

RESEARCH ARTICLE

UHRF2 mRNA Expression is Low in Malignant Glioma but Silencing Inhibits the Growth of U251 Glioma Cells *in vitro*

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Abstract

UHRF2 is a member of the ubiquitin plant homeo domain RING finger family, which has been proven to be frequently up-regulated in colorectal cancer cells and play a role as an oncogene in breast cancer cells. However, the role of UHRF2 in glioma cells remains unclear. In this study, we performed real-time quantitative PCR on 32 pathologically confirmed glioma samples (grade I, 4 cases; grade II, 11 cases; grade III, 10 cases; and grade IV, 7 cases; according to the 2007 WHO classification system) and four glioma cell lines (A172, U251, U373, and U87). The expression of UHRF2 mRNA was significantly lower in the grade III and grade IV groups compared with the noncancerous brain tissue group, whereas its expression was high in A172, U251, and U373 glioma cell lines. An *in vitro* assay was performed to investigate the functions of UHRF2. Using a lentivirus-based RNA interference (RNAi) approach, we down-regulated UHRF2 expression in the U251 glioma cell line. This down-regulation led to the inhibition of cell proliferation, an increase in cell apoptosis, and a change of cell cycle distribution, in which S stage cells decreased and G2/M stage cells increased. Our results suggest that UHRF2 may be closely related to tumorigenesis and the development of gliomas.

Keywords: UHRF2 - NIRF - ubiquitin PHD RING finger family - malignant glioma

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Introduction

Malignant glioma, especially glioblastoma multiforme, is the most frequent primary brain tumor and the most malignant neoplasm, with predominant astrocytic differentiation (Louis et al., 2007). Patients with this most aggressive glioma variant have a median survival of only 15 months (Wen et al., 2008). Over the past 10 years, there has been an increasing understanding of the molecular biology of gliomas, such as O-6-methylguanine-DNA methyltransferase (MGMT) methylation status, epidermal growth factor receptor (EGFR) pathway alterations, and isocitrate dehydrogenase (IDH) mutations, which have been determined to be diagnostic, prognostic, and predictive markers (Jansen et al., 2010). With the rapid development of systems biology, increasing numbers of key cancer genes and their correlated proteins have been identified using bioinformatics.

UHRF2, also named NIRF (Np95/ RNF107), is a member of the UHRF family. The official full name of UHRF2 is ubiquitin-like with plant homeo domain (PHD) and ring finger domains 2, E3 ubiquitin protein ligase. Genes of the UHRF family are considered to act as oncogenes and are suitable drug targets for cancer therapy (Bronner et al., 2007). UHRF2 has been reported to be a ubiquitin ligase that interacts with the CDK2-cyclin E complex and induces G1 arrest of the HEK293 human

embryonic kidney cell line (Li et al., 2004; Mori et al., 2004). Studies on UHRF2 have demonstrated that it constitutes a nodal point in the cell cycle network (Mori et al., 2011). Because this protein possesses a ubiquitin-like (UBL) domain, tandem Tudor domain (TTD), plant homeo domain (PHD) finger domain, SET and RING-associated (SRA) domain, and new gene (RING) finger domain at the C-terminus, its functions are more complex than an ordinary cell cycle hub protein (Glaab et al., 2010; Mori et al., 2012). The SRA domain in UHRF2 is correlated with methyl-binding function (Johnson et al., 2007). The UHRF2 gene has been mapped to chromosome 9p24, in which many studies have proposed the presence of multiple tumor-associated genes (Kocarnik et al., 2010).

The expression of UHRF2 and the function of UHRF2 have not been previously studied in gliomas, whereas UHRF2 has been proven to be frequently up-regulated in colorectal cancer cells and to play a role as an oncogene in breast cancer cells (Wang et al., 2012; Wu et al., 2012). This is the first investigation of UHRF2 mRNA expression in gliomas and its functions in the glioma cell line U251.

Materials and Methods

Patient samples

The Ethics Committee of Soochow University approved this study. All of the experiments complied

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with the current laws of our country. A total of 32 glioma patients were enrolled; all gave written informed consent. Between March 2009 and September 2010, the patients underwent surgical resection of tumors at the Department of Neurosurgery of the First Affiliated Hospital of Soochow University (grade I, 4 cases; grade II, 11 cases; grade III, 10 cases; and grade IV, 7 cases, according to the 2007 WHO classification system). Samples of cancer tissue were collected during surgery. No patients received chemotherapy or radiotherapy before surgery. The patients included 17 men and 15 women. The mean ages of the patients at the time of surgery were 38 years (men) and 41 years (women). Five adult noncancerous brain tissue samples were obtained from surgical resections of five brain trauma patients for whom a partial resection of normal brain tissue was required as decompression treatment to reduce increased intracranial pressure, with the permission of each patient's family. All tissue samples were collected, snap-frozen in liquid nitrogen, and stored until experimental use.

Cells and cell culture

Five human glioma cancer cell lines were used in this study. The human glioma cell lines U251, SHG44, and U87MG, derived from glioblastoma multiforme (GBM), were provided by our Brain and Nerve Research Laboratory. The glioblastoma-derived cell line A172 and the human glioblastoma-astrocytoma cell line U373MG were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (CBTCCAS, Shanghai, China). The five cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco) at 37°C under a humidified atmosphere of 5% CO₂.

Real-time quantitative reverse transcription polymerase chain reaction

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to analyze patient samples and cell lines. Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was prepared from 2–6 µg of total RNA using superscript II reverse transcriptase (Invitrogen) and random hexamer primers. Using 2 µl of cDNA, real-time PCR was performed to detect UHRF2 using SYBR Green Mixture (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. The specific primer pairs were as follows: UHRF2-primer (sense, 5'-GGACCTTCCAATCAGCC-3'; antisense, 5'-TTCAAACCAAGCACCAA-3'; product size 120 bp); and the internal control gene was GAPDH (GAPDH primer sense, 5'-TGACTTCAACAGCGACACCCA-3'; antisense, 5'-CACCTGTTGCTGTAGCCAAA-3'; product size 121 bp). Real-time PCR conditions included an initial denaturation at 95°C for 15 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. The data were analyzed using GraphPad PRISM4.0 software (GraphPad, La Jolla, CA, USA). The results were presented as CT values, which were defined as the threshold PCR cycle number at which an amplified product was first detected. Briefly, GAPDH was used for the normalization of the

quantity of RNA used. Its C_T value was then subtracted from the C_T value of the UHRF2 gene to obtain a ΔC_T difference value. The difference ($\Delta \Delta C_T$) between the ΔC_T value of each sample for the gene target and the ΔC_T value of the calibrator (Z5) was determined. The Z5 calibrator was chosen because it showed the lowest expression value in the noncancerous brain tissue samples. The relative quantitative value was expressed as $2^{-\Delta \Delta C_T}$, representing the amount of UHRF2 expression (normalized to an endogenous reference) relative to the Z5 calibrator. Procedures on all specimens were run in triplicate, and the values were averaged.

UHRF2-siRNA lentiviral infection in U251 cells

Five RNAi candidate sequences to target human UHRF2 (GenBank ID: NM152896) were designed by Genechem Co. Ltd. (Shanghai, China) and cloned into a pGCL-GFP vector. One targeting sequence (5'-GAUCCUGGCUUUGGAAUAUTT-3') showed the best interference efficiency in 293T cells, as revealed by western blotting (GeneChem). This sequence was selected to knock down endogenous UHRF2 in U251 glioma cells. The lentiviral vector expressing only GFP was used as a negative control. The recombinant virus was packaged using a lentiviral vector expression system (Genechem). For lentiviral infection, U251 cells were grown to 70–80% confluence and infected with UHRF2-siRNA lentivirus or control lentivirus at a multiplicity of infection (MOI) of 10 using an enhanced infection solution. After 24 h of transfection, the medium was replaced with fresh DMEM medium and incubated for a further 48 h at 37°C under a humidified atmosphere of 5% CO₂. To determine the infection efficiency, cells expressing GFP protein were observed using fluorescence microscopy (CKX41 inverted microscope, Olympus, Shinjuku, Tokyo, Japan) 5 days after infection.

Cell growth assay

Cell growth was measured via multiparametric high-content screening (Cellomics Inc., Pittsburgh, PA, USA). Ten days after being infected with either negative control (NC) lentivirus or UHRF2-siRNA lentivirus, human glioma U251 cells were seeded at 2000 cells per well in 96-well plates and incubated at 37°C with 5% CO₂ for 5 days. Plates were processed using ArrayScan™ HCS software (Cellomics) and kept at 4°C for up to 24 h before each day's analysis. The system is a computerized, automated, fluorescence-imaging microscope that automatically identifies infected cells and reports the intensity and distribution of fluorescence in each individual cell. Images were acquired for each fluorescence channel using suitable filters and a 20× objective. In each well, at least 800 cells were analyzed.

Flow cytometry to examine cell cycle progression

Different cell cycle phases (G0/G1, S, and G2/M phase) are characterized by different DNA contents; the DNA contents of cell cycle phases are reflected by varying propidium iodide (PI) fluorescence intensities. In brief, cells infected with UHRF2-siRNA lentivirus or NC lentivirus were harvested by trypsinization,

washed with PBS, and fixed in 70% ethanol at 4°C. Cells were collected by centrifugation, resuspended in PBS containing 40 µg/ml PI and incubated at 4°C for 30 min in the dark. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA). The fractions of cells in the G0/G1, S, and G2/M phases were analyzed using dedicated software (Becton–Dickinson).

Analysis of apoptosis

The apoptosis of the cells infected with UHRF2-siRNA lentivirus or NC lentivirus was assayed by staining with Annexin V-APC (BD Biosciences, USA) and detected by flow cytometry. Cells were collected and washed with ice-cold PBS 5 days after transfection and stained with 100 µl binding buffer containing 5 µl Annexin V-APC at room temperature in the dark for 10–15 min. For each experiment, 20,000 cells were analyzed using the FACSCalibur flow cytometer. All of the experiments were performed in triplicate.

Statistical analyses

The relative levels of UHRF2 mRNA expression, $2^{-\Delta\Delta CT}$, as measured using RT-PCR in noncancerous brain tissue samples and glioma tissue samples, were compared using unpaired t tests on log-transformed values. For the cell growth assay, cell cycle distribution, and apoptosis, the differences between the two groups were analyzed using Student's t-test. The data were presented as the mean ± standard deviation (SD) of these observations. The statistical analyses were conducted in SAS (Version 9.2; SAS Institute, Cary, NC, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

The expression of UHRF2 mRNA in five glioma cell lines

Semi-quantitative RT-PCR was performed to detect the expression of UHRF2 mRNA in the following glioma cell lines: U87MG, U251, SHG44, A172, and U373. UHRF2 mRNA was expressed in all of these cell lines, except SHG44 (Figure 1A).

The relative expression levels of UHRF2 mRNA in glioma tissue samples, noncancerous brain tissue samples, and glioma cell lines

Real-time quantitative PCR was performed to detect the relative expression levels of UHRF2 mRNA among noncancerous brain tissue samples, glioma tissue samples, and glioma cell lines using Z5 as the reference. The geometric means were 2.2 (noncancerous brain tissue group), 0.7 (grade I group), 1.3 (grade II group), 0.8 (grade III group), 0.3 (grade IV group), and 7.7 (glioma cell line group) after logarithmic transformation. There was no significant difference in UHRF2 expression between the noncancerous brain tissue group and grade I ($P=0.06$) and grade II groups ($P=0.17$). The expression levels of UHRF2 were significantly lower in the grade III ($P=0.02$) and grade IV groups ($P<0.01$) compared with the noncancerous brain tissue group. However, UHRF2 expression was significantly higher in glioma cell lines,

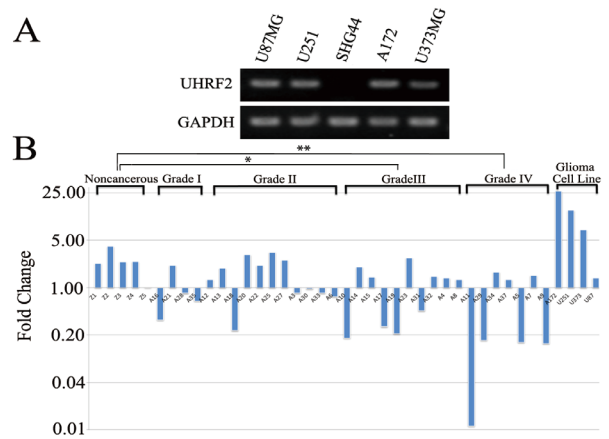


Figure 1. Detection of UHRF2 mRNA in Glioma Cell Lines, Noncancerous Brain Tissue and Glioma Tissue Samples. A, UHRF2 was expressed in U87MG, U251, A172, and U373MG glioma cell lines but not in SHG44. B, Expression differences were shown using real-time quantitative PCR. UHRF2 expression was significantly lower in the grade III ($P=0.02$) and grade IV groups ($P<0.01$) compared with the noncancerous brain tissue group. Each value is expressed as the mean of duplicate experiments. The reference, Z5, was considered to have a value of 1

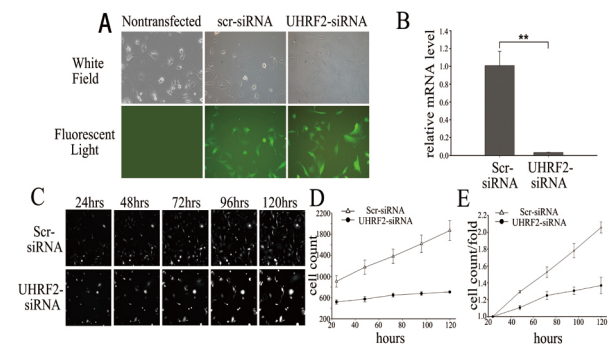


Figure 2. Lentivirus-mediated RNAi Decreased UHRF2 Expression in U251 Cells and Inhibited Their Growth Rate. A, The glioma cell line U251 was infected with UHRF2-siRNA or NC lentivirus or not transfected and observed using a fluorescence microscope and optical microscope at the 5th day after infection. Cells infected with lentivirus were labeled with GFP. Representative images of the cultures are shown (×200). B, Real-time quantitative PCR was performed to compare UHRF2 mRNA levels between the scr-siRNA group and UHRF2-siRNA group. The data shown are the mean results ± SD of a representative experiment performed in triplicate. $**P < 0.01$. C, High-content cell-imaging assays were applied to acquire raw images (unprocessed by software algorithm) of cell growth at different time points. D, A cell count graph of the scr-siRNA group and UHRF2-siRNA group is shown. E, A cell count/fold graph of the scr-siRNA group and UHRF2-siRNA group is shown

except for U87MG, compared with the noncancerous brain tissue group (Figure 1B).

Infection efficiency of human U251 glioma cells using UHRF2-siRNA lentivirus labeled with GFP

The human U251 glioma cells were divided into three groups: the nontransfected group, the NC lentivirus-infected group (scr-siRNA), and the UHRF2-siRNA lentivirus-infected group. After the transfection procedure, cells were cultured for 5 days before being observed under

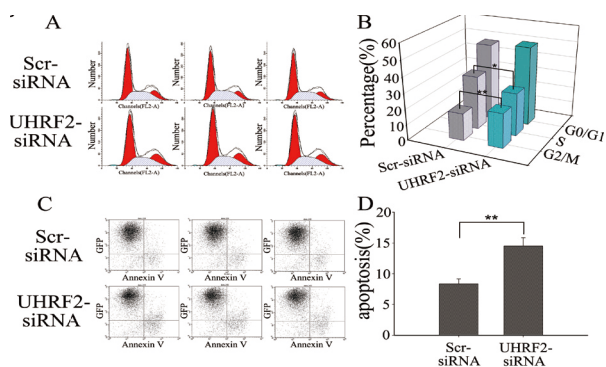


Figure 3. Down-regulation of UHRF2 Arrested Human U251 Glioma Cells in the G2/M Phase and Induced Apoptosis. A, Cell cycle distribution was compared by flow cytometric analysis of the NC lentivirus group and UHRF2-siRNA group. The experiment was performed in triplicate. B, UHRF2 gene silencing in U251 cells induced cell cycle arrest in G2/M phase 48 hours after infection. C, After Annexin-V staining, flow cytometry was performed to compare the apoptotic rates of the NC lentivirus group and UHRF2-siRNA group. The experiment was performed in triplicate. D, The bar graph indicates that the apoptotic percentage of UHRF2-siRNA-targeted cells increased significantly compared with the NC lentivirus group. ** $P < 0.01$

a fluorescence microscope. The transfection efficiency was greater than 80% in both the scr-siRNA group and the UHRF2-siRNA group, and no GFP-labeled cells were observed in the nontransfected group. No significant difference was observed between the nontransfected group and the scr-siRNA group, indicating that the transfection process itself had no effect on cell growth (Figure 2A). QRT-PCR analysis showed that the UHRF2 mRNA levels were significantly lower in the UHRF2-siRNA group than in the scr-siRNA group ($P < 0.01$, Figure 2B).

Down-regulation of UHRF2 suppressed human U251 glioma cell growth

To determine whether the down-regulation of UHRF2 had an influence on cell growth *in vitro*, the proliferation of U251 glioma cells was evaluated using the Cellomics system, which automatically identifies GFP-infected cells and reports the intensity and distribution of fluorescence in each individual cell every 24 hours. The cell growth of the scr-siRNA group and the UHRF2-siRNA group was observed for 120 hours (Figure 2C). The results showed that compared with the scr-siRNA group, the proliferation of the UHRF2-siRNA group was significantly inhibited (Figure 2D). The percentage of GFP-labeled cells in U251 cells infected with UHRF2-siRNA lentivirus decreased by approximately 70% at 120 hours compared with the scr-siRNA group ($P < 0.01$, Figure 2E).

UHRF2 gene silencing arrested human U251 glioma cells in the G2/M phase

Flow cytometry was used to investigate the cell cycle distribution of U251 glioma cells infected with NC lentivirus or UHRF2-siRNA lentivirus. Cells were collected 48 hours after infection. The experiments were performed in triplicate. The results showed that the percentage of GFP-infected cells in S phase decreased significantly in the UHRF2-siRNA group compared with

the scr-siRNA group ($P = 0.02$), while the percentage of GFP-infected cells in G2/M phase increased markedly ($P < 0.01$, Figure 3A,B). There was no significant difference in the percentage of GFP-infected cells in G1 phase between the two groups ($P > 0.05$). These results suggest that the down-regulation of UHRF2 arrests U251 glioma cells in the G2/M phase of the cell cycle.

Down-regulation of UHRF2 induced the apoptosis of human U251 glioma cells

We explored the apoptotic rate between U251 glioma cells infected with NC lentivirus or UHRF2-siRNA lentivirus. Flow cytometry analysis showed that the apoptotic rate of GFP-infected U251 cells treated with UHRF2-siRNA ($14.51 \pm 1.35\%$) was significantly higher than the cells treated with scr-siRNA ($8.35 \pm 0.82\%$) ($P < 0.01$, Figure 3C,D). This result indicates that knockdown of UHRF2 gene expression induces the apoptosis of human U251 glioma cells.

Discussion

The UHRF family, including UHRF1 and UHRF2, is considered to be involved in carcinogenesis (Bronner et al., 2007). The UHRF2 gene is localized at chromosome 9p24.1 (Mori et al., 2002). It has been reported that the frequency of deletion at 9p23–p24 is highest in 73 tumor types (Knuutila et al., 1999). Because many important genes, such as JAK2, IL33, and microRNA 101–2, are located at 9p24.1, it is reasonable to hypothesize that its deletion is closely related to human disease, especially malignant disease.

In our study, we detected the expression of UHRF2 mRNA in 32 glioma samples using RT-PCR. Our results showed no significant difference between the noncancerous brain tissue group and the grade I and grade II groups, but UHRF2 mRNA expression was significantly lower in the grade III and grade IV groups compared with the noncancerous brain-tissue group. The results were consistent with DNA copy number profiles of the UHRF2 gene in a glioblastoma dataset (Network CGAR, 2008). Using a cancer outlier profile analysis (COPA), DNA copy number loss of the UHRF2 gene was identified in a variety of malignancies related to the brain (Kotliarov et al., 2006).

However, the expression of UHRF2 mRNA in the glioma cell lines A172, U251, and U373MG but not U87MG was high. The contradictory results between glioma tissue and glioma cell lines may lead to a better understanding of tumor biology. The cell lines used in this study are classical ones; they have been used in glioma studies for many years. The transcriptome and genotypic characterization analyses suggested that the *in vitro* serum-containing medium could cause some uncertain changes in these cell lines, which have been passaged for a long time (Lee et al., 2006). In fact, the dynamics of tumor biology are rather complicated, especially regarding glioma. The malignant progression of glioma includes extreme genome instability and mutation in tumor cells (Hanahan et al., 2011). Glioblastoma represents the most heterogeneous tumor; it is reasonable to hypothesize

that the typical glioma cell line, such as U251, may have undergone mutations during culture. In contrast, the microenvironment, including hypoxic conditions, vascular niche, and the matrix, is very important for the survival of glioma cells (Gilbertson et al., 2007; Oliver et al., 2009; Ulrich et al., 2009). It is possible that these factors may have inhibited the mRNA expression of UHRF2 in glioma cells *in vivo*, which led to contradictory results *in vitro*. Although the expression data showed contradictions, the function of UHRF2 in glioma cell lines has not been studied previously. This research represents the first time the role of UHRF2 has been studied in the U251 glioma cell line.

Like many controversial genes, the role of the UHRF2 gene in tumor tissue is complex. It has been reported that UHRF2 may play important roles in early human development (Kiessling et al., 2009). The overexpression of UHRF2 induces G1 arrest of the HEK293 human embryonic kidney cell line, which indicates that it could behave as an inhibitory factor in cell proliferation (Just, 2012). Other reports have elucidated its oncogenic role in tumor cells. UHRF2 knockdown inhibited the growth of breast cancer cells, specifically with 9p24 amplification, and reduced colorectal cell proliferation and migration (Wang et al., 2012; Wu et al., 2012). Decreased expression of let-7a induced an enhancement of UHRF2, which may contribute to lung carcinogenesis (He et al., 2009). In our study, the results that knockdown of UHRF2 expression inhibited the cell growth of the U251 glioma cell line indicates its tumorigenic role in this cell line.

Cell cycle deregulation, which sustains proliferative signaling and enables replicative immortality, is a feature of malignant tumor cells (Nakayama et al., 2006; Malumbres et al., 2009). Our study showed that knockdown of UHRF2 expression arrests U251 glioma cells in the G2/M phase. The mechanism underlying this result is intricate. The structure of the UHRF2 protein has been identified by X-ray crystallographic and nuclear magnetic resonance (NMR) analyses (Ren et al., 2009). As previously noted, UHRF2 contains five functional domains. The ubiquitin-like domain and ring finger structure lay the foundation for its ubiquitin-proteasome system (Iwata et al., 2009). This system targets cyclins and maintains the orderly progression of the cell cycle (Nakayama et al., 2006). UHRF2 is capable of ubiquitinating two different classes of G1 cyclins, D1 and E1, and two tumor suppressors, p53 and pRB. In HEK293 cells, a human embryonic kidney cell line, the overexpression of UHRF2 induces G1 arrest (Li et al., 2004). However, the results of our study indicate a different role for UHRF2 in the deregulated cell cycle of glioma cells. The protein-protein interaction network shows that UHRF2 forms complexes with cyclins (A, B, D, and E), cyclin-dependent kinases (CDK1, CDK2, CDK4, and CDK6), proliferating cell nuclear antigen (PCNA), p53, pRB, chromatin proteins, chromatin modifiers (HDAC1 and DNMTs), and hepatitis B virus core protein (Mori et al., 2011; Qian et al., 2012). The actual mechanism may be hidden under this complex interaction network.

In addition to its cell cycle machinery and ubiquitin-proteasome system, its epigenetic system is even more

complicated (Gibney et al., 2010). The UHRF2 protein can bind methylated DNA through its SRA domain and H3K9me3 through its TTD-PHD region (Fournier et al., 2011; Parry et al., 2011; Pichler et al., 2011). These processes are important for UHRF2 to be involved in controlling the expression of multiple genes through chromatin and transcriptional regulation (Zhang et al., 2011). In our study, the down-regulation of UHRF2 induced the apoptosis of U251 glioma cells. Because UHRF2 is so widely connected with modification of the epigenetic information of histones and DNA, it is plausible that abrogation could lead to impaired cellular homeostasis and even to cancer cells.

In conclusion, our study provides some evidence that UHRF2 plays an important role in tumorigenesis and the development of gliomas. Further clarification of the molecular mechanism by which UHRF2 influences glioma cells is significant in the field of cancer research. Because UHRF2 constitutes a nodal point for the cellular information flows that influence diverse cellular functions, further research on UHRF2 has the potential to significantly contribute to the advancement of cancer research and treatment.

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