ANTXR1 Facilitates Tumor Growth in Glioma via Deactivating MAPK Signaling Pathway

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Research

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Abstract

Background: Gliomas are commonly known as primary brain tumors and associated with frequent recurrence and an unsatisfactory prognosis in despite of extensive research in the underlying molecular mechanisms. In this study, we aimed to examine the role of ANT XR1 in glioma tumorigenesis and explore its downstream regulatory mechanism.

Methods: We detected overexpression of ANT XR1 in glioma cell lines (SHG-44 and U251) in comparison with those in normal brain tissues.

Result: Glioma cell growth and migratory ability were dramatically impaired as a result of silencing *ANT XR1* by shANT XR1 lentivirus. ANT XR1 blockade also accelerated cell apoptosis since ANT XR1 held back apoptosis via targeting G2 phase during cell mitosis. *In vivo* xenograft models verified *in vitro* findings above that the solid tumors stripped from mice were much lighter and smaller after depletion of ANT XR1 than controls. We also mechanically probed the downstream pathways and disclosed that overexpression of ANT XR1 abrogated the levels of MAPK9 and apoptosis-related protein HTRA2, but augmenting CCND1 and CDK6 levels in glioma cells. Our findings allow us to demonstrate that ANT XR1 acts as a tumor promoter in glioma induction through attenuating MAPK9-mediated gene transcription and HTRA2-induced apoptosis but intensifying CCND1-mediated proliferation.

Conclusion: Together, we declare that ANT XR1 plays an indispensable role in glioma tumorigenesis via deactivating MAPK signaling and apoptosis pathway but activating PI3K/AKT-mediated cell growth. Our study provide a valuable clue to targeting ANT XR1 as a molecular target and a promising anticancer agent in glioma clinical therapeutics.

Background

Brain tumors are associated with significant morbidity and mortality due to aggressiveness of local growth and metastasis outside the central nervous system [1]. Glioma was estimated to comprise 28% of brain and other central nervous system tumors, and 80% of malignant brain tumors [2], which has been considered as a primary malignant brain tumor. It is thought to originate from neuroglial stem cells and categorized as astrocytic, oligodendroglial or ependymal tumors based on the histopathologic features [3]. Typically, glioma is characterized by high degree of infiltrative growth which contributes to frequent recurrence [4]. Despite the role of molecular markers in determination of low-grade gliomas has been generally confirmed, i.e., *IDH1*- and *IDH2*- mutations, it is not necessarily true in the high-grade glioma and the specific molecular markers still remain further investigations [5, 6]. To date, the therapeutic approach of glioma is usually surgical resection in conjunction with chemotherapy, radiotherapy, immunotherapy or gene therapy. However, the combined treatments were not always effective and glioma patients in many cases suffered from high risk of recurrence and a very poor prognosis [7]. Therefore, it still matters to seek for advances in therapeutic strategies of gliomas based on its molecular pathogenesis.

ANTXR1 (Anthrax toxin receptor 1), also known as Tumor endothelial marker 8 (TEM8), is encoded on chromosome 2 in human as a type I transmembrane protein and is a highly-conserved cellular receptor for anthrax toxin secreted by *Bacillus anthracis* [8, 9]. The tripartite anthrax toxin is composed of protective antigen (PA), lethal factor and edema factor [10], among which the PA component binds to ANTXR1 and then the complex mediates delivery of toxin to cytoplasm. Through binding collagen types I and VI, ANTXR1 also acts a pivotal role in endothelial cell attachment and migration [11, 12], and serves as a promoter in the process of pathological angiogenesis. ANTXR1 was originally found upregulated in the endothelial cells of colorectal cancer during tumor-related vasculature [13], and subsequently was identified overexpressed in various cancers, such as gallbladder carcinomas, breast cancer [14] and prostate cancer [15], in comparison with that in normal tissues [16]. A previous study suggested that the mutation in *ANTXR1* gene would lead to disorganized angiogenesis in response to the inhibitory effect on downstream signaling pathways in infantile hemangioma [17]. Besides, downregulation of ANTXR1 slowed down tumor growth in a few cancer models, including melanoma, breast cancer, colorectal cancer and lung cancer [18]. However, little is known about the role of ANTXR1 in the development of glioma.

In this study, we thus exploited a series of *in vitro* and *in vivo* experiments to disclose and identify biological functions of ANTXR1 in glioma progression, through which we aimed to evaluate the possibility of ANTXR1 to serve as a novel molecular therapy target. Our findings indicate the ANTXR1-mediated apoptosis via deactivating MAPK signaling pathway and underscore the vital role of ANTXR1 in glioma progression, as well as its further potential application as a targeted molecular agent.

Methods

Clinical samples and immunohistochemical staining

Tissue microarrays of glioma samples from 134 patients (15-80 years old, glioma histopathological grade I, II and III) and normal brain samples from 24 people were from Jiangxi Provincial People's Hospital Affiliated to Nanchang University. The usage of all samples were approved with permission from all patients and followed the International Ethical Guidelines for Biomedical Research Involving Human Subjects issued by the Council for International Organization of Medical Sciences (CIOMS). The histopathological grading of the glioma tissue microarrays here was based on the 2016 World Health Organization classification of tumors of the central nervous system [19]. In terms of immunohistochemical staining, we firstly baked the slides of glioma tissues and matched normal brain tissues in oven at 65 °C for 30 min followed by dewaxing and rinsing them for several times. After retrieving the antigen by disposing with citric acid antigen at 100°C for 10 min, we blocked the slides using 3% H₂O₂ for 5 min. ANTXR1 antibody (1:250, Cat. #ab21270, Abcam) was next added on the slides at 37°C for 1 h and then the second antibody. The staining was finished after treatment of DAB and hematoxylin. Then we photographed the slides and evaluated the staining percentage and the intensity, according to which we assigned values to each slide and determined the outcomes of immunohistochemistry. Specifically, the assignment score of a slide was the integer from 1 to 4 when the staining percentage was less than 25%, 50%, 75% and 100%, respectively. Similarly, the staining intensity

was also quantified and scored from 0 to 3 based on the color depth with 0 signifying no dyeing and 1-3 light yellow, pale brown and dark brown.

Constructing lentiviral vector

We applied human gene *ANTXR1* as the template and then designed RNA interference target sequences (5'-ATCCGTCAAGGCCTAGAAGAA-3', 5'-GACAGTAAGGATCATGTGTTT-3', 5'-CTATTCAGAGAGGGAGGCTAA-3'). Followed by the treatment of restriction endonuclease Age I and EcoR I (Cat. #R3552L and R3101L, NEB), cDNA encompassing interference sequence was linked with the linear BR-V108 vector (Shanghai Yibeirui Biomedical science and Technology Co., Ltd.), which were then transfected into *E.coli* receptor cells (Cat. #CB104-03, TIANGEN). PCR technique was subsequently carried out to select and verify positive clones. High-purity plasmids carrying interference sequence were then extracted utilizing EndoFree midi Plasmid Kit (Cat. #DP118-2, TIANGEN). In the end, we co-transfected Helper 1.0 and Helper 2.0 plasmids, as well as BR-V108 into 293T cells to successfully construct lentiviral vector.

Lentiviral transfection and qRT-PCR

Human glioma cell lines SHG-44 and U251 have been prepared, thawed from freezing and then cultured in medium (90% DMEM + 10% FBS). Cells were incubated for 24 h under 5% CO₂ at 37°C, after which transfected by shANTXR1 or shCtrl lentivirus for 72 h and photographed through fluorescence microscopy. Subsequently, the total of RNA was extracted from both cell lines and quantified using Trizol (Cat. #T9424-100m, Sigma) and Nanodrop 2000/2000C, respectively. After reverse transcription using Hiscript QRT supermix for qPCR (+gDNA WIPER) (Cat. #R123-01, Vazyme), we obtained cDNA and then amplified by means of qRT-PCR using Hiscript QRT supermix for qPCR (+gDNA WIPER) (Cat. #R123-01, Vazyme) and AceQ qPCR SYBR Green Master Mix (Cat. #Q111-02, Vazyme). We next applied $2^{-\Delta\Delta CT}$ method to measure GAPDH as controls. The sequences of primers used were as described below:

ANTXR1, upstream sequence 5'-AGGTGCTAAGTTGGAAAAGGC-3' and downstream sequence 5'-TGTTGAGATTTTCGCGGCTC-3';

GAPDH, upstream sequence 5'-TGACTTCAACAGCGACACCCA-3' and downstream sequence 5'-CACCCTGTTGCTGTAGCCAAA-3'.

Western blotting

Glioma cells were prepared and lysed by 1 × cold lysis buffer. Total proteins from cells were then extracted applying BCA protein detection kit (Cat. #23225, HyClone-Pierce). After separated by 10% SDS-PAGE, protein extraction was shifted to a polyvinylidene difluoride membrane and incubated using blocking liquid for 1 h. We next probed the blots using primary antibodies of ANTXR1 (1:1000, Cat. #ab21270, Abcam), AKT (1:2000, Cat. #SAB4500796, Sigma), P-AKT (1:500, Cat. #AF887-sp, R&D), CCND1 (1:5000, Cat. #ab134175, Abcam), CDK6 (1:1000, Cat. #ab151247, Abcam), MAPK9 (1:3000, Cat. #ab76125, Abcam) and GAPDH (1:3000, Cat. #AP0063, Bioworld), and goat anti-rabbit IgG (HRP)

(1:3000, Cat. #A0208, Beyotime) as secondary antibody. The signal of membranes was finally detected utilizing immobilon Western Chemiluminescent HRP Substrate (Cat. #RPN2232, Millipore).

MTT assay

Cell proliferation of SHG-44 and U251 cell lines was detected via MTT assay. To put it simply, cell suspension (2000 cell/well) was prepared after treatment of trypsin and then cultured in 96-well plates. Cell plates were treated and stained by MTT solution (20 μ L 5 mg/mL) 4 h before culture termination. At the end of cultivation, we discarded culture medium, disposed with 100 μ L DMSO and shook for 5 min. For the next 5 consecutive days, the absorption values of cells were obtained at OD490 nm with a Microplate Reader and thus viability of lentivirus-transfected cells was finally acquired.

Flow cytometry

We employed flow cytometry to examine cell apoptosis and cell cycle of both glioma cell lines. At the very beginning, cells were trypsinized and rinsed in cold PBS (pH=7.2~7.4). As to the detection of apoptosis, cells were then re-rinsed with 1 $\times$ binding buffer and stained by Annexin V-APC for 15 min from light. The percentage of cell apoptotic rate was thus determined by FACSCalibur (BD Biosciences). In terms of cell cycle detection, we fixed cells suspension with cold ethanol (70%) at 4°C for at least 1 h and then removed ethanol. Following centrifugation and re-rinse of PBS, cells were stained using PI solution (40 $\times$, 2 mg/mL: 100 $\times$ RNase, 10 mg/mL: 1 $\times$ PBS =25:10:1000) for 30 min. Lastly, FACSCalibur (BD Biosciences) was employed to detect cell cycle distribution (G1, S and G2).

Wound-healing assay

Glioma SHG-44 and U251 cells were seeded into 6-well dishes. After cell growing for 72 h, we scratched a wound across the cell layer using a 96-wounding replicator (Cat. #VP408FH, VP scientific) and then rinsed slightly for 2-3 times. SHG-44 cells were cultured for 8 h and 24 h while U251 cells for 24 h and 48 h, following which we took fluorescence micrographs and accordingly calculated the cell migration rate.

Apoptosis pathway assay

Apoptosis-related proteins and signaling pathways involved in this research were determined utilizing Human apoptosis antibody array (Cat. #ab134001, Abcam). Simply, after U251 cell samples were washed and lysed, we measured the concentration of the whole proteins via BCA protein assay kit (HyClone-Pierce). The proteins were thus incubated with blocked antibody array membrane throughout the night at 4°C, followed by continued incubation with Streptavidin-HRP for 1 h. Subsequently, we employed enhanced chemiluminescence (ECL) (Amersham) for visualizing proteins and got the gray results by ImageJ software.

***In vivo* tumorigenesis**

Mice used for constructing xenograft models were 28-day-old and BALB/c nude females (Beijing Vitalriver Experimental Animal Technology Co., Ltd), which were randomly separated (shANTXR1 vs. shCtrl groups) and subcutaneously injected with 0.2 mL (2×10^7 cells/mL) lentivirus-transfected SHG-44 cell suspensions. The body weight, as well as tumor length and width (for tumor volume) of each mouse model was first measured after 35 days of the construction of xenograft models, since which the measurement was executed every seven days for another four weeks. On the day of the last measurement, we disposed the experimental mice using 0.7% sodium pentobarbital (10 µL/g, SIGMA) and determined the fluorescence expression *in vivo* through a Perkin Elmer IVIS Spectrum (Waltham). In the end, we executed all mice and removed the solid tumors for Ki-67 staining (primary antibody: Ki-67, 1:200, Cat. #ab16667; secondary antibody: HRP, 1:400, Cat. #ab6721, Abcam). Animal experiments in this research were carried out on a protocol approved by Ethics committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University.

Statistical analysis

We performed cell experiments in triplicate and expressed the data as mean $\pm$ SD. Differences between two groups were examined by *t*-test and Mann-Whitney *U* test, with sign test used for the difference of ANTXR1 expression. Spearman correlation test was exploited to estimate the relationship between ANTXR1 level and clinical characteristics, while Kaplan-Meier survival analysis was detecting how high/low level of ANTXR1 affected overall survival of glioma patients. We run all analysis and plotting in SPSS 20.0 and GraphPad Prism software 7.0 with $P < 0.05$ as statistical significant.

Results

Overexpression of *ANTXR1* gene in glioma tissues

The expression patterns of ANTXR1 in glioma tissues and normal brain tissues were explored through immunohistochemistry analysis. Sign test was subsequently employed to detect the difference between glioma tissues and normal brain tissues. The results showed that expression of ANTXR1 was upregulated in glioma tissues in comparison with that in normal tissues ($P < 0.001$) (**Figure 1A, Table 1**). In regard to clinicopathologic characteristics of the patients with glioma, we also found a significantly positive correlation between the malignant grade of glioma and the expression level of ANTXR1 ($P < 0.01$) (**Tables 2-3**). ANTXR1 expression was also more likely to upregulate in the recurrent glioma tissues than in the non-recurrent ones ($P < 0.001$) (**Tables 2-3**). However, we failed to determine an age- or sex-specific difference in the expression levels of ANTXR1 in patients with glioma ($P > 0.181$) (**Table 2**). We next utilized Kaplan-Meier survival analysis and found that a higher expression of ANTXR1 predicated a lower survival probability of the glioma patients ($P < 0.05$) (**Figure 1B**).

Establishment of ANTXR1-knockdown model *in vitro*

To construct ANTXR1-knockdown model in glioma cell lines, we first packaged shRNA targeting ANTXR1 in lentivirus vector which was labeled by green fluorescent protein (GFP) and established ANTXR1-

silenced lentivirus. Then we transfected ANT XR1-shRNA (shANT XR1) or shCtrl lentivirus into SHG-44 and U251 cell lines. The two transfected cell lines were observed 72h after lentiviral transfection under fluorescence microscope and the photomicrographs demonstrated a >80% fluorescence efficiency with cells in good conditions (**Figure 2A**). Subsequently, the inhibitory effects of lentiviral transfection on the expression of ANT XR1 mRNA level were detected by means of qRT-PCR, with the knockdown efficiency of ANT XR1 in SHG-44 and U251 cells as high as 62.3% ($P < 0.001$) and 60.8% ($P < 0.001$), respectively (**Figure 2B**). The following western blotting verified this finding and demonstrated that ANT XR1 protein was notably downregulated in both glioma cell lines in comparison with controls (**Figure 2C**). Collectively, the ANT XR1-knockdown models were successfully established and thus prepared for the following experiments.

Silencing of ANT XR1 hampered *in vitro* growth and migration of glioma cells

We next monitored and compared the capacity of cell growth and migration between ANT XR1-silenced groups and controls in two cell lines. The Celigo cell counting assay unraveled that both cell lines exhibited significantly slower cell proliferation rate in the first 5 consecutive days (SHG-44 cells, $P < 0.001$, fold change = -2.0; U251 cells, $P < 0.001$, fold change = -7.0) in comparison with those in shCtrl group (**Figure 3A**), indicating a remarkably restriction in cell growth resulted by ANT XR1 knockdown. Thus, we applied flow cytometry to detect cell apoptosis and cell cycle in SHG-44 and U251 cell lines. The results proposed that cell apoptosis was significantly enhanced in shANT XR1 groups (SHG-44 cells, $P < 0.001$, fold change = 9.1; U251 cells, $P < 0.001$, fold change = 3.1) compared with controls (**Figure 3B**). With regard to cell cycle, we disclosed that knockdown of ANT XR1 significantly increased the percentage of cells in G1 phrase in SHG-44 cell line ($P < 0.01$) through flow cytometry but it was not true in U251 cell line. However, silencing of ANT XR1 significantly reduced the proportion of cells in S phrase (SHG-44 cells, $P < 0.001$; U251 cells, $P < 0.01$) and increased these in G2 phrase in shANT XR1 groups of both cell lines ($P < 0.001$) (**Figure 3C**), which denoted that ANT XR1 held back apoptosis via targeting G2 phrase during cell mitosis.

The ability of tumor cell migration was then detected and quantified in both cell lines. The results of wound-healing assay displayed that the cell migration rate (24 h) was lessened by 57% ($P < 0.01$) after harboring shANT XR1 in SHG-44 cells, while it also dropped by the same count in shANT XR1 group (48 h) compared with controls in U251 cells ($P < 0.001$) (**Figure 4A**). The following transwell assay indicated similar outcomes that knocking down ANT XR1 was associated with 63% ($P < 0.001$) and 54% ($P < 0.001$) reduction of migration ability in SHG-44 and U251 cells, respectively (**Figure 4B**). On the whole, our data pointed out ANT XR1 downexpression substantially suppressed glioma cell growth and migration in experimental cell lines *in vitro*.

Underlying molecular mechanism of ANT XR1 modulating apoptosis in glioma cells

The increment of cell apoptosis rate allowed us for a further human apoptosis antibody array. We perceived that among the human apoptosis-related proteins, HTRA was upregulated with other proteins downregulated (i.e., Bcl-2, Bcl-w, cIAP-2, HSP60, HSP70, IGF-I, IGF-II, Livin, sTNF-R2, TNF- α , TNF- β , TRAILR-

3, TRAILR-4 and XIAP) after knockdown of ANT XR1 in the U251 cell line (**Figure 5A-C**). Western blotting was thereafter employed in U251 tumor cells and the outcomes exhibited an involvement of the upstream MAPK9 signaling pathway. Specifically, the transfection of shANTXR1 had a bearing on overexpression of MAPK9 but downregulation of P-AKT, CCND1 and CDK6, while the expression level of AKT barely changed in the U251 cell line (**Figure 5D**).

Depletion of ANT XR1 refrained glioma progression *in vivo*

To verify the effect ANT XR1 exerted on glioma progression *in vivo*, we subcutaneously injected SHG-44 cells that were transfected with shCtrl or shANTXR1 into nude mice to construct xenograft models and monitored the development of potential tumors. The tumor in mice models of the negative controls grew much faster and the volumes were much larger than those in the shANTXR1 group ($P < 0.05$) (**Figure 6A**). The outcomes of bioluminescence photographing were shown in **Figure 6B** and total bioluminescent intensity of controls was significantly higher than that in shANTXR1 group ($P < 0.01$). Strikingly, tumor weight of controls also outweighed that after treatment with ANT XR1 lentivirus ($P < 0.01$) (**Figure 6C**). Ki-67 staining proved that this proliferation protein marker was notably downexpressed followed by the depletion of ANT XR1 in comparison with that in control group (**Figure 6D**). As a consequence, disruption of ANT XR1 evoked considerable suppression on *in vivo* tumorigenesis of glioma.

Discussion

Unlike other cancers locating noncerebral tissues, glioma is derived from lesions in the primary central nervous system and is seriously threatening mental health. Patients with glioma were also more likely to be cognitively impaired and suffer from more psychological symptoms [20], highlighting the urgent need for more effective treatments of this intractable disorder. This research displayed that in human clinical glioma tissues, ANT XR1 expression was correlated with the severity of the disorder indicated by the clinicopathologic features. Followed by ANT XR1-lentivirus treatment in glioma cell lines, cell apoptosis were dramatically promoted *in vitro*, while cell growth and proliferation were limited with similar circumstances encountered in the ability of cell movement and migration. Further western blotting on the regulatory signaling and the downstream apoptosis pathway unveiled the involvement of MAPK signaling pathway and the alterations of apoptosis-related proteins owing to the silencing of ANT XR1. *In vivo* trials verified the aforementioned findings and ANT XR1 has shown its effectiveness in advancing glioma development. In light of these discoveries, we proposed that ANT XR1 participated in and modulated the process of tumor growth through deactivating MAPK pathway and activating downstream apoptosis pathway in glioma patients.

Consistently, researchers had announced the increased expression level of ANT XR1 and declared that miR-26b-3p acted as its critical upstream modulator in glioma progression [21]. The present study tried to unfold and trace its downstream regulatory mechanisms. The findings here implied the pivotal role of ANT XR1 in MAPK signaling pathway as its upstream deactivator since downregulation of MAPK9 was greatly attributable to the presence of ANT XR1. MAPK (mitogen-activated protein kinase) signaling

cascades include several signaling components (RAS-MAPK) and have been suggested to participate in mediating cellular signal transmissions, thus regulating cell growth and proliferation [22]. This pathway is involved in numerous biological processes and its dysregulation was commonly found in many syndromes and disorders, including a number of human tumors [23, 24]. MAPK9, also known as JNK2, was a member of MAPKs targeting specific transcription factors and thus modulating gene expression during the cell-cycle process [25]. In colitis-induced colorectal carcinoma, MAPK9 functioned as a tumor suppressor and inflammation-triggered inactivation of MAPK9 would result in abnormalities of MAPK9-dependent cell cycle [26]. MAPK9 deficiency was also found to support the development of breast cancer [27]. Nevertheless, the carcinostasis of MAPK9 was not always true. In melanoma, MAPK9 played a positively role in cell proliferation and its activity was of the essence in tumorigenesis [28].

Additionally, ANT XR1 shortage also led to reduction of AKT phosphorylation, implying that silencing of ANT XR1 blocked tumorigenesis through inactivating PI3K/AKT signaling pathway and further misregulating CDKs. In our experiments, CDK6, which was particularly needed for G1/S transition, were found overexpressed in glioma cell lines while lack of ANT XR1 lessened CDK6 expression. This result proposed an ANT XR1-mediated association between MAPK9 inactivation and the expression of cell cycle-related proteins. CCND1, also named cyclin D1, functioned as a regulatory subunit of CDK4 or CDK6, both of which were vital regulators in the stepwise progression of cell cycle [29]. CCND1 was considered to be a pan-cancer actor and dysregulation of CCND1 had linkage with pathogenesis of human tumors, e.g., prostate cancer [30], retinoblastoma [31] and head and neck squamous cell carcinoma [32]. Amplification of *CCND1* also conferred unfavorable prognosis in malignant tumors [33]. In agreement with this, we also demonstrated a ANT XR1-mediated cell cycle disruption and growth promotion via activating PI3K/AKT signaling in glioma patients.

Regarding the downstream apoptosis pathway, we declared a series of decreases in apoptosis-related proteins but an increase in HTRA2 following depletion of ANT XR1. HTRA2 (high temperature requirement A2), locating in the mitochondrial inter membrane, has been reported to bind apoptosis inhibitory factors and evoke cell apoptosis to maintain mitochondrial homeostasis, which acknowledgedly served as a favorable target for anticancer agents [34, 35]. In prostate cancer, a reduction in HTRA2 abrogated cell apoptotic activity through interacting with C-terminus of ITGA7, a primary laminin receptor [36]. Intracerebral hemorrhage in rat models gave rise to upregulation of HTRA2 and initiated HTRA2-triggered neuronal apoptosis [37]. Researchers previously announced that HTRA2 performed its pro-apoptotic function by interacting with XIAP (X-linked inhibitor of apoptosis protein) in a caspase-dependent manner [38]. Consistent with this notion, our study also detected overexpression of XIAP but downexpression of HTRA2, whereas the case was exactly the opposite after silencing ANT XR1. We hence drew the conclusion that ANT XR1-mediated dysregulation of MAPK signaling in glioma cells contributed to abnormalities in apoptotic pathway by downregulating HTRA2.

Conclusions

In summary, we investigated the ANT XR1 role and its downstream molecular mechanisms in regulating malignant biological behaviors of glioma. Our results sounded ANT XR1 acted as a tumor promoter in glioma induction via deactivating MAPK signaling and apoptosis pathway but activating PI3K/AKT-mediated cell growth, specifically, attenuating MAPK9-mediated gene transcription and HTRA2-induced apoptosis but intensifying CCND1-mediated proliferation. Findings from this study, in conjunction with previous work on ANT XR1 can provide a promising option as anticancer agents for the clinical therapeutics of glioma.

List Of Abbreviations

ANT XR1: Anthrax toxin receptor 1; TEM8: Tumor endothelial marker 8; PA: protective antigen; MAPK: mitogen-activated protein kinase.

Declarations

Ethics approval and consent to participate

Animal experiments in this research were carried out on a protocol approved by Ethics committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University.

The study protocol was approved by the Research Ethics Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University. The usage of all samples were approved with permission from all patients and followed the International Ethical Guidelines for Biomedical Research Involving Human Subjects issued by the Council for International Organization of Medical Sciences (CIOMS).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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None.

Authors' contributions

Dengfeng Wan, Aijun Liang and Chaoyang Zhou have made substantial contributions to the concept and design of this study. Jianzhong Zhang, Jingxing Leng, Bin Xi and Bin Zhou, conducted the experiments. Yu Yang, Ronglan Zhu, Liangchen Zhong and Xingxing Jiang conducted data analysis. Aijun Liang and Chaoyang Zhou produced the manuscript. All authors have approved the final draft.

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Tables

Table 1 The difference in expression patterns of ANTXR1 between glioma tissues and normal brain tissues suggested by immunohistochemistry analysis.

ANTXR1 expression	Glioma tissue		Normal tissue		<i>P</i> value
	Cases	Percentage	Cases	Percentage	
Low	69	51.5%	23	100%	< 0.001
High	65	48.5%	0	-	

Table 2 Differences in the expression levels of ANTXR1 in patients with different glioma characteristics.

Features	No. of patients	ANTXR1 expression		<i>P</i> value
		Low	High	
All patients	134	69	65	
Age (years)				0.934
≤43	66	34	32	
>43	67	35	32	
Gender				0.181
Male	88	49	39	
Female	46	20	26	
Tumor recurrence				< 0.001
No	52	39	13	
Yes	82	30	52	
Grade				< 0.01
II	72	46	26	
III	48	17	31	
IV	14	6	8	

Note, the cut-off age point (43 years old) was the median age of the patients whose ages ranged from 15-80 years old.

Table 3 Results of Spearman correlation analysis between ANTXR1 expression and two tumor characteristics in patients with glioma.

Tumor characteristics	Spearman correlation test		
	N	Correlation coefficient	<i>P</i> value
Grade	134	0.248	< 0.01
Tumor recurrence	134	0.375	< 0.001

Figures

Figure 1

Overexpression of ANT XR1 is related with poor prognosis in glioma. (A) Representative photomicrographs at 200 × and 400 × magnification of ANT XR1 expression in glioma tissues (grade II and III) and adjacent normal tissue by immunohistochemical staining. (B) Kaplan-Meier curves of overall survival showing patients with higher ANT XR1 expression level had lower survival probability. Significance was obtained using the log rank test. ANT XR1, anthrax toxin receptor 1. *P < 0.05.

Figure 2

ANTXR1 expression is reduced in glioma cell lines after lentiviral transfection. (A) Representative photomicrographs at 100× magnification of lentiviral transfection in SHG-44 and U251 cells using fluorescence imaging. (B-C) The expression level of ANTXR1 mRNA and ANTXR1 protein in both cell lines by qRT-PCR and western blotting, respectively. ANTXR1, anthrax toxin receptor 1; shANTXR1, cells

transfected by ANT XR1-targeting shRNA; shCtrl, cells transfected by control shRNA; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (n ≥ 3). ***P < 0.001.

Figure 3

Growth suppression of glioma cells after the transfection of shANTXR1 lentivirus. (A) The photomicrographs of a same field of view (40×) under the microscope for the first 5 consecutive days. The comparison of cell numbers obtained through Celigo cell counting assay (shANTXR1 vs. shCtrl) in

SHG-44 and U251 cell lines. (B) Cells were stained and the rate of cell apoptosis in two cell lines was obtained through flow cytometry. (C) The distribution of cell cycle in three phrases estimated by flow cytometry. ANT XR1, anthrax toxin receptor 1; shANT XR1, cells transfected by ANT XR1-targeting shRNA; shCtrl, cells transfected by control shRNA. **P < 0.01, ***P < 0.001.

Figure 4

Migration inhibition in shANTXR1-harboring glioma cells. (A) Microphotographs obtained from wound-healing assay and comparison of cell migration between shANTXR1-harboring cells and controls in both cell lines. (B) Representative photographs (200 ×) of tumor cells after transwell assay and the comparative results of cell migration between the experimental shANTXR1 group and shCtrl group in SHG-44 and U251 cell lines. ANT XR1, anthrax toxin receptor 1; shANTXR1, cells transfected by ANT XR1-targeting shRNA; shCtrl, cells transfected by control shRNA. ***P < 0.001.

Figure 5

Molecular mechanisms underlying ANTXR1 action in glioma cells. (A-B) The expression level of apoptosis-related proteins in U251 cells treated with shCtrl or shANTXR1 through human apoptosis antibody array. The red box labeled the antibody spots where the signal was significantly reinforced and the green boxes referred to the markedly reduced signals following the transfection of shANTXR1. (C) Comparisons in gray values of 15 apoptosis-related protein markers that were significantly dysregulated in the U251 tumor cells. (D) A western blotting-based detection in the expression of proteins involved in MAPK9 signaling pathway in U251 cells. ANTXR1, anthrax toxin receptor 1; shANTXR1, cells transfected by ANTXR1-targeting shRNA; shCtrl, cells transfected by control shRNA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *P < 0.05, **P < 0.01.

Figure 6

Depleting ANT XR1 in vivo alleviated glioma progression. (A) The quantified comparison of xenograft tumor volumes (shCtrl vs. shANT XR1). (B) Bioluminescence photographs on tumors in mice models after subcutaneous injection of SHG-44 cells, along with the comparison in the total bioluminescent intensity of tumors between the shANT XR1 group and controls. (C) The removed solid tumors taken out from mice models and the comparison in tumor weight. (D) Microphotographs of cells after Ki-67 staining assay in shCtrl and shANT XR1 groups of SHG-44 cells (200× and 400×, respectively). ANT XR1, anthrax toxin receptor 1; shANT XR1, cells transfected by ANT XR1-targeting shRNA; shCtrl, cells transfected by control shRNA. *P < 0.05, **P < 0.01.