Loss of ATRX suppresses ATM dependent DNA damage repair by modulating H3K9me3 to enhance temozolomide sensitivity in glioma

Bo Han, Jinquan Cai, Weida Gao, Xiangqi Meng, Fei Gao, Pengfei Wu, Chunbin Duan, Ruijia Wang, Magafurov Dinislam, Lin Lin, Chunsheng Kang, Chuanlu Jiang

Department of Neurosurgery, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China
Chinese Glioma Cooperative Group (CGCG), Beijing 100050, China
Neuroscience Institute, Heilongjiang Academy of Medical Sciences, Harbin 150086, China
Department of Laboratory Diagnosis, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China
Department of Neurosurgery, Tianjin Medical University General Hospital, Lab of Neuro-oncology, Tianjin Neurological Institute, Key Laboratory of Post-Neuroinjury Neuro-repair and Regeneration in Central Nervous System, Ministry of Education and Tianjin City, Tianjin, 300052, China

Abstract

Mutations in ATRX constitute the most prevalent genetic abnormalities in gliomas. The presence of ATRX mutations in glioma serves as a marker of better prognosis with longer patient survival although the underlying mechanisms are poorly understood. In the present study, we found that ATRX biological function was significantly involved in DNA replication and repair. CRISPR/Cas9-mediated genetic inactivation of ATRX induced inhibition of cell proliferation, invasion and vasculogenic mimicry. In addition, temozolomide (TMZ) treatment induced greater DNA damage and apoptotic changes in ATRX knockout glioma cells. Moreover, we confirmed that ATRX knockout resulted in a failure to trigger ATM phosphorylation and finally restrained the activation of downstream proteins of the ATM pathway. The ATM-associated DNA repair pathway was extensively compromised in ATRX knockout cells owing to decreased histone H3K9me3 availability. Public databases also showed that patients with low ATRX expression exhibited preferable overall survival and profited more from TMZ treatment. These data suggest that ATRX is involved in DNA damage repair by regulating the ATM pathway and might serve as a prognostic maker in predicting TMZ chemosensitivity.

1. Introduction

Glioma constitutes the most malignant intracranial tumor occurs in adults and its high invasiveness and malignancy severely shortens patient overall survival. Standard treatment involves surgery, radiation and chemotherapy with TMZ. Patients with GBM receiving treatment only exhibit a median of less than 14 months overall survival [1]. A major aspect attributed to the unfavorable outcome is the development of chemoresistance by glioma. Therefore, better understanding of the mechanisms involved in GBM chemoresistance is urgently needed.

Mutations of alpha thalassemia/mental retardation syndrome X-linked (ATRX), which encodes a SWI/SNF-like protein, were first discovered in patients bearing the X-linked alpha thalassemia/mental retardation syndrome [2,3]. In isocitrate dehydrogenase (IDH)-wild-type infiltrating gliomas, ATRX alterations were...
associated with favorable outcomes [4]. Our previous studies showed that ATRX expression was associated with the DNA methylation level of chromosome ends in glioma and knockdown of ATRX by short hairpin RNA restrained the migration and proliferation but induced apoptosis of glioma cell [5,6]. ATRX deficiency also impaired non-homologous end joining and increased sensitivity to DNA-damaging agents that induce double-stranded DNA breaks in glioma [7]. However, the mechanism of ATRX in regulating glioma malignancy and chemoresistance requires further research.

Ataxia telangiectasia mutated (ATM) kinase functions as a critical protein in sensing and repairing DNA double-stranded breaks (DSBs) such as those induced by TMZ [8–10]. Previous studies had revealed that ATM activation caused by self-inflicted DNA DSBs could promote stemness of patient-derived glioma cells and control glioma chemoresistance by regulating 3-methylpurine-DNA glycosylase [11,12]. However, the activation of ATM is regulated by multiple factors including Tip60 acetyltransferase activity and histone H3 lysine 9 trimethylation (H3K9me3) status [13], such that the loss of H3K9me3 caused ATM phosphorylation deficiency [14]. Notably, the ADD domain facilitates ATRX binding to histone H3K9me3 and preserving trimethylation [15,16]. These studies suggested that ATRX might regulate DNA damage by modulating the activity of ATM.

In the current study, we aimed to explore whether ATRX knockout (KO)-mediated increased sensitivity to a DNA-damaging agent was associated with the ATM pathway. We confirmed that ATRX biological function correlated with DNA repair. To explore the effect of ATRX loss in glioma cells, we established ATRX KO glioma cell lines. We observed a reduction in proliferation and migration and increased sensitivity to TMZ among CRISPR/Cas9-treated ATRX KO glioma cells. In addition, increased chemosensitivity to TMZ was confirmed by western blot and surveyor assay analysis. CRISPR/Cas9/ATRX-sgRNA plasmid pairs and lentiviruses were synthesized and purchased from Genechem Company (Shanghai, China).

Cells for transfection were seeded in 6-well plates at 70%–80% confluence. Plasmid carrying nonsense normal control sequence or full length KDM4D was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 48 h, the cells were treated with TMZ for further experiments.

2.3. CRISPR/Cas9-mediated ATRX-knockout and plasmid transfection

For ATRX knockout, viruses containing the CAS9 gene were transfected to cells with infection duration of another 48 h. Then, the supernatant was changed to fresh culture medium containing 3 μg/ml puromycin for 7 days. The cells were subsequently infected with virus carrying small guide (sg) RNAs designed for ATRX. The infection proceeded for 24 h and ATRX gene expression and KO was confirmed by western blot and surveyor assay analysis.

2.4. Surveyor assay

Surveyor assay was carried out following the manufacturer’s instruction in the Knockout and Mutation Detection Kit (Genesci, Shanghai, China). In brief, cells transfected with Cas9 and sgRNA lentivirus were incubated at 37 °C for 72 h before genomic DNA extraction. Total genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Purified genomic DNA (100 ng) was mixed with 2× Taq Plus Master Mix, specific primer pairs designed for the CRISPR target site (Supplementary Table S1), and ddH2O to a final volume of 25 μl. The PCR procedure was carried out with 94 °C, 5 min for 1 cycle, 94 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s for 40 cycles, 72 °C, 5 min, and 98 °C, 3 min, then cooled to room temperature. The KO-group product (200 ng) was mixed with 200 ng NC-group product, to which 1 μl Detcase and 2 μl Detcase Buffer was added followed by ddH2O to a final volume of 10 μl, and incubated at 45 °C for 15 min. Then, 2 μl Stop Buffer was added into the mixture. Final products were analyzed by agarose gel electrophoresis to detect the mutation.

2.5. Immunoblotting

Western blot (WB), immunofluorescence (IF), and immunohistochemistry (IHC) assays were performed as previously described [17]. Rabbit anti-ATRX (1:1000, Abcam, Cambridge, UK), rabbit anti-ATM, anti-p-ATM, anti-p-RAD50, anti-p-p53, anti-γH2AX, anti-p-Chk1, anti-p-Chk2, anti-p-p53, anti-Histone H3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-H3K9me3 (1:1000, Active Motif), rabbit anti-TRIM28 mouse anti-GAPDH, anti-p-ATM, anti-p-RAD50, anti-p-p53, anti-γH2AX, anti-p-Chk1, anti-p-Chk2, anti-p-p53, anti-Histone H3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-H3K9me3 (1:1000, Active Motif), rabbit anti-TRIM28 mouse anti-GAPDH,
and anti-β-actin (1:1000, ABclonal, Cambridge, MA, USA) were used along with horseradish peroxidase-labeled secondary antibody (1:4000, Zsbio, Beijing, China) in WB. Rabbit antibodies anti-ATRX, anti-p-ATM, anti-p-RAD50 (1:200, Cell Signaling Technology), anti-PDGFRα (1:200, Boster Bio, Pleasanton, CA, USA), mouse anti-H3K9me3 (1:200, Active Motif, Carlsbad, CA, USA), with horseradish peroxidase-labeled secondary antibodies (1:200, Boster) and 594-labeled secondary antibody (1:1000, Invitrogen), were used for immunoprecipitation (IP) and rabbit anti-ATRX (1:50, Cell Signaling Technology) were used for immunoprecipitation.

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (TaKaRa, Otsu, Japan). The cDNAs were synthesized with a PrimeScript RT reagents kit (TaKaRa) according to manufacturer’s instructions. Endogenous mRNA levels of DNM3TA, RAD50 and PDGFRα were determined using the SYBR PrimeScript RT-PCR Kit (Roche, Kumamoto, Japan). The cDNAs were synthesized with a PrimeScript RT reagents kit (TaKaRa) according to manufacturer’s instructions. Endogenous mRNA levels of DNM3TA, RAD50 and PDGFRα were determined using the SYBR PrimeScript RT-PCR Kit (Roche, Roswell, GA, USA). qRT-PCR data were analyzed using the 2^{-ΔΔCt} method.

2.7. Chromatin immunoprecipitation (ChIP) and ChIP-sequencing (ChIP-Seq) analysis

ChIP experiments were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore, 17-295) and the anti-ATRX antibody (Cell Signaling Technology, 14820S) following the manufacturer's protocol for Pure Proteome Protein A/G Mix Magnetic Beads (Merck Millipore, Billerica, MA, USA) and the samples were analyzed using WB. Rabbit anti-ATM (1:50 Abcam), anti-Mouse H3K9me3 (1:50, Active Motif), and rabbit anti-ATRX (1:50, Cell Signaling Technology) were used for immunoprecipitation.

2.8. Functional annotation

GO and KEGG Pathway analysis were performed using the online database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.ncifcrf.gov/) [18], ClueGO and BiNGO (plugin for Cytoscape) [19].

2.9. Cell cycle analysis and apoptosis detection

For cell cycle analysis, cells were collected and fixed in ice-cold 75% ethanol for 12 h, then centrifuged at 1500 rpm for 10 min. The supernatant was discarded and cells were washed with ice-cold PBS twice. Cells were resuspended in 500 μl propidium iodide (BD) staining buffer for 30 min at room temperature. Stained cells were analyzed using FACS Canto II (BD).

The apoptosis assay was applied in U251, LN229 and HG6 cells after ATRX knockout using allophycocyanin/propidium iodide (BD) and analyzed on FACS Canto II (BD).

2.10. F-actin staining assay and 5-ethyl-2'-deoxyuridine (EdU) labeling

F-actin staining was performed using TRITC-labeled Phalloidin (Yeasen, Shanghai, China). In brief, cells were seeded on tissue culture-treated glass coverslips. After 24 h, cells were washed in pre-warmed PBS twice, fixed with 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100. After washing with PBS, cells were blocked with 5% bovine serum albumin and incubated with 100 nM phalloidin at 37 °C followed by DAPI staining. Cells were finally observed using a fluorescence microscope (Nikon C2, Tokyo, Japan).

For EdU labeling, cells were seeded into tissue-culture treated slides (Nest, Rahway, NJ, USA) overnight in cell culture medium for 12 h. Then, cells were incubated with 5 μM EdU (Ribobio, Guangzhou, China) for 4 h. The cells were fixed and labeled with Apollo 567 (Ribobio) and Hoechst according to the manufacturer's protocol.

2.11. Vascular mimicry assay cell viability analysis, soft agar colony formation, and colony formation assay

For the vascular mimicry assay, 3 × 10^4 cells were seeded into 48-well dishes pre-coated with Matrigel. Tube formation was assessed using a phase-contrast microscope 12 h after seeding. CCK-8 (Dojindo, Kumamoto, Japan) was used to determine the number of viable cells according to the manufacturer’s protocol. Cell viability was measured by optical absorbance on an Epoch plate reader (Biotek Instruments, Winooski, VT, USA).

For the soft agar assay, 24-well plates were pre-coated with 0.5 ml 1 × concentration complete 1640 medium (Gibco, Gaithersburg, MD, USA) containing 0.6% agarose (Yeason) and 10% FBS. Then, tumor cells were suspended in 1 ml complete 1640 medium containing 0.35% agarose. When the bottom layer of agarose was coagulated, fully-suspend cells were placed into the upper layer and incubated at 37 °C with 5% CO₂ for 3 weeks. Cell colonies were stained with 0.1% crystal violet for 15 min and observed using a Bio-Rad ChemiDoc™ imaging system (Berkeley, CA, USA).

For colony formation assays, 6-well plates were seeded with 500 cells as indicated. The assays were stopped when the colonies could be observed clearly with the naked eye. Then, cells were fixed with methanol and stained with 0.1% crystal violet for recording colony numbers.

2.12. Transwell invasion assay and comet assay

The transwell invasive assay was performed in 24-well cell culture chambers with transwell inserts (Corning) (8-μm pore) pre-coated with Matrigel. In brief, cells with ATRX-WT or ATRX KO were seeded at a density of 5 × 10^4 cells per upper well in 200 μl culture medium (DMEM/F12, 4% FBS), with the lower chambers filled with 500 μl medium (DMEM/F12, 50% FBS). After 24 h, the upper surface was removed by scrubbing with a cotton-tipped stick, whereas the lower surface was fixed with methanol for 5 min, air-dried, and stained with hematoxylin and eosin (H&E). All experiments were performed in triplicate.
For the comet assay, cells were seeded in 12-well plates overnight and then treated with TMZ for 12 h and collected. Then, the cells were treated per manufacturer introduction (Trevigen, Gaithersburg, MD, USA) and stained with 1× SYBGreen (Sangon, Shanghai, China).

2.13. Luciferase reporter assay

Cells were seeded at 4 × 10^4 cells/well in 24-well plates and allowed to settle overnight. The next day, cells were transfected with pGL4.74, pGL4.34, pGL4.39, pGL4.42, pGL4.33, pGL4.44, pGL4.45, pGL4.43, pGL4.40, pGL4.48, pGL4.37, pGL4.30, pGL4.41, pGL4.29, pGL4.52, or pGL4.47 plasmid and co-transfected with pGL4.75 for 48 h, after which cell lysates were prepared and quantified using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Both firefly and Renilla luciferase activities were calculated and the firefly/Renilla luciferase activity was recorded as fold induction.

2.14. Tumor xenograft study

In brief, ATRX KO and control cells (3 × 10^5 cells per mouse in 3 μl total) transfected with luciferase lentivirus were injected into the intracranial space of 5-week-old female nude mice purchased from Beijing Vital River Laboratory Animal Technology as described previously [17]. After 14, 21, 28 and 42 days, tumors were measured by bioluminescence using an IVIS Lumina Imaging System (Xenogen, Alameda, CA, USA). After tumor bioluminescence imaging signal reached 1.5 × 10^8 p/sec/cm^2/sr, TMZ (60 mg/kg) was intraperitoneally injected daily for 6 days. After 7 days treatment with TMZ, tumors were re-measured by bioluminescence. Cryosections (4-mm) were stained with H&E and used for IHC. The protocol for animal studies was approved by the Clinical Research Ethics Committee of the 2nd Affiliated Hospital of Harbin Medical University.

2.15. Statistics

According to the cutoff value (the most significant split) [20], the student t-test and Chi-square test were used to determine the significance of differences between the two groups. Overall survival curves were plotted according to the Kaplan-Meier method and the log-rank test was applied for comparison. All differences were considered statistically significant at the level of P < 0.05. Statistics were performed using SPSS Graduate Pack 19.0 statistical software (SPSS, Chicago, IL, USA) or GraphPad Prime 15.0 (Lajolla, CA, USA).

Fig. 1. ATRX is involved in the DNA damage repair process. (A–B) GO and KEGG pathway analysis for ATRX in glioblastoma shows that ATRX function involves DNA replication, Cell cycle, and DNA repair. (C) CRISPR-Cas9 schematic showing the Cas9 protein, sgRNA sequences and binding sites. Western blots show the protein level of ATRX is reduced in KO group compared to NC group after transfection with lentivirus.
3. Results

3.1. ATRX is involved in the DNA damage repair process

To explore the biological function of glioma with distinct ATRX expression levels, we analyzed GBM datasets from TCGA. We identified the genes correlated with ATRX and annotated the biological process and signaling pathways using GO and KEGG pathway analysis. The genes correlating with ATRX expression were strongly involved in “chromatin organization” and “DNA process”, especially the cell cycle. Additionally, similar results were also verified in ATRX-related pathways: the genes were mostly enriched in the cell cycle and DNA repair-associated pathways such as DNA replication and mismatch repair (Fig. 1A and B).

To observe the ATRX function in glioma, we utilized CRISPR/Cas9 technology to create a highly specific genomic scissors targeting ATRX (Fig. 1C, Supplementary Fig. S1A, B). After cells were infected with Lenti-cas9 and ATRX-targeted lentivirus, we performed surveyor and WB assays to confirm the efficiency of ATRX knockout (Fig. 1C, Supplementary Fig. S1C).

3.2. ATRX KO restrains glioma cell malignant behaviors

Searches of TCGA public data showed that ATRX expression exhibited positive correlation with genes regulating the ATM signaling pathway including ATM, RAD50, and CHK1 (Fig. 2A, Supplementary Fig. S2A). To further investigate the role of ATRX in glioma cells, we carried out RNA Expression Microarray analysis for ATRX NC and ATRX KO LN229 cells. We subjected the genes exhibiting association with ATRX KO (Student t-test $P < 0.05$, $|\text{Fold change}| > 2$) to GO analysis. We found that ATRX correlated genes were involved in the regulation of cell cycle, regulation of mesenchymal stem cell differentiation, DNA replication, and DNA-dependent RNA replication (Supplementary Fig. S2B, C). Similarly, the down regulated genes in ATRX KO cells included some ZNF transcriptional factor family genes and some genes involved in DNA repair, stemness, cell proliferation, and oncogenic programs (Fig. 2B). We then used qPCR to verify the changes in glioma cells (Fig. 2C). These observations suggested that ATRX potentially playing an important role in gliomagenesis.

To better understand the functions of ATRX in tumorigenesis, we performed several assays to evaluate the effect of ATRX knockout.

Fig. 2. ATRX knockout restrains glioblastoma cell malignant behaviors. (A) ATRX expression level positively correlates with those of genes that regulate DNA damage including ATM, BRAC1, and RAD51. (B) Heatmap of the differentially-expressed genes associates with tumorigenesis between NC cells ($n = 3$) and KO cells ($n = 3$) including ZNF transactivators, DNA repair markers, stem markers, and oncogenes. (C) QRT-PCR reveals mRNA alteration corresponding to that determined by expression microarray in glioma cells. (D) Neurosphere formation assay shows ATRX KO inhibits the formation and growth of glioma spheres. (E) Western blots demonstrate that the protein level of a stem markers are downregulated in ATRX KO stem-like glioma cells. (F, G) EdU staining assay and soft agar assay are performed to evaluate the proliferative ability and transformation abilities between ATRX KO and ATRX NC cells.
ATRX depletion reduced the growth of glioma stem-like cells and restrained the protein expression of stem markers (Fig. 2D and E). ATRX knockout also could downregulate the activity of transactors in glioma cells (Supplementary Fig. S2D). Reduced DNA-replication and capacity of transformation were also detected via EdU assay, soft agar assay, and colony formation assay (Fig. 2F and G, Supplementary Fig. S2E). In addition, we also observed filopodia formation and lamellipodia disappearance (Supplementary Fig. S2F). These results indicated that ATRX KO restrained glioma cell malignant behaviors.

3.3. ATRX binds widely throughout the genome and regulates DNA replication

Previous studies have presented ATRX as a chromatin remodeler in governing genomic stability through the regulation of repetitive sequences [21]. To investigate the distribution of ATRX protein across the genome, we carried out whole-genome ChIP-Seq (Supplementary Fig. S3A). The results indicated ATRX as being distributed widely throughout the genome and ATRX-bound peaks were mainly distributed in gene bodies and transcription start sites (Fig. 3A and B Supplementary Fig. S3B, C, D). We entered the genes adjacent to ATRX-binding peaks into BiNGO and observed their predominant enrichment in DNA processing including “cell cycle”, “DNA repair,” and “DNA metabolic process” (Fig. 3C). ClueGO analysis also showed these genes as being involved in certain tumor associated signaling pathways and tumor cell biological behaviors (Fig. 3D, Supplementary Fig. S3E). These results indicated ATRX as potentially functioning as a monitor in regulating the cell cycle and DNA repair in glioma cells.

3.4. ATRX loss increases tumor sensitivity to TMZ

TMZ, the major chemotherapeutic agent used for GBM treatment, causes DNA damage in tumor cells. To explore whether ATRX loss affords chemosensitivity in glioma cells, we first carried out a CCK-8 assay to assess cell viability under TMZ treatments. ATRX KO glioma cells were more vulnerable to TMZ (Fig. 4A), suggesting that ATRX loss increased glioma cell sensitivity to TMZ. Similar results were revealed in the tube-formation assay (Supplementary Fig. S4A). Additionally, we confirmed the presence of DNA damage through labeling of γ-H2AX puncta in the nucleus, with the result indicating TMZ caused more DNA damage in ATRX KO than ATRX NC cells (Fig. 4B). ATRX KO cell migration ability was reduced even without TMZ treatment, with corresponded to our previous result (Supplementary Fig. S4B). TMZ also induced increased apoptosis in ATRX KO cells at different concentrations (Supplementary Fig. S4C).

The strengthened chemosensitivity was further confirmed through PI staining followed by cell cycle analysis. Specifically, we showed impaired cell cycle progression with G2/M arrest in both ATRX NC and ATRX KO cells. However, the population of cells in G2/
M arrest was notably decreased in ATRX KO cells, which was discrepant compared with the previous result that TMZ caused G2/M arrest (Fig. 4C). To explore whether TMZ caused increased levels of DNA damage in ATRX KO cells, we carried out a Neutral Comet Assay to explore DNA damage. Notably, we found that ATRX KO cells suffered enhanced DNA damage when exposed to TMZ compared with ATRX NC cells (Fig. 4D), which indicated that the reduced G2/M arrest in ATRX KO cells was not caused by completion of DNA repair. Overall, our data supported the conclusion that ATRX KO glioma cells were more sensitive to TMZ treatment, TMZ induced increased levels of DNA damage in ATRX KO cells, and ATRX KO cells failed to trigger cell cycle check points when exposed to TMZ.

3.5. ATRX depletion inhibits TMZ-induced ATM phosphorylation through demethylation of H3K9me3

Our previous results demonstrated ATRX-related genes were involved in cell cycle regulation and DNA damage repair (Fig. 1A), and we also observed ATRX KO increased the chemosensitivity of glioma cell. In addition, ATRX mRNA expression had positive correlation with genes regulating the ATM signaling pathway (Fig. 2A). This pathway functions as a key factor in governing DNA repair. Activation of the ATM pathway could result in the activation of checkpoint proteins and repair of DNA damage [22]. Notably, ATM also regulated tumorigenicity, stemness, and chemoresistance in GBM [12]. To verify whether the increased chemosensitivity was regulated by deactivation of the ATM protein in ATRX KO cells, we tested the phosphorylation level of ATM and the expressive level of downstream proteins phosphorylated by ATM. We observed reduced phosphorylation levels of ATM signaling pathway in ATRX KO cells compared to ATRX NC cells after TMZ treatment (Fig. 5A). As ATM phosphorylation was followed by its acetylation, we carried out immunoprecipitation assays to measure the ATM acetylation level. The results indicated ATRX KO cells had reduced ATM acetylation levels (Fig. 5B). ATM acetylation is regulated by various factors including H3K9me3. Therefore, we used KDM4D, a demethylase of histone H3K9, to test the influence of histone H3K9me3 on the acetylation of ATM. The results showed that overexpression KDM4D exerted a similar influence as ATRX KO (Supplementary Fig. S5A, B). By blocking ATM function with the ATM inhibitor KU-55933, TMZ induced higher level of γ-H2A.X, indicating that blocking ATM generated more TMZ-related DNA damage (Supplementary Fig. SSC).

We next compared the peaks identified in the ChIP-Seq assay with the H3K9me3 ChiP-Seq peaks downloaded from public databases. We found that ATRX binding peaks coincided with H3K9me3 binding peaks, further confirming wide ATRX binding to H3K9me3 in the genome (Fig. 3C). To determine whether ATRX KO influenced the trimethylation of histone H3K9, we applied WB and IF. The results showed reduction of H3K9me3 levels in ATRX KO cells whereas TMZ treatment had little influence on H3K9me3 (Fig. 5D, Supplementary Fig. S5D). As ATRX could bind H3K9me3 through the ADD domain to preserve and facilitate H3K9me3, we suspected ATRX KO glioma cells as potentially possessing a similar mechanism for H3K9me3 reduction. We therefore assessed the binding between H3K9me3 and ATRX in the nucleus. We found that H3K9me3 failed to bind to ATRX in ATRX KO cells (as expected), as well as to a methyltransferase complex component known to bind to ATRX for preserving and facilitating H3K9me3 (Fig. 5E).

Fig. 4. ATRX loss enhances tumor sensitivity to TMZ. (A) CCK-8 assay demonstrates stronger effects of TMZ on ATRX knockout glioma cells in restraining tumor proliferation. (B) TMZ induces more γ-H2A.X foci in ATRX KO cells. Western blot showing TMZ induction of higher level γ-H2A.X in ATRX KO cells. (C) Representative glioma cell cycle profiles of ATRX KO cells assessed by propidium iodide staining. ATRX KO cells have smaller numbers of cells in G2/M phase arrest compared to normal cells following treatment with TMZ. (D) Comet assay confirms that TMZ induces more DNA damage in ATRX KO cells.
3.6. ATRX knockout inhibits glioma formation and increases chemosensitivity in vivo

Despite the suppressive effect of ATRX KO on GBM cells in vitro, we expanded our investigation to explore whether ATRX KO exhibited similar tumor-depressive effects in vivo. We transplanted ATRX KO and ATRX NC LN229 cells in 5-week-old female nude mice. IVIS showed ATRX KO restrained tumor growth and prolonged mouse survival compared with the control group (Supplementary Fig. S6A, B). Moreover, ATRX KO cells were more sensitive to TMZ treatment than the control group (Fig. 6A). The ATRX KO group showed reduced levels of Ki-67, a biomarker of proliferation, as well as other oncogene proteins such as PDGFRA (Fig. 6B Supplementary Fig. S6B). This group also showed reduced level of histone H3K9me3, phosphorylated ATM and RAD50 (Fig. 6B).

To better understand the effect of ATRX on patient overall survival, we analyzed publicly available microarray datasets. We did not observe confirmed ATRX expression correlations among TCGA subtypes in 3 databases (Supplementary Fig. S6C). However, ATRX expression negatively correlated with patient overall survival. Additionally, patients with high ATRX expression had shorter overall survival than those with low ATRX expression; moreover, patients in the ATRX-high expression group gained no significant benefit from TMZ treatment (Supplementary Fig. S6D).

4. Discussion

In the present study, we identified that ATRX knockout inhibited glioma cell growth and invasion, reduced transcriptional activity, and increased sensitivity to TMZ treatment. Furthermore, ATRX KO mediated reduction of histone H3K9me3, inhibited the acetylation of ATM, and impaired activation of the ATM signaling pathway induced by TMZ treatment. Deactivation of the ATM pathway in turn increased glioma cell chemosensitivity.

Emerging studies have demonstrated ATRX exhibited multiple functions in cancers. ATRX loss could result in alternative lengthening of telomeres, causing genomic instability [21]. Our former studies have shown ATRX mRNA expression, combined with IDH mutation and Ki-67 status, as potentially being used to reclassify patients with astrocytic tumors into 3 groups presenting different outcomes [5,6]. In the present study, we observed ATRX biological...
function involved in DNA replication process, cell cycle regulation, and DNA damage repair. In particular, CRISPR-Cas9-mediated ATRX KO inhibited GBM cell proliferation, invasion and angiogenesis.

TMZ constitutes a first-line drug recommended for chemotherapy of patients with glioma [23]. A major consequence of TMZ treatment in glioma cells is the induction of DNA damage. In the present study, ATRX KO further increased glioma cell sensitivity to TMZ. Specifically, in ATRX KO GBM cells, TMZ treatment caused more severe DNA damage and reduced G2/M phase arrest. Conversely, in glioma cells, activation of the ATM pathway could repair the DNA damage induced by TMZ [24]. ATM serves as the key protein in the ATM pathway and plays an important role in regulation of DNA repair [25]. Accordingly, activation of the ATM protein itself could reverse or reduce the TMZ effect on DNA damage [26,27]. Notably, in the present study we found ATRX expression levels as correlating with ATM, RAD50, and other key components in the ATM signaling pathway, with similar mRNA alterations being observed in glioma cells. Furthermore, GO and KEGG pathway analysis indicated ATRX gene function as being involved in chromosome organization, chromatin organization, cell cycle, and DNA repair.

ATM-mediated DNA damage repair is regulated by ATM phosphorylation, with phosphorylated ATM able to activate downstream phosphorylation to initiate DNA damage repair [28]. ATM phosphorylation is mediated by its acetylation level [29], whereas histone H3K9me3 is required for the acetylation of ATM in response to DNA damage. Exposed histone H3K9me3 could regulate the acetylation of ATM following DNA damage upon chromatin dissociation [13]. ATRX could form a complex to facilitate the deposition and maintenance of H3K9me3 [16]. ATRX is recruited on PCH by the interaction between H3K9me3 and the ADD domain, a cysteine-rich domain conserved in ATRX. Mutation in this domain disrupts ATRX binding to H3K9me3, which may lead to a compromised high order structure of PCH, followed by chromosome missegregation and apoptosis in neuroprogenitor cells [15]. In mouse embryonic stem cells, ATRX KO cells failed to deposit H3.3 at these sites, leading to loss of the H3K9me3 heterochromatin modification, loss of repression, and aberrant allelic expression [30]. In the present study, we found that reduced binding between H3K9me3 and ATRX in ATRX KO cells could inhibit TMZ-induced ATM acetylation. A similar phenomenon was triggered by exogenous expression of the H3K9me3-specific demethylation enzyme KDM4D. Moreover, similar results were observed in vivo in ATRX KO cells. We

![Fig. 6. ATRX knockout inhibits glioma formation and increases chemosensitivity in vivo. (A) TMZ reduces larger tumor volume in ATRX KO cells in vivo. (B) Hematoxylin and eosin (H&E) staining and ATRX, Ki-67, H3K9me3, p-ATM, and p-RAD50 immunohistochemistry are carried out to assess the inhibitory effects in ATRX KO glioma cells. (C) ATRX loss blocks activation of the ATM pathway to increase tumor sensitivity to TMZ.](image)
considered ATRX KO glioma cells as potentially being more vulnerable to TMZ resulting from ATRX deficiency-mediated histone H3K9me3, as ATRX might function in preserving the trimethylated status of H3K9. In turn, TMZ-induced acetylation of tone H3K9me3, as ATRX might function in preserving the tri-vulnerable to TMZ resulting from ATRX de

Studies have shown the potential for ATRX to serve as a prognostic marker in cancers [31]. Our data suggested that the overall survival of patients with GBM was correlated to ATRX expression level. In addition, patients with lower ATRX expression benefited more from TMZ chemotherapy. These results implied the potential for ATRX to play roles in regulating glioma chemoresistance. Thus, our findings described a new mechanism of chemoresistance in the subgroup of GBM with high ATRX expression GBM and suggested ATRX as a promising target in predicting patient sensitivity to TMZ.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

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