Targeting To Hedgehog Signalling Pathway Increase Sensitivity Anticancer Effects of Arsenic Trioxide Mediated By miR-326

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Research Article

Keywords: Glioblastoma multiforme, temozolomide, Arsenic trioxide, the hedgehog signalling pathway, miR-326

DOI: https://doi.org/10.21203/rs.3.rs-340883/v1

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Abstract

Background: The radical cure of Glioblastoma multiforme (GBM) is a troublesome medical problem, owing to its resistance to temozolomide chemotherapy and very poor surgical results or high relapse rate. Resistance to temozolomide emerges from numerous signalling pathways that are altered in GBM, especially the hedgehog signalling pathway. Hence, further research is urgent needed to identify more effective treatment modalities.

Methods: We evaluated the effect of ATO on viability, cell proliferation, colony formation production. Flow cytometer assesses the degree of apoptosis, and Western blot analysis the expression of hedgehog signalling pathway proteins (Gli1, Gli2 and SMO). Moreover, use database (CGGA and TCGA) to inquire the relationship between Arrb1 expression level and miR-326 expression level in different levels of gliomas. Finally, The methylation sequencing level of CpG in Arrb1 gene with the survival period of nude mice gives a good explanation to the results of the Immunohistochemica.

Results: Flow cytometer showed that the ATO caused apoptosis increased in a dose-dependent manner. Western blot analysis revealed the low expression of Gli1, Gli2 and SMO as well as the mRNA levels (included FOXM1). Arrb1 expression level was positively related with miR-326 expression level in different levels of gliomas from databases (CGGA and TCGA). Immunohistochemical analysis showed that ATO downregulated the expression of SMO, GLI1 and Arrb1. The methylation level of CpG in Arrb1 gene was significantly reduced and the survival period of nude mice was prolonged by ATO.

Conclusion: Our results showed that the cytotoxicity of ATO could be regulated by the SMO via Hh signalling pathway as well as miR-326, presenting a promising potential therapy for patients with GBM.

Background

Glioblastoma multiforme (GBM) are the most common and aggressive primary brain tumors[1]. On the basis of anaplastic features, gliomas are assigned WHO grades I to IV, in which WHO IV indicating the most malignant behavior[2]. GBM, WHO grade IV, is considered incurable with a median survival of one year after diagnosis despite aggressive adjuvant therapeutics[3,4]. Thus, more effective therapeutic strategies are urgently needed. Arsenic trioxide (ATO) is a FDA approved drug clinically used in the treatment of acute promyelocytic leukemia (APL)[5]. The advantageous effects of ATO have been also examined in gliomas[6]. Although several studies had indicated the proposed targets such as PI3K/Akt, Notch and HSP[7–9], the molecular mechanisms of ATO actions on glioma cells growth and survival are yet not entirely elucidated.

Hedgehog (Hh) signalling pathway, which plays a critical role in stem cell maintenance and cell growth, is activated in gliomas and implicated in the tumorgenicity of gliomas[10,11]. This pathway activation is triggered upon Hh ligands (Sonic, Indian and Desert Hh) binding to Patched (Patch) receptor, thus relieving Patch suppression on Smoothened (SMO)[11]. Activated SMO promotes the transcription of
downstream target genes such as GLI1, PTCH1 and FOXM1 which involve in glioma progression including inhibition of apoptosis, facilitation of cell proliferation and stem cell maintenance\textsuperscript{[12, 13]}. Therefore, chemical antagonist of Hh signalling would produce promising effects against gliomas. Previous studies indicated that ATO inhibited Hh-dependent transcription via direct binding to GLI1 in Ewing sarcoma\textsuperscript{1} or promoting GLI2 degradation in Medulloblastoma \textsuperscript{2}\textsuperscript{[14]}. Additionally, the inhibitory effect of ATO on GLI2 expression level was also detected in glioma neurosphere\textsuperscript{[7]}. However, no studies test the possible ATO’s mechanism of actions via targeting SMO, a key protein in Hh signalling transduction in gliomas. Here, we explored this possibility and the molecular mechanism of ATO modulation on SMO in vitro and in vivo.

In the present study, we found that ATO inhibited the proliferation and promoted apoptosis of glioma cells in vitro and in vivo. These cytotoxic effects were mediated by downregulation of SMO, which was direct targeted by miR-326. Together, Our results revealed that the ATO combined with miR-326 still be an attractive therapeutic method for the treatment of GBM.

**Methods**

**Glioma cell lines culture and drug preparation**

Human U87MG, U251, LN229 and SNB19 glioma cell lines were purchased from the Chinese Academy of Sciences Cell Bank, Shanghai. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal Bovine serum (Gibco), 100U/ml penicillin and 100µg/ml streptomycin. Glioma cell lines were cultured in the humidified incubator with 5% CO2 at 37°C. ATO powder (Sigma, St Louis, Mo-Aldrich) was dissolved in 1mM sodium hydroxide (NaOH) and diluted to different concentrations with phosphate-buffered saline (PBS).

**Cytotoxic assay**

The cytotoxic effects of ATO was assessed in human glioma cell lines by MTT assay. Briefly, glioma cells were seeded at a density of $2 \times 10^4$ per well onto 96-well plates in triplicates. The cells were allowed for attach and grow for 24 h, subsequently exposed to different ATO concentrations for 48 h. After ATO treatment, MTT was added to each single well which was incubated for 4 h at 37°C. The absorbance was read at 540 nm.

**Proliferation assay**

BrdU assay was performed to test the proliferation of glioma cells. Two cell lines, LN229 and U87MG, were treated with DMSO or 5 µm ATO for 72 h. The cells then fixed with 4% formaldehyde for 15 mins followed by permeabilization with 1% Triton for another 15 mins. 2 N HCL was used to denature the glioma cell proteins, which was blocked by incubation with 5% NGS for 15 mins. The cells were incubated with Anti-BrdU antibody (Sigma, B2531) at 1:500 dilution, then cy-3 conjugated secondary antibody separated by PBS washing for 3 times. The cell nucleus were counterstain with 4', 6-diamidino-2-
phenylindole (DAPI) in dark room. The proliferation activity of glioma cells was visualized and pictured by fluorescence microscopy.

**Colony formation assay**

Glioma cells were plated in 6-well plates at a density of $2 \times 10^3$ cells per well for 24 h. Then, Glioma cells were treated with 5 µm or 10 µm ATO for 72 h. An equivalent volume of DMSO was used as the control. The medium containing ATO was removed and glioma cells were washed in PBS for 3 times. Glioma cells were continue cultured in fresh medium in the absence of ATO or DMSO. Twelve days later, the colonies of glioma cells were graphed. Each experiment was performed in triplicate.

**Evaluation of cell apoptosis**

Apoptosis was assessed via annexin V labeling. U87MG and LN229 cell lines were treated with 5 µm, 10 µm ATO or DMSO as a control for 24 h before annexin V labeling. The procedure of annexin V labeling involved the addition of 10 µl FITC annexin V and 5 µl propidium iodide (BD Pharmingen) to resuspended culture and incubated for 15 mins. The stained cells were then analyzed by flow cytometry (FACSCanto II, BD Biosciences)

**Luciferase Reporter Assay**

Reporter containing 8×GLI-binding sites downstream of the luciferase gene was used to assess the transcriptional activity of Hh signalling pathway as described in previous study[^15]. Briefly, U87MG and LN229 glioma cells were treated with DMSO, 10 µm ATO or 10 µg/ml Shh for 24 h. The treatment with DMSO was considered as negative control, while Shh activating the Hh signalling pathway positive control.

**Quantitative real-time polymerase chain reaction**

Quantitative Real-time Polymerase Chain Reaction (q-RT PCR) was performed in triplicate to investigate the expression level of miR-326 and Hh signalling pathway targets. After ATO treatment for 24 h, RNA was extracted from glioma cells, reversed transcribed into cDNA according to the instructions. The cDNA levels were analyzed by q-RT PCR in LightCycler2.0 (Roche Diagnostics). PCR primer sequences were as follows: glyceraldehyde 3-phosphatedehydrogenase (GAPDH) U6 as internal reference.

MIR338 primer F: 5'TGGTATTTTTTTT T3'AGTTGT GG; R: 5'AA A AAAACCTAACCTAAAACTTCCC3'.

SOCS3 3 pairs of primers are: D1-F 5'TGGGTTGTGAATGTTTTTTTTTTTTT3', D1-R 5'CCAAATTCTTTACC AAC CCTAA C3'; D2-F 5'GGAATYGGGAGGTT TTTTA3 ', D2-R; 5' ATCACTACTCAACA AA AAAAA3'; D3-F 5'TAAATATTA TAAGAAGGGTYGTYG3; D3-R AA CRACCA AC RATAACCC.

ARRB1 Primer F: 5'TGTTAGGTATTATTTYGGGGT T3', R:CCCCAAAATCRACR TTC3.

**Western blot analysis**
Western blot was performed as described in previous study. In brief, proteins were extracted from glioma cells treated with 5 µM or 10 µM ATO for 24 h. DMSO was used as negative control. Total cellular proteins were separated on SDS-polyacrylamide gel by electrophoresis and transferred onto PVDF membranes. The proteins containing membranes were probed with primary antibodies overnight. The primary antibodies used in this study included anti-Gli1 (1:1000; CST), anti-Gli2 (1:1000; CST), anti-SMO (1:1000; Abcam) and anti-GAPDH (1:1000; Santa Cruz Biotechnology).

Nude mouse tumor intracranial model and ATO treatment

Animal experiments were according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and followed the guidelines of Harbin Medical University Institutional Animal Care and Use Committee. U87MG were injected intracranially into 5 weeks old BALB/c-nude female mice. After 7 days, mice were treated with 10 µg/g ATO or equivalent amount of vehicle (DMSO) intraperitoneally every 2 days for 2 weeks. Tumors growth were detected by fluorescent images using an IVIS Lumina Imaging System (Xenogen).

Statistical analyses

All statistical tests were performed using the GraphPad Prism 8.0 software. IC\textsubscript{50} values were determined by using a nonlinear sigmoidal dose-response curve fit. The survival curve was analyzed by the log-rank test. Statistical significance were evaluated by two-tailed Student's t test. P < 0.05 was considered significant.

Results

ATO Has Cytotoxic Effects On Glioma Cells

We evaluated the cytotoxic effects of ATO on four human GBM cell lines (U87MG, U251, LN229 and SNB19 cells) via MTT assay. Glioma cells were treated with ATO at a dosage ranging from 1 µM to 50 µM for 24 h or 48 h. We observed that ATO inhibited glioma cells survival in a concentration and time dependent manner (Figure 1 A-D). The average IC\textsubscript{50} of most glioma cell lines was within 5 µM ~ 10 µM (U87MG: 10 µM, U251: 5 µM, LN229: 1 µM, SNB19: 2 µM) (Figure 1 A-D). Thus, ATO was treated at concentration of 5 µM or 10 µM in the following tests use U87MG and LN229.

ATO Suppresses the Proliferation and Colony Formation Capability Of Glioma Cells

While most studies pointed out the ATO effects on apoptosis of cancer cells, we detected whether the cytotoxic effect of ATO on glioma cells was mediated, in part, by decreased proliferation. Results in EdU labeling showed that, after treatment with 5 µM ATO for 24 h, the cell proliferative levels of both LN229 and U87MG cell lines were significantly reduced when compared with control group (Figure 2A). Moreover, the colony formation ability of LN229 and U87MG cell lines was also suppressed by ATO treatment at 5 µM, and even more pronounced effects at 10 µM concentration (Figure 2B). These data indicated that ATO was suppressed both the proliferative activities and colony formation of glioma cells.
ATO Promotes the Apoptosis Of Glioma Cells

Induction of apoptosis may also account for the anti-glioma effects of ATO. Flow cytometry with Annexin V apoptosis detection kit was performed to measure the apoptosis in two cell lines, U87MG and LN229. We observed that ATO treatment resulted in significant increase in the percentage of cells in early apoptosis phase (Figure 3A). Although no differences in late apoptosis were observed between ATO group and DMSO group at 5 μm level, 10 μm ATO treatment obviously promoted apoptosis in U87MG and LN229 cell lines (Figure 3A). We further identified the increase in apoptosis by ATO under electron microscopy. As shown in Figure 3 B, ATO significantly decreased glioma cells growth at 5 μm concentration.

ATO Suppresses the Expression Of SMO and GLI1/2

We examined whether ATO inhibit GLI activity by measuring the GLI-responsive luciferase promoter pGL38xGLI, which contains 8 GLI DNA-binding sites. Cells were treated with DMSO, Shh (as positive control) or ATO for 24 h. The results showed an inhibitory effects of ATO on GLI transcriptional activity in U87MG and LN229 glioma cell lines (Figure 4 A-B, P < 0.05). We also estimate ATO on the critical proteins of Hh signalling pathway including SMO, GLI, GLI2 and its downstream target FOXM1 by qRT-PCR. We observed that, compared with DMSO group, the mRNA levels of SMO, GLI, GLI2 and FOXM1 were all significantly reduced in U87MG and LN229 glioma cell lines treated with ATO (Figure 4 C-D, P < 0.05). Additionally, ATO suppressed the protein expression of SMO, GLI and GLI2 at the dosage of both 5 μm and 10 μm (Figure 4E, P < 0.05). These data indicated that ATO targeted SMO, GLI and GLI2 at mRNA and protein levels in Hh signalling to inhibit glioma cells growth.

ATO Inhibit Mir-326 Expression In Glioma Cells

We have previously identified that SMO could be silenced by miR-326 through direct binding at 3'UTR\[16\]. To investigate whether ATO modulated SMO expression was mediated by miR-326, we treated glioma cells with DMSO, 5μM or 10 μM ATO for 24 h in three glioma cell lines. We found that the expression level of miR-326 was upregulated by ATO in a dose dependent manner in U87MG and LN229 glioma cell lines (Figure 5 A, P < 0.05). Previous studies indicated that expression of miR-326 is positively correlated to the expression of its host gene Arrb1\[17\]. This positive correlation was pointed to be due to the expression of miR-326 and Arrb 1 was controlled under the same promoter\[18, 19\]. Moreover, ATO has been reported to inhibit DNMT1 expression in previous studies\[20, 21\]. DNMT1 is DNA methyltransferase which can methylate CG nucleotide sites on DNA sequence, thus inactivate the transcriptional activity of Arrb 1\[22\]. Consequently, miR-326 expression level was also downregulated. Results showed that the expression level of miR-326 was positively correlated with Arrb 1 in WHO IV glioma samples from both CGGA and TCGA databases (Figure 5B, C, P < 0.0001). Furthermore, Arrb 1 expression exhibited a glioma grades dependent manner with the lowest expression in WHO IV samples (Figure 5D, E, P < 0.0001). These data indicated that ATO might inhibit miR-326 expression via increasing Arrb 1 methylation due to relieving its inhibitory effect on DNMT1.
ATO Downregulates the Methylation Level Of Arrb 1

Traditional studies of DNA methylation mainly focus on CpG sites at a gene’s promoter and CpG methylation often silences gene expression\(^2\). Thus, we further elucidate whether ATO modulate Arrb 1 methylation in LN229 cell line. Results showed that the methylation level of Arrb 1 was lower in ATO treated glioma cells than that in DMSO group (Figure 6A, B). This result provided evidences that ATO upregulated miR-326 expression via decreasing Arrb 1 methylation level in glioma cells.

ATO Inhibited Tumor Growth In Vivo and Prolonged Survival

ATO suppressed the activity of Hh signalling pathway in glioma cells, we evaluated the anti-tumor effects of ATO in vivo. The results showed that ATO notably decreased glioma volume of U87MG xenograft nude mice (Figure 7A). The survival period analyzed by Kaplan–Meier curve in ATO treated group was also significantly enhanced (Figure 6B, \(P < 0.01\)). Moreover, ATO inhibit the expression of SMO, GLI and Arrb1 of coronal brain sections by H&E staining, was the same trend to that in vitro (Figure 7C).

Discussion

WHO IV malignant gliomas, GBM, has been known as invincible on the basis of aggressiveness and shorter lifetime. ATO has remarkably enhanced therapeutic efficacy in treating both newly diagnosed and relapsed patients suffering from APL. The effects of ATO was not only used in the treatment of APL, but also applied in GBM for better treatment and longer life in recent years. We evaluated ATO’s cytotoxic effects(5 µm – 10 µm) by MTT. Moreover, ATO could also reduce proliferation and colony formation of GBM, and observe the increase in the early apoptosis phase measured by electron microscopy at 5 µm concentration. Additionally, we also estimated ATO acted on the key proteins of Hh signalling pathway including SMO, GLI and GLI2 as well as reduced their expressions. Meanwhile, we found the low expression of downstream target FOXM1 by qRT-PCR. We further investigate the mechanism of ATO actions on miR-326 expression. MiR-326 gene is intragenic located in the first intron of the host gene beta arrestin (Arrb1)\(^1\). MiR-326 expression also increased in three different cell lines(U87MG and LN229) with the ATO concentration increased. We obtained positive correlation between the Arrb1 expression level and miR-326 expression level in different levels of gliomas from databases(CGGA and TCGA) by spearman correlation coefficient. Simultaneously, immunohistochemical analysis showed that ATO could downregulated the expression of SMO, GLI1 and Arrb1. Further, the methylation level of CpG in Arrb1 gene was significantly reduced and the survival period of nude mice was prolonged by ATO. From protein level to gene level changes in Hh signalling pathway, our data showed that ATO influenced the function of Hh signalling pathway proteins and the methylation level of CpG in Arrb1 gene furthermore improved nude mice survival period.

Our explore manifested that there was quite obviously ATO had dose-dependent effect and smaller doses had the same effect in some aspects. Arsenic compounds have been used for many years in medicine but it was not a milestone until a small dose was found to be beneficial for medical treatment. Soon after
ATO was used in the treatment of various tumors and also aware of low side effects at low doses. ATO could degrade multiple proteins by NFkB pathway and alter the targeted protein domain (the portion of MDS1 and EVI1) then change proper protein folding, structure, or function. Moreover, ATO could increase activation of p38 MAPK and ERK pathway induce autophagy subsequently lead to apoptosis finally[23].

We focus on transmembrane protein SMO and downstream effectors GLI proteins. Most of its effects in cells were arsenic can inhibit the Hh signalling pathway by direct binding the critical cysteine residues in GLI zinc finger domains[23]. Multiple studies demonstrated that Hh signalling involved in tumorigenic behaviors of glioma cells[10, 24, 25]. ATO targets Hh signalling pathway at the level of GLI 1/2 proteins[7, 18].

Further studies shows that the ATO/vincristine(VCR) combination triggers caspase-dependent apoptosis than monotherapy. ATO reflects antitumor activity against sarcoma, especially in combination with VCR via the overexpression of the antiapoptotic protein Bcl-2 which is the key activation molecules of apoptotic cell death. However, it just involves ATO-mediated Gli1 transcriptional activity lacking further depth study about Hh signalling pathway[26]. And previous studies had already concerned about blocking the Hh signalling pathway that selective knockdown of SMO to inhibit Hh pathway is more effective.

Moreover, SMO small interfering RNA could effective block Hh signalling pathway that mediated a two- to more than fivefold reduction of SMO and Gli1 gene expression[27].

In this study, we combinational therapies using miR-326 to synergistically cooperate with ATO for glioma cells lines and tumor-bearing rat, whereby the miRNA could enhance ATO’s anti-glioma effect via the SMO/GLI1 pathway, and ATO was shown also to regulate miRNA expression in turn. As well as previous studies which not only provided via the SHH/GLI1 pathway but also the evidence for potential target to treat gliomas[28]. In addition to these, SMO was upregulated in gliomas and was associated with tumor grade and survival period, and database-related miR-326 affected the activity of Hh signalling mediated by SMO and also regulated the self-renewal ability and stemness and partially prompted differentiation in glioma stem cells[29]. Potential values in predicting poor overall survival in glioma patients with high pathological grades[30]. Downregulation of these miRNAs allows high levels of Hh-dependent gene expression leading to tumor cell proliferation miRNA are crucial post-transcriptional regulators of gene expression and control cell differentiation and proliferation[31]. Increasing evidence suggests that the importance of ATO in regulating Hh signalling pathway and its future potential goes beyond treatment.

**Conclusions**

Our study demonstrated that More evidences manifested that ATO could apply to the treatment of varieties of tumors including GBM. To be important, if the medicine can treat the disease, it is preferred to surgery/radio-chemotherapy. we achieve the purpose of treatment by reducing the expression of Hh signalling pathway proteins and related genes. And miR-326 may become an indicator for predicting GBM patient prognosis. We will use smaller doses of ATO for the future study of GBM.

**Abbreviations**
GBM: Glioblastoma multiforme

CGGA and TCGA: The Chinese Glioma Genome Atlas and The Cancer Genome Atlas

ATO: Arsenic trioxide

Hh: Hedgehog signalling pathway

SMO: Smoothened

NaOH: sodium hydroxide

PBS: phosphate-buffered saline

Declarations

Ethics declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by The Harbin Medical University Innovation Research Fund; Outstanding Youth Project of Heilongjiang Natural Science Foundation.

Acknowledgement

Not applicable

Author Contributions
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Figures
Figure 1

The cytotoxic effect of ATO on human glioblastoma cell line. (A) Human glioblastoma cell line U87MG, U251, LN229 and SNB19 cells were cultured in 96 well plates. The survival rate was estimated by MTT assay. The cell counts U87MG death were significantly increased with the treatment of ATO at 24h and 48h when compared with control group. The cytotoxic effect of ATO was dependent on the concentration and treatment time of ATO. (B) After the treatment of ATO for 24h and 48h, the survival viability of U251 was decreased. (C) The death counts of LN229 were remarkably increased after the treatment of ATO for 24h and 48h. (D) ATO induced the death of SNB19.
Figure 2

ATO had inhibitory effects on the proliferation and colony formation of LN229, U251 and U87MG human glioma cell lines. (A) This picture showed the Edu staining for estimating malignant glioma cell proliferation. The blue signal (DAPI) signed nucleus and the green signal signed FITC. The green signal (DNA replication) significantly decreased with the treatment of 5μM ATO. Compared with DMSO group, U251 glioma cell proliferation obviously decreased in ATO treatment group. (B) ATO disrupted the colony formation of U87MG and LN229 glioma cell line. The inhibitory effect of ATO with 10μM concentration was stronger than 5μM.

Figure 3

ATO promotes the apoptosis of malignant glioma cells. (A) The flow cytometry analysis showed the percentage of Annexin V positive cells (Q2, Q4) for U87MG and LN229 increased after the treatment with 5μM or 10μM ATO. ATO also facilitated the early and late apoptosis of LN229 glioma cell line. (B) ATO with low concentration (5μM) had antitumor effects on SNB19 and LN229 glioma cells.
Figure 4

ATO inhibits the expression of key proteins in Hedgehog/GLI pathway. (A-B) Compared with DMSO group, the mRNA levels of GLI in U87MG cell line and LN229 cell line were significantly reduced in ATO group, while lower than Shh positive group. (C-D) SMO, GLI1, GLI2 and FOXM1 levels in U87MG cell line and LN229 cell line were obviously decreased with the treatment of ATO. FOXM1 is the target gene of Hedgehog/GLI pathway. (E) This Figure showed the western blot analysis results. The expression levels of GLI1, GLI2 and SMO proteins in 5μM ATO group were lower than DMSO group. The expression levels of these three proteins in 10μM ATO were higher than DMSO group and 5μM ATO group.
Figure 5

ATO upregulates the expression level of miR-326. (A) ATO promoted the expression of miR-326 in U87MG, LN229 and U251 cell lines. Moreover, miR-326 level in 10μM ATO was higher than that in 5μM ATO. (B) CGGA database analysis showed that ARRB1 expression level was positively related with miR-326 expression level in gliomas. (C) TCGA database analysis results also demonstrated that the expression level of ARRB1 was positively correlated with miR-326 expression level in gliomas. (D) It was shown that
the expression level of ARRB1 in GBM was lower than that in LGGs which data was from CGGA database. (E) TCGA database analysis results also demonstrated that the expression level of ARRB1 was decreased with the glioma grades increased.

Figure 6

ATO suppresses the methylation level of ARRB1 in glioma cells. (A-B) The methylation level of CpG in ARRB1 gene was significantly reduced after treatment with ATO when compared with DMSO group.

Figure 7

ATO has anti-tumor effects on malignant glioma cells in vivo mediated by dysregulated SMO/GLI1 expression. (A) Bioluminescence imaging of the whole body showed that the intensity and area of U87-luciferase were diminished by ATO treatment. (B) The survival period of nude mice was indicated by Kaplan–Meier curve. Compared with DMSO injection group, the survival period of malignant glioma tumor xenograft nude mice was remarkably improved by twenty-days-ATO injection. (C) Xenograft Tumors in brain were collected and fixed by formalin after being sacrificed. Immunohistochemical (IHC) analysis showed that ATO downregulated the expression of SMO and GLI1, upregulated the expression of ARRB1 in xenograft tumor.