from the glioma cell line U87 using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using the SuperScript II RNase H-Reverse Transcriptase System (Invitrogen) and then amplified using the specific primer sets. The primers (Invitrogen) used are as follows:

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**Statistical analysis**

The quantitative RT-PCR and Western blot data were analysed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). The p-values were calculated using the Student’s t-test for the comparison between glioma and normal brain tissues.

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*Corresponding authors
Email: xylancet@yahoo.com.cn; min.li@uth.tmc.edu

1 Department of Neurosurgery, Xiangya Hospital, Central South University, Changsha, China
2 The Vivian L. Smith Department of Neurosurgery, The University of Texas Medical School at Houston, Houston, TX, USA

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were cultured in RPMI media 1640 supplemented with 10% FBS and penicillin G (100 U/mL)/streptomycin (100 μg/mL), at 37°C in a humidified atmosphere containing 5% CO₂.

**Transfection**

U87 cells were seeded (2 × 10⁵ cells/well) on six-well plates. On the day of transfection, the cells were harvested and transfected with the recombinant plasmid pZsGreen1-C1/ARID1A or empty vector using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were treated with 0.5 μg/mL puromycin (Sigma–Aldrich Corp., St Louis, MO, USA), and the medium was replaced 24 h later and every 2 days thereafter. The stable clones were maintained with 0.25 μg/mL of puromycin after 7–10 days, and they were then isolated and cultured in 96-well plates for subsequent study. U87 cells transfected with the empty vector and the vector containing ARID1A were designated as U87-vector and ARID1A+, respectively.

**Cell viability and proliferation assays**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the evaluation of U87 cell proliferation in all groups. The cells were seeded on 96-well microplates (approximately 1 × 10⁴ cells/well). After 48 h, 20 μL MTT was added to each tube, and the samples were incubated for 15 min at room temperature in the dark. Subsequently, 400 μL of binding buffer was added to each tube, and the samples were analysed using a BD fluorescence cytometer and CellQuest software within 1 h.

**Western blot analysis**

Cells were solubilized in cold RIPA lysis buffer and then separated with 5% SDS-PAGE. After SDS-PAGE, proteins were transferred from the gel to a PVDF membrane. Membranes were blocked in 5% non-fat-dried milk in PBS for 2 h and then incubated overnight with ARID1A, pAKT and pS6K antibodies obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), with GAPDH as a control. After incubation with the appropriate secondary antibody, immune complexes were detected using an ECL kit. Results were visualized by autoradiography using preflashed Kodak XAR film.

**Apoptosis assay**

To determine cell apoptosis, we used the annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) double-staining assay. After harvesting, the cells were washed twice with cold PBS and resuspended at 1 × 10⁵ cells/mL in annexin V binding buffer (BD Biosciences). Then, 100 μL of the solution (1 × 10⁵ cells) was transferred to a 5-mL culture tube. After 5 μL annexin V-FITC (BD Biosciences) and 5 μL PI (BD Biosciences) were added, the mixture was incubated for 15 min at room temperature. In the dark. Subsequently, 400 μL of binding buffer was added to each tube, and the samples were analysed using a BD fluorescence cytometer and CellQuest software within 1 h.

**Table 1** ARID1A mRNA levels in human glioma tissues with different pathological features

<table>
<thead>
<tr>
<th>Pathological features</th>
<th>No. of cases</th>
<th>ARID1A, mean ± SD</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td><strong>Tissue type</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>1 ± 0.12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glioma</td>
<td>55</td>
<td>0.38 ± 0.31</td>
<td></td>
</tr>
<tr>
<td><strong>WHO grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>0.89 ± 0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>II</td>
<td>18</td>
<td>0.41 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>0.27 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>0.10 ± 0.04</td>
<td></td>
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Statistical analyses

Statistical analysis was performed using SPSS 17.0 statistical software. Data are expressed as mean ± SEM. The statistical significance of the differences was determined by ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered significant.

**Results**

**Expression of ARID1A decreased in gliomas**

The associations between quantitative RT-PCR analysis of ARID1A gene expression and grade of gliomas are described in Table 1. ARID1A mRNA levels were examined in five normal brain tissue and 55 glioma tissue samples. Our results showed that the ARID1A level in tumour samples (0.38 ± 0.31) was significantly lower than that in normal brain tissues (1 ± 0.12; P < 0.05). A significant association was found between ARID1A mRNA down-regulation and the pathological grade according to the WHO criteria. ARID1A mRNA decreased with the advancement of WHO grades I–IV (P < 0.05) (Figure 1a).

We further determined the ARID1A protein expression in normal brain tissues and glioma tissues. As shown in Figure 1b, ARID1A protein expression level decreased from the normal brain tissues to the glioma tissues. We further investigated whether ARID1A protein expression correlated with the WHO grade. Our data demonstrated that the ARID1A protein expression was highest in grade I and lowest in grade IV. The data are in agreement with the results of quantitative RT-PCR and indicates a close correlation of ARID1A protein expression with the WHO grade.

**Expression of ARID1A in ARID1A+, U87-vector and U87 cells**

To determine whether ARID1A was successfully transfected in U87 cells, quantitative RT-PCR and Western blotting were performed to determine the mRNA and protein expression of ARID1A in ARID1A+, U87-vector and U87 cells.

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**Figure 1**: (a) Quantitative RT-PCR of gliomas and normal brain tissues. A significant down-regulation of ARID1A mRNA expression was observed in gliomas. (b) Expression of ARID1A protein in glioma and normal brain tissues by Western blot analysis. *N* refers to normal brain tissues; GAPDH was used as a control for equal protein loading. Values are mean ± SD. *P* < 0.05 in comparison with normal brain tissues; **P** < 0.01 in comparison with normal brain tissues.

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ARID1A in experimental and control groups. Our results indicate that the mRNA level in ARID1A+ cells (2.59 ± 0.03) was significantly up-regulated compared with that in U87-vector (0.89 ± 0.05) and U87 cells (1 ± 0.19; \( P < 0.01 \); Figure 2a). The protein level of ARID1A was also remarkably increased in ARID1A+ U87 cells when compared with that in U87-vector and U87 cells (Figure 2b).

**ARID1A inhibits glioma cell proliferation**

We compared the proliferation of U87, U87-vector and ARID1A+ cells using the MTT assay. Compared with U87 and U87-vector cells, proliferation of ARID1A+ cells was significantly inhibited by ARID1A at 48 h after transfection (\( P < 0.05 \); Figure 3). Our results indicated that ARID1A could inhibit U87 cell proliferation.

**Overexpression of ARID1A arrests cell cycle in the G1/S phase**

To study the link between cell cycle control and ARID1A in U87 cells, we assessed the cell cycle distribution by flow cytometry at 48 h after transfection in U87, U87-vector and ARID1A+ cells. As shown in Figure 4, ARID1A+ cells exhibited a significant increase in the cell fraction in the G1 phase (U87 37.83 ± 3.34%, U87-vector 40.23 ± 2.45% and ARID1A+ 66.31 ± 1.47%, \( P < 0.05 \)) and a corresponding reduction in the fraction of cells in the S phase (U87 38.43 ± 2.15%, U87-vector 37.51 ± 3.14% and ARID1A+ 24.57 ± 3.15%, \( P < 0.05 \)) and G2-M phase (U87 23.74 ± 2.42%, U87-vector 22.26 ± 3.26% and ARID1A+ 9.12 ± 0.48%, \( P < 0.05 \)). Our data implied that ARID1A is involved in the regulation of cell cycle arrest in U87 cells, which may represent a potential mechanism of ARID1A-induced cell proliferation inhibition.

**ARID1A induces U87 cell apoptosis**

The flow cytometry results demonstrated that early-stage apoptotic cells (annexin V+/PI−) and late-stage apoptotic cells (annexin V+/PI+) were increased (\( P < 0.05 \); Figure 5) in ARID1A+ cells when compared with those in untreated U87 cells and U87-vector cells. These results indicate that ARID1A induces apoptosis in human malignant glioma U87 cells.

**ARID1A inhibits U87 cell proliferation by the phosphatidylinositol 3-kinase (PI3K) pathway**

We then investigated the potential signalling pathways involved in ARID1A-inhibited cell proliferation. As shown in Figure 6, the phosphorylation...
ARID1A is a tumour suppressor and inhibits glioma cell proliferation via the PI3K pathway. Head Neck Oncol. 2013 Jan 14;5(1):6.

Discussion
The relationship between ARID1A and gliomagenesis has not been extensively investigated. Limited studies on ARID1A mutations in brain tumours were performed by next-generation sequencing. Mutations were observed in 3 of 125 (2%) medulloblastomas, but no mutations were observed among the 34 glioblastomas tested. Another study discovered one ARID1A mutation in a set of 22 medulloblastomas using Sanger sequencing, but there has been no reported study on ARID1A expression in brain tumours. In this study, we investigated ARID1A expression at both the mRNA and protein levels using a larger number of human glioma tissue samples, and the relationship between ARID1A and pathological characteristics was analysed. The results showed that ARID1A mRNA and protein expression in glioma tissues was significantly lower than that in the normal brain tissues. As the pathological grade (WHO classification 2007) increased, the mRNA and protein level decreased. This result was useful in identifying gliomas from normal tissues and low-grade tumours from malignant types. Therefore, ARID1A may serve as a novel biomarker for glioma diagnosis. Furthermore, these results suggest that ARID1A may be a suppressor during the genesis or progression of glioma. We found that the overexpression of ARID1A not only inhibits cell proliferation but also mediates cell cycle progression and apoptosis in glioma cells (Figure 7).

**Figure 4:** (a) Overexpression of ARID1A induced cell cycle arrest at G1/S phase. (b–d) Cell cycle distribution in ARID1A+, U87-vector and U87 cells by FACS Calibur flow cytometry.

**Figure 5:** Effects of ARID1A on cell death of U87 cells. ARID1A+, U87-vector and U87 cells were stained with annexin V-FITC and PI and assayed using flow cytometry. The apoptotic cells, which included necrotic cells, were increased in the ARID1A+ group. **P < 0.05 compared with U87 cells.
of ARID1A reduced cell proliferation using an MTT assay, which may be partly attributed to the suppressive effect of ARID1A on cell cycle progression. Overexpression of ARID1A increased the cell population in the G1 phase and reduced the cell population in the S and G2-M phases, suggesting an inhibition of cell cycle progression from the G1 phase to the S phase. The importance of ARID1A in cell proliferation control has been explored in the MC3T3-E1 preosteoblast line, which shows that ARID1A is essential for normal cell cycle arrest. ARID1A-depleted cells fail to undergo normal cell cycle arrest on induction17–19. These results, together with our data, suggest that ARID1A significantly impacts cell cycle progression in gliomas.

Our results also indicate that overexpression of ARID1A partly induced apoptotic cell death in U87 cells. As the population of apoptotic cells increased, the viable cells decreased, leading to reduced cell proliferation in ARID1A+ U87 cells. AKT has emerged as a central player involved in the regulation of cell proliferation, growth, differentiation, migration and apoptosis in many human cancers through the PI3K pathway20,21. The downstream effects of AKT activation are primarily conveyed by TSC2 and S6 kinase signalling in gliomas, which mainly enhance proliferation rather than inhibit apoptosis22,23. In recent research, ARID1A has been found to suppress the cell proliferation of endometrial cancer cell lines by regulating the activity of the PI3K pathway, and the phosphorylation of several downstream targets (PDK1, AKT, GSK3, TSC2, p70S6K and ACC) is significantly up-regulated in tumours with ARID1A mutations24. We thus examined the effect of ARID1A on phosphoprotein levels of core members in the PI3K pathway using Western blot, and we found that the phosphorylation of AKT and S6K is down-regulated in the ARID1A overexpression group. These results demonstrated that ARID1A

![Figure 6](image1.png) **Figure 6**: Western blot assays of phosphorylation of AKT and S6K expression in ARID1A+, U87-vector and U87 cells. The expression of phosphorylation of AKT and S6K was down-regulated.

![Figure 7](image2.png) **Figure 7**: Working model of ARID1A-mediated glioma cell growth inhibition. Overexpression of ARID1A inhibits cell proliferation and arrests cell cycle progression in the G1/S phase, and induces cell apoptosis in glioma cells. This inhibitory effect of ARID1A is mediated through the PI3K pathway by down-regulating pAKT and pS6K.
may inhibit cell proliferation by the PI3K pathway. Because the PI3K pathway is a common target for therapy\textsuperscript{25,26}, these results may be useful for clinical translation.

In conclusion, our data provide compelling evidence that ARID1A may be a novel therapeutic and diagnostic target for human glioma. The expression of ARID1A is inversely correlated with the grade of the glioma. Overexpression of ARID1A inhibits cell proliferation through the PI3K pathway, suggesting that ARID1A is a tumour suppressor in gliomas.

References