

## microRNA-214-mediated UBC9 expression in glioma

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**It has been reported that ubiquitin-conjugating enzyme 9 (Ubc9), the unique enzyme2 in the sumoylation pathway, is up-regulated in many cancers. However, the expression and regulation of UBC9 in glioma remains unknown. In this study, we found that Ubc9 was up-regulated in glioma tissues and cell lines compared to a normal control. UBC9 knockdown by small interfering RNA (siRNA) affected cell proliferation and apoptosis in T98G cells. Further experiments revealed that microRNA (miR)-214 directly targeted the 3' untranslated region (UTR) of UBC9 and that there was an inverse relationship between the expression levels of miR-214 and UBC9 protein in glioma tissues and cells. miR-214 overexpression suppressed the endogenous UBC9 protein and affected T98G cell proliferation. These findings suggest that miR-214 reduction facilitates UBC9 expression and is involved in the regulation of glioma cell proliferation. [BMB Reports 2012; 45(11): 641-646]**

### INTRODUCTION

Post-translational modifications (PTMs) of proteins play an important role in protein function through the regulation of their structure, activity, degradation, localization and/or interactions. The covalent attachment of small ubiquitin-like modifiers (SUMOs) to different protein substrates, or sumoylation modification, is an important regulator of the functions of many proteins (1, 2). The SUMO polypeptides share a similar structure with ubiquitin, and their conjugation to protein substrates occurs through a similar enzymatic cascade, which includes a sequential action of an E1 activating enzyme, an E2 conjugating enzyme and an E3 protein ligase (3, 4). Unlike ubiquitination, which usually targets substrates to degradation in the proteasome pathway, sumoylation is involved in various cellular processes including cell cycle regulation, gene transcription, subcellular localization, protein-protein interaction, stress response, apoptosis, cell differentiation and the maintenance of

genome integrity (5). As the unique E2 conjugating enzyme, UBC9 is essential for the sumoylation process, and it transfers the activated SUMO polypeptides to protein substrates (6). Recently, many important proteins, including tumor suppressors, oncoproteins and apoptosis-related proteins, have been identified as substrates for sumoylation or shown to interact with UBC9 with their expression localization or activities regulated by UBC9 (7). Some reports also reveal that UBC9 plays a role in tumorigenesis (8, 9). Therefore, UBC9 dysregulation may influence cancer cell growth and development. UBC9 is ubiquitously expressed in all human organs and tissues, and it is frequently up-regulated in tumors. For example, UBC9 is up-regulated in primary colon and prostate cancer (10), ovarian carcinoma (8) and breast cancer (11, 12). However, little is known about the molecular mechanism of UBC9 up-regulation in cancer; only miR-30e was identified as a negative regulator of UBC9 expression in breast cancer (11, 12).

MicroRNAs (miRNAs) are a class of endogenously expressed small noncoding RNAs, which are usually 18-24 nucleotides long and regulate gene expression by mRNA degradation or translation inhibition (13). Recent studies have revealed an important role for miRNAs in tumorigenesis. They function as tumor suppressors or oncogenes by regulating target genes expression (14).

Glioma is the most common type of primary brain tumor (15), yet the expression and regulation of UBC9 in glioma has not been reported. In this study, we show that UBC9 is up-regulated in human glioma tissues and T98G cells. To explore the function of UBC9 in glioma, we knocked down UBC9 in T98G cells and analyzed the role of UBC9 in glioma cellular processes. We then combined computational and experimental approaches to evaluate miR-214 regulation of UBC9 expression in glioma. Meanwhile, we examined miR-214 expression in glioma tissues and cells and examined the effect of miR-214 overexpression on glioma cell proliferation.

### RESULTS

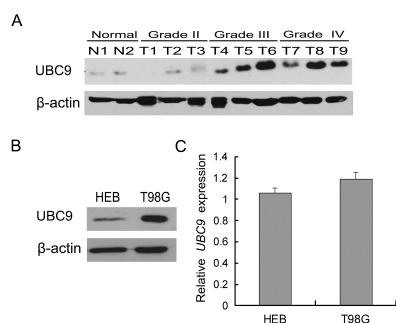
#### UBC9 is up-regulated in human glioma tissues and cells

To detect the expression pattern of UBC9 protein in glioma tissues, western blotting was performed with total protein extracted from 11 primary glioma samples, including three grade II, three grade III and three grade IV glioma tissue samples and two normal brain tissue samples. As shown in Fig. 1A, UBC9

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**Fig. 1.** *UBC9* is up-regulated in glioma cells. (A) A representative western blot showing *UBC9* protein levels in two normal brain tissues (N1, N2) and nine glioma tissues (T1-T3 grade II, T4-T6 grade III and T7-T9 grade IV).  $\beta$ -actin was used as a loading control. (B) A representative western blot showing the *UBC9* protein level in normal glial HEB cells and glioma T98G cells.  $\beta$ -actin was used as a loading control. (C) Real-time PCR analysis of the *UBC9* mRNA level in HEB and T98G cells. RNA input was normalized to human *GAPDH*. The values represented the mean  $\pm$  standard deviation (SD) of triplicate wells.

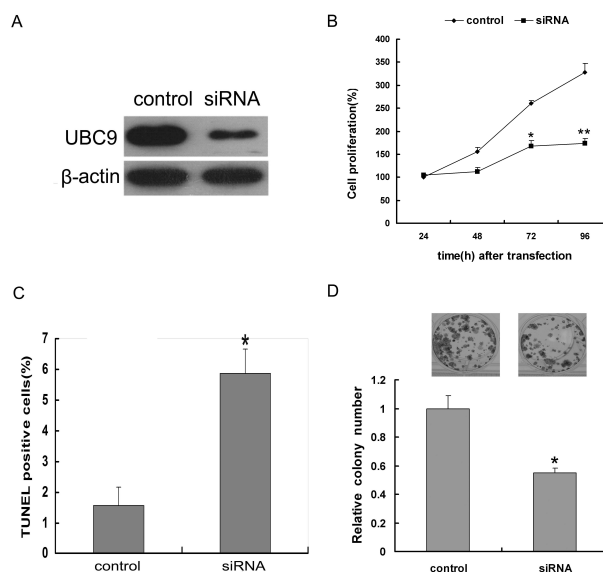
protein was up-regulated in the glioma tissues when compared to the normal brain tissues. We then examined the *UBC9* protein concentration in normal glial HEB cells and glioma T98G cells and found that it was markedly increased in T98G cells when compared to HEB cells (Fig. 1B), indicating that *UBC9* is significantly up-regulated in T98G cells. Meanwhile, the *UBC9* mRNA level exhibited a small difference between HEB and T98G cells (Fig. 1C).

### *UBC9* knockdown affects T98G cell proliferation

To investigate the function of up-regulated *UBC9* in T98G cells, we transfected T98G cells with *UBC9* siRNA and analyzed the cell phenotype induced by *UBC9* knockdown. As shown in Fig. 2A, endogenous *UBC9* protein was significantly reduced by siRNA in T98G cells. To determine the role of *UBC9* knockdown on cell growth, we performed an MTT assay and found that T98G cells exhibited significant growth suppression compared to control cells (Fig. 2B). However, a TUNEL assay showed that *UBC9* reduction promoted cell apoptosis compared to the control (Fig. 2C). Meanwhile, a colony-forming assay showed that T98G cells transfected with *UBC9* siRNA formed fewer colonies than did control cells (Fig. 2D).

### miR-214 directly targets the 3'UTR of *UBC9*

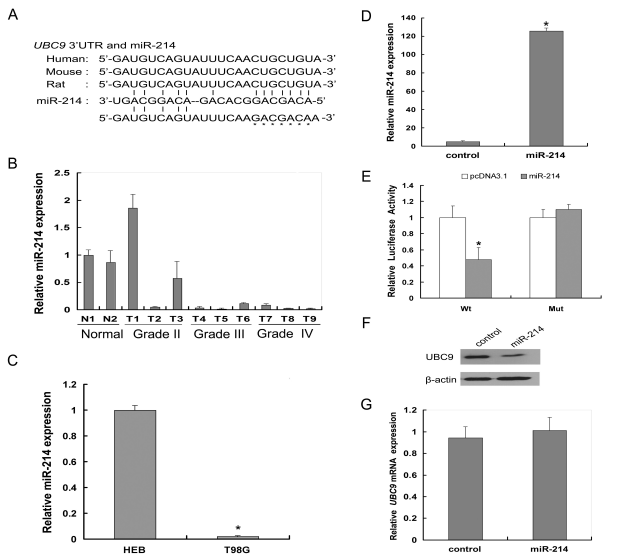
The regulation of gene expression occurs at several levels, including gene amplification, transcriptional regulation, post-transcriptional regulation and translational regulation. Due to the small difference in the *UBC9* mRNA expression level between HEB and T98G cells, we hypothesized that *UBC9* expression in T98G cells is post-transcriptionally and/or translationally regulated. In this study, we focused on miRNAs that could regulate *UBC9* expression. To identify miRNAs that reg-



**Fig. 2.** *UBC9* knockdown affects cell proliferation and apoptosis in T98G cells. (A) A representative western blot showing that *UBC9* protein was reduced by siRNA in T98G cells.  $\beta$ -actin was used as a loading control. (B) An MTT assay of T98G cells following treatment with miRNA control or siRNA. The data are expressed as the percentage of cell proliferation compared to the initial absorbance at 24 h post-transfection. (C) A TUNEL assay of T98G cells 48 h after transfection with control or siRNA. The percentage of apoptotic cells was calculated and plotted in a histogram. (D) A colony-forming assay of T98G cells after transfection with control or siRNA. Two weeks later, the colonies were stained with MTT, the histogram shows the relative colony number compared to the control cells. The values represented the mean  $\pm$  standard deviation (SD) of triplicate wells. The asterisks indicate \* $P < 0.05$  and \*\* $P < 0.01$  compared to control.

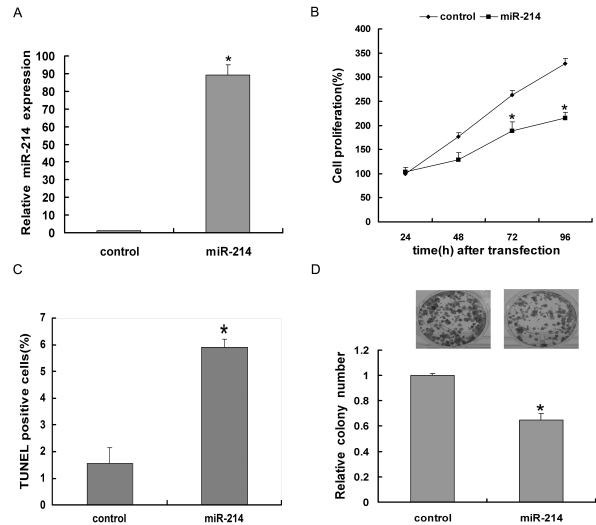
ulate *UBC9* expression in glioma cells, we used a bioinformatics analysis database and found that the 3'UTR of *UBC9* contained a typical miR-214 binding site that is highly conserved among mammals (Fig. 3A). Real-time PCR performed with the total RNA extracted from the same 11 primary glioma samples showed that miR-214 was expressed at a lower level in the tumor tissues than in the normal brain tissue samples (Fig. 3B). Additionally, miR-214 expression was significantly decreased in glioma T98G cells when compared to normal glial HEB cells (Fig. 3C), suggesting that an inverse relationship exists between the expression of miR-214 and *UBC9* in glioma tissues and cells.

To examine whether miR-214 directly targets the 3'UTR of *UBC9* mRNA, we first confirmed that ectopic expression in 293ET cells generated mature miR-214 using real-time PCR (Fig. 3D) and then evaluated the effect of miR-214 on *UBC9* expression. miR-214 was subjected to validation by luciferase reporter assays in 293ET cells. 293ET cells transfected with miR-214 expression plasmids displayed a reduction of approximately 50% in luciferase activity compared to those trans-



**Fig. 3.** miR-214 down-regulates *UBC9* by targeting the 3'UTR of *UBC9*. (A) Schematic representation of the miR-214 binding site in the 3'UTR of *UBC9*. The mutant sequence of the miR-214 binding site is identical to the wild-type sequence marked by asterisks. (B) A real-time PCR analysis of the miR-214 level in the same two normal brain tissues (N1, N2) and nine glioma tissues (T1-T3 grade II, T4-T6 grade III and T7-T9 grade IV). The RNA input was normalized to *U6*. (C) A real-time PCR analysis of miR-214 level in HEB and T98G cells. RNA input was normalized to *U6*. (D) Overexpression of miR-214 in 293ET cells was measured by real-time PCR 48 h after transfection with the miR-214 expression plasmid. RNA input was normalized to *U6*. (E) Luciferase reporter assay. 293ET cells were transfected with the luciferase reporter constructs containing a wild-type or mutant sequence in the miR-214 binding site and the miR-214 expression plasmid. The relative luciferase activities were normalized with the Renilla luciferase activities. The values are expressed as percentages of relative luciferase activity of the pcDNA3.1-luc plasmid. (F) A representative western blot of endogenous *UBC9* protein in T98G cells transfected with miR-214 mimics.  $\beta$ -actin was used as a loading control. (G) A real-time PCR analysis of the effect of miR-214 on *UBC9* mRNA. RNA input was normalized to *GAPDH*. The data are presented as the mean  $\pm$  standard deviation (SD) of triplicate wells. The asterisks indicate \* $P < 0.05$  compared to control.

fectured with negative control vectors. When the hypothesized miR-214 binding site in the 3'UTR of *UBC9* mRNA was mutated, luciferase activity was restored and was similar to the control level (Fig. 3E), suggesting that miR-214 directly targets the binding site in the 3'UTR of *UBC9*. To verify that miR-214 regulates *UBC9* protein expression, miR-214 mimics were transfected into T98G cells, and the protein and total RNA were extracted 48 h after transfection. Western blotting demonstrated that there was a significant reduction in the *UBC9* protein level after miR-214 overexpression (Fig. 3F), while real-time PCR indicated that miR-214 overexpression had little effect on *UBC9* mRNA (Fig. 3G), indicating that miR-214 negatively regulated *UBC9* expression by inhibiting translation.



**Fig. 4.** miR-214 affects cell proliferation and apoptosis. (A) A real-time PCR analysis of miR-214 overexpression in T98G cells 48 h post-transfection. RNA input was normalized to *U6*. (B) An MTT assay of T98G cells after treatment with control or miR-214 mimics as above. (C) TUNEL assays were performed 48 h post-transfection with control or miR-214 as above. (D) Colony-forming assay. T98G cells were transfected with control or miR-214 mimics as above. The histogram shows the relative colony number compared to the control cells. The values represented the mean  $\pm$  standard deviation (SD) of triplicate wells. The asterisks indicate \* $P < 0.05$  compared to control.

### miR-214 affects T98G cell proliferation

To determine the effect of miR-214 on tumor cellular processes, we transfected T98G cells with miR-214 mimics. Real-time PCR demonstrated that mature miR-214 significantly increased after miR-214 transfection (Fig. 4A). We then examined T98G cell proliferation using the MTT assay. As shown in Fig. 4B, T98G cells transfected with miR-214 exhibited significant growth suppression compared to those transfected with negative control miRNA. Moreover, miR-214 overexpression promoted T98G cell apoptosis compared to the control group (Fig. 4C), and a colony-forming assay showed that the miR-214-transfected cells formed fewer colonies than control cells (Fig. 4D).

### DISCUSSION

Sumoylation is an important post-translational modification and plays a key role in diverse cellular processes. Similar to ubiquitination, sumoylation also occurs through an enzymatic cascade to conjugate the SUMO peptide to substrates. Dysregulation of *UBC9*, the unique enzyme 2 in the sumoylation pathway, may lead to alterations in the sumoylation process or may only affect the sumoylation modification of some key proteins, ultimately impacting cell growth and cancer development. In our study, we found that *UBC9* was up-regu-

lated in human glioma tissues and T98G cells. To our knowledge, this is the first study to evaluate *UBC9* expression in glioma. Previous studies have reported that *UBC9* up-regulation exists in many cancer specimens (10-12). Additionally, *UBC9* suppression has been reported to induce cell growth inhibition, whereas ectopic *UBC9* expression has been reported to enhance tumor growth in animal models (8), suggesting that *UBC9* may play a key role in tumorigenesis. In our study, we also found that *UBC9* down-regulation induced cell growth suppression.

The regulation of gene expression occurs on many levels, including transcriptional, post-transcriptional and translational. According to the small difference in *UBC9* mRNA expression between HEB cells and T98G cells, we speculate that miRNAs may be involved in the regulation of *UBC9* expression in glioma cells. To evaluate miRNA regulation of *UBC9* expression, we first analyzed the 3'UTR of *UBC9* and identified a conserved miR-214 binding site in the 3'UTR sequence. Meanwhile, real-time PCR indicated that miR-214 expression inversely correlated with *UBC9* protein expression in glioma tissues and cells, suggesting that miR-214 may negatively regulate *UBC9* expression. A luciferase reporter assay indicated that miR-214 directly interacts with this binding site. Real-time PCR and western blotting revealed that miR-214 specifically suppressed *UBC9* expression at the translational level. Furthermore, miR-214 overexpression and *UBC9* knockdown had a similar effect on cell proliferation. In our study, we found that ectopic miR-214 expression induced *UBC9* reduction and cell growth inhibition that was less than that caused by *UBC9* siRNA transfection, suggesting that other miRNAs or factors may be involved in the regulation of *UBC9* expression in glioma cells.

miRNAs can function as tumor suppressors or oncogenes (14). Aberrant miRNA expression is thought to play broad roles in tumorigenesis. An increasing number of miRNAs are being shown to play a role in cancer development through the examination of miRNA expression in clinical samples (16,17). The miR-214 expression pattern has been shown to vary in different cancer cells (18-21). In our study, we found that ectopic miR-214 expression caused cell growth inhibition, suggesting that miR-214 may act as a tumor suppressor gene, possibly by suppressing tumor-promoting factors in glioma cells, in accordance with the study on miR-214 in cervical cancer (22). miR-214 down-regulation in glioma tissues and cell lines supports this notion.

Previous studies have shown that Twist-1 regulates the expression of the miR-199a/214 cluster and that miR-214 was deleted in breast cancer (23, 24). Future studies are necessary to determine whether *UBC9* expression is transcriptionally regulated, how miR-214 is down-regulated in glioma and how many miRNAs and other factors are involved in the regulation of *UBC9* expression. These future studies will be important not only for understanding the regulation of *UBC9* expression in cancer but also for providing some clues for cancer therapy.

## MATERIALS AND METHODS

### Human tissue samples

All human glioma tissue and normal brain tissue samples were obtained from the Department of Neurosurgery, Beijing Tiantan Hospital. The tumor samples were classified according to the third edition on the histological grades of tumors of the nervous system published by the WHO in 2000. This study was approved by the institutional ethics committee.

### Cell lines and cell culture

The human glioma cell line T98G was purchased from American Type Culture Collection (ATCC). The human embryonic brain (HEB) cell line was kindly provided by Dr. Guangmei Yan (Zhongshan School of Medicine, Sun Yat-Sen University, China). The human 293ET cell line was a gift from Dr. Chengyu Jiang (Peking Union Medical College, China). All of the cells were cultured according to the guidelines recommended by ATCC, and the cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>-95% air.

### Plasmids and oligomers

The *UBC9* 3'UTR was amplified from normal human genomic DNA by PCR. The fragment was cloned into the *Xho* I and *Xba* I sites of pcDNA3.1-LUC vector, which was then named *UBC9*-Wt. The pcDNA3.1-LUC vector was modified from pcDNA3.1 (+) (Invitrogen, USA) as previously described (25). The *UBC9* mutant 3'UTR plasmid with an altered miR-214 binding site was generated by bridge PCR and named *UBC9*-Mut. For the miRNA expression plasmid, the genomic sequence of the pre-miRNA was amplified from human genomic DNA and then cloned into the *Bam*HI and *Xho* I sites of the pcDNA3.1 (+) vector. All constructs were confirmed by DNA sequencing. The sequence of human *UBC9* siRNA was 5'-GGCCAGCCAUCACAAUCAA-3' and the negative control sequence was 5'-AACCUAGAAGGACCGUUUGUG-3'. The mature miR-214 mimic sequence was 5'-ACAGCAGGCACA GACAGGCAGU-3', and the negative miRNA control sequence was 5'-UCACAGUGAACCGGUCUCUUU-3'. All of these RNA oligomers were synthesized by the Shanghai GenePharma Company (China).

### miRNA and siRNA transfection

Mature miR-214 and siRNA were transfected into T98G cells using lipofectamine 2,000 (Invitrogen, USA), according to the manufacturer's instructions, at a final concentration of 100 nM.

### RNA extraction and real-time PCR

Total RNA was isolated from human tissue samples or cells using TRIZOL (Invitrogen, USA). Reverse transcription was performed using the First-Strand cDNA kit (TransGen Biotech, China) according to the manufacturer's protocol. Stem-loop RT-PCR for the mature miRNAs was performed as previously described (26). Real-time PCR was performed using the SYBR

Green PCR Master Mix kit (Takara, Japan) according to the manufacturer's instructions on the ABI Prism 7500 sequence detection system. The expression of mRNA and miRNA was quantified with real-time PCR using the following primers: GAPDH, forward 5'-GAGAAGGCTGGGGCTCATTTGCA-3', reverse 5'-TTGGCCAGGGGTGCTAAGCAGT-3', *UBC9*, forward 5'-ATGGAGGAAAGACCACCCATTTG-3', reverse 5'-CCACGGAGTCCCTTTCTTTCC-3', U6, forward 5'-GCGCGTCGTGAAGCGTTC-3', miR-214, forward 5'-ACAGCAGGCACA GACAGG-3', universal reverse primer for miRNA 5'-GTGCAG GGTCCGAGGT-3'. The RNA input was normalized to the level of human GAPDH mRNA for protein-coding genes, while that of the endogenous snRNA U6 was normalized to mature miRNA.

#### Luciferase reporter assay

Twenty-four hours before transfection, 293ET cells were plated in a 24-well plate. The wt/mut *UBC9*-3'UTR (200 ng), empty pcDNA3.1/pcDNA3.1-miR-214 (1 µg) and pRL-TK (50 ng) plasmids were transfected into 293ET cells using the Vigorous transfection reagent (Vigorous, China). After 48 h, the cells were processed for luciferase assays using the Dual Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. The data were normalized by dividing firefly luciferase activity with that of Renilla luciferase.

#### Western blotting

The protocol for western blotting was described previously (25). Immunoblots were performed with the following antibodies: anti-*UBC9* (Cat. AB75854, Abcam, USA), anti-β-actin (Cat. A5441, Sigma, USA).

#### Cell proliferation and apoptosis assay

The dimethyl thiazolyl diphenyltetrazolium (MTT) assay was performed as described previously (27).

After transfection with miRNA or siRNA for 48 h, a terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) assay was performed with the In Situ Cell Death Detection Kit (Roche, Switzerland) according to the manufacturer's instructions.

#### Colony-forming assay

A total of  $2 \times 10^3$  cells (transfected with miRNA or siRNA) were seeded per 12-well plate and each treatment was carried out in triplicate. During colony growth, the culture medium was replaced every 3 days. Two weeks later, 200 µl of 5 mg/ml MTT was added per well to the medium to stain the colonies.

#### Databases and bioinformatics analysis

The miRNA sequence was obtained from miRBase (<http://microma.sanger.ac.uk/sequences/>), and *UBC9*-targeting miRNAs were predicted at the PicTar website ([http://pictar.mdc-berlin.de/cgi-bin/new\\_PicTar\\_vertebrate.cgi](http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi)).

#### Statistical analysis

Data are shown as the mean ± standard deviation (SD). Statistical significance was determined by Student's *t*-test, and all tests were two-sided. Differences were considered to be statistically significant at  $P < 0.05$ .

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