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Original Article

Knockdown of apoptosis repressor with caspase recruitment domain (ARC) increases the sensitivity of human glioma cell line U251MG to VM-26

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Abstract: Previous studies have demonstrated that apoptosis repressor with caspase recruitment domain (ARC) is up-regulated in many forms of malignant tumors and low levels of ARC protein were expressed in normal human brain tissue. Little is known expression of ARC in glioma. Here, we found that ARC protein was highly expressed in primary human glioma when compared with normal brain tissues. A decrease in cell viability and an increase in apoptosis were observed in U251MG cells after ARC was knocked down. Knockdown of ARC was confirmed by western blotting. Knockdown of ARC promoted caspase-8, caspase-3 activation and Bax accumulation. These results indicate that ARC has a anti-apoptosis function in glioma.

Keywords: ARC, glioma, apoptosis, VM-26 sensitivity

Introduction

Malignant gliomas are the most common and deadly primary brain tumors. Approximately 7 cases per 100,000 individuals are diagnosed with glioma worldwide every year [1, 2]. Although survival of patients with glioblastoma multiform (GBM, a grade IV glioma, as classified by the World Heath Organization) has improved due to therapeutic developments, the prognosis for patients with GBM remains poor. The median survival of the patients is approximately 1 year [3]. Therefore, it is critical to explore the molecular mechanism underlying GBM and to find new approaches that can complement and improve current therapeutic strategies for GBM.

Apoptosis is a counterbalance to cell proliferation. The disruption of apoptosis or programmed cell death is critical for carcinogenesis, including glioma. Many researchers have proved an increase in anti-apoptotic proteins and a decrease in pro-apoptotic proteins in the progression of GBM [4]. For example, the expression of several endogenous apoptosis inhibitors including Bcl-2, survivin, cIAP are upregulated in GBM [5, 6]. Apoptosis repressor with caspase recruitment domain (ARC) is also an endogenous inhibitor of apoptosis [7]. It can not only suppress death-inducing signal complex (DISC) formation by binding to Fas, FADD and procaspase-8, but also inhibits Bax translocation to mitochondria by interacting with Bax [8, 9]. Previous studies have revealed that ARC is expressed in many human cancer cell lines and primary human cancers [10, 11]. Moreover, overexpression of ARC in cancer cell lines (Hela and Hs578T cells) can inhibit chemical- and radiation-induced apoptosis and knockdown of ARC in cancer cell lines can promote chemical- and radiation-induced apoptosis [11, 12]. However, whether ARC has an effect on the development of GBM remains unknown. In this study, first, we compared the expression of ARC between GBM and normal brain tissue. Second, we constructed a plasmid that encoded a hairpin sequence of siRNA targeting ARC and stably transfected the vector into glioma cell line U251MG in which the expression level of ARC was dominantly decreased compared with the control vector. Reduced expression of ARC increased the sensitivity of U251MG to anti-

cancer drug Vm-26 *in vitro* and might promote activation of caspase 8, caspase-3 and translocation of Bax.

Materials and methods

Clinical samples

All clinical samples described here were gained from patients who had given informed consent. Samples were collected immediately after surgical resection, snap frozen and stored at -80 ℃. Human GBM, pituitary adenomas and meningioma tissues were obtained from Department of Neurosurgery, Tianjin Huanhu Hospital. Normal brain tissues were obtained by collecting donations from individuals who died in traffic accidents and confirmed to be free of any prior pathologically detectable conditions. The study was approved by Research Ethics Committee in our Hospital.

Reagent

ARC antibody, Bax antibody, cleaved-Caspase-3 antibody, Caspase-8 antibody, β-actin antibody, secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Santa cruz, R&D and Caymen (USA). Lipofectamine 2000 was purchased from Invitrogen. MTT was purchased from Sigma (St. Louis, USA). All other reagents were purchased from Promega or Takara.

Plasmid construction

DEQOR (a web-based tool at http://cluster-1.mpi-cbg.de/Deqor/deqor.html) was used to design the siRNA targeting ARC. The mRNA target sequence of the siRNA to ARC is at nucleotide positions 692-710 (5'-AGGGACGAGTCCGAAGT-3') of the human ARC mRNA. The DNA designed to encode a hairpin sequence of siRNA targeting ARC was synthesized by the Augct Company (China) and inserted into the pSilencer/vector for stable expression. Plasmids were verified by DNA sequencing. pSilencer/ARC-siRNA plasmid was transfected into U251MG cells using Lipofectamine 2000. The ARC expression level was tested at 48 hours (h) after the initial transfection by western blot analysis.

Cell culture

LN229, U251MG, LN308, U87MG, A-172 U373MG cells and SH-SY5Y neuroblastoma cells were preserved in Institute of Neurosurgery, Tianjin Huanhu Hospital and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 μg/mL streptomycin, kept in 5% CO_2 in a humid incubator at 37 ℃. The culture medium was changed every 48 h.

Transfection

Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For transient transfection experiments, U251MG cells were transfected with plasmids and incubated for 48h.

After U251MG cells were transfected with pSilencer/ARC-siRNA plasmid or pSilencer/scrambled-ARC-siRNA plasmid, stable cell clones (named A-/U251MG and S/U251MG, respectively) were selected with 400 μg/mL of G418 for 4~5weeks and were maintained with antibiotics throughout the culturing period [13].

Drug treatment

VM-26 (Teniposide) was provided by Beijing Double-crane Pharmaceutical Beijing, China. Agent was dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mg/mL and store at -20°C. A stock was directly diluted with the media of U251MG cells to produce final concentrations of VM-26. U251MG, A-/U251MG or S/U251MG cells were seeded in 96-well plates or in 25cm^2 flasks in triplicate. Twelve hours after seeding the cells, VM-26 was added to the culture media at a final concentration of 2 μg/mL. Then, the proliferation, survival and apoptosis of glioma cells were measured by MTT assay or flow cytometry.

MTT assay

U251MG cells and the cells treated with A-/U251MG or S/U251MG were plated in 96-well and incubated at 37℃ for at least 12h. The cells were then exposed to Vm-26. After 72h, 20 μl of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was added to every well and incubated at 37℃ for 4h, then the medium was removed and 100 μl DMSO was added. The absorbance at 570 nm was detected using μQuant Universal Microplate Spectrophotometer (Biotek instrument, Winooski, USA) after the precipitated formazan

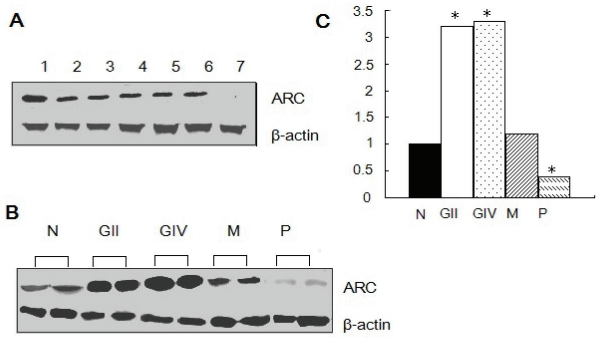

Figure 1. Expression of ARC protein in human glioma cell lines, GBM, pituitary tumor, meningioma, and normal brain tissue. A. ARC protein expression in human glioma cell lines was analyzed by immunoblotting. SH-SY5Y neuroblastoma cells was used as a negative control. Lane 1, U87MG; Lane 2, LN229; Lane 3, A-172; Lane 4, U251MG; Lane 5, U373MG; Lane 6, LN308; Lane7, SH-SY5Y B. The expression of ARC in GBM, Pituitary tumor, Meningioma, and normal brain tissue was detected by Western blotting. β-actin was used as a control. N: normal brain tissue, GII: grade II glioma, GIV: grade IV glioma, M: meningioma, P: pituitary tumor C. Relative expression level of ARC was quantified by LabWorkstrade; Image Acquisition and software, and the relative ARC levels were shown. N: normal brain tissue, GII: grade II glioma, GIV: grade IV glioma, M: Meningioma, P: Pituitary tumor. An asterisk (*) denoted $P < 0.05$.

had dissolved. Each experiment was carried out in triplicate and repeated for three times, and mean ± standard deviation of A570 was shown in the figure.

Apoptosis analysis

To quantify whether knockdown of ARC increased drug-induced apoptosis, apoptosis was evaluated by flow cytometry analysis. Untreated U251MG cells, the cells treated with A-/U251MG and S/U251MG were incubated in 6-well plates ($1X10^6$ cells/well) in the medium with or without 2 μg/mL Vm-26 for 72h. Apoptosis was detected using the Annexin V-FITC/propidium iodide (PI, Invitrogen company, USA). Briefly, the cells were harvested and then resuspended in 1 ml of buffer followed by addition of 5 μl Annexin V and 10 μl PI. Cells were incubated in the dark at room temperature for 15min. Cell death was determined using a flow cytometer (BD company). Data were obtained and analyzed by Flowjo software.

Western blot analysis

Cells was washed twice in PBS, then directly lysed with radio immunoprecipitation assay (RIPA) buffer (1% P-40, 1 mM Tris-HCL, 1 mM MgCl2 and 0.1% SDS) containing protease inhibitors for 20~30 min at 4℃ and then were repeatedly blown with sample injector 20~30 times in order to make cells broken. Frozen brain samples were thawed and homogenized (6-8 strokes) in RIPA with a homogenizer (Ingenieurburo CAT, M zipperer GmbH, Germany). The lysis or brain homogenate was centrifuged at 11,000g for 10 min at 4℃. The supernatant was added equal volume of sample buffer (50mM Tris-HCL, 4% SDS, 0.01% Bromophenol blue, 20% glycerol and 2% 2-mercaptoethanol, pH = 6.8) and the mixture was heat-denatured and placed on ice for 5 min, respectively. The concentration of protein was determined using BCA protein assay kit. Equal amount of protein was loaded onto 12% or 15% Tris-glycine SDS-PAGE gel and separated at 80~120 volts for 1.5~2h. The protein was then transferred to nitrocellulose membrane and blocked with 5% milk in TBST buffer. Anti-ARC antibody, anti-Caspase-8 antibody, anti-Bax antibody and anti-Caspase-3 were detected for ARC, Caspase-8, Bax, Caspase-3, respectively. An anti-β-actin antibody was used as a control for the equal loading of sample.

Statistical Analysis

Data were expressed as the mean ± SEM and were analyzed from three independent experiments. The signification of difference between values was analyzed by one-way ANOVA test followed by Dunnet's multiple comparison. Statistical significance was set as $P < 0.05$.

Results

ARC protein was expressed in several glioma cell lines and increased in primary human glioma

Because suppression of apoptosis is a fundamental mechanism in the development of human GBM, we hypothesized that ARC was up-regulated in glioma cell lines. To assess this hypothesis, ARC was detected in 6 glioma cell lines-- LN229, U251MG, LN308, U87MG, A-172, U373MG and SH-SY5Y neuroblastoma cells was used as a negative control [14] by western blotting. Human ARC has an alterna-

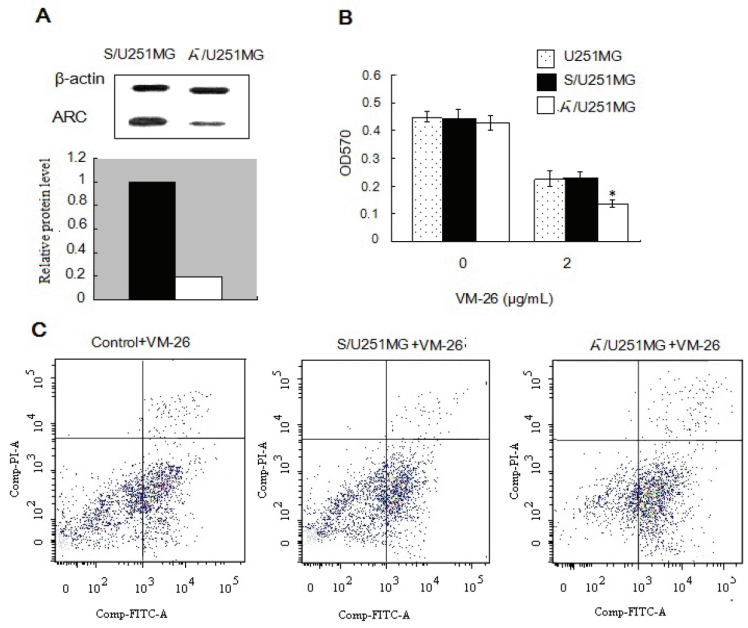

Figure 2. Endogenous ARC in U251MG was effectively knocked down and increases the sensitivity of human glioma cell line U251MG to VM-26. A. Analysis of ARC protein expression in A-/U251MG cell lines by Western blot with anti-ARC antibody, β-actin was used as a control. Relative expression level of ARC was quantified by LabWorkstrade; Image Acquisition and software, and the relative ARC levels are shown. B. The relative cell viability was tested by MTT assay. 72h after untreated U251MG cells, the cells treated with A-/U251MG and S/U251MG were exposed to VM-26, an MTT assay was performed as described in the materials and methods. The results shown were representative of three independent experiments, the histograms represented average and error bars represented standard deviation. An asterisk (*) denoted $P < 0.05$. C. 72h after U251MG, A-/U251MG and S/U251MG were exposed to VM-26, adherent cells were collected by trypsinization and analyzed in three sets of experiments. An Annexin-V-FITC and PI double staining flow cytometry analysis showed that the cells treated with A-/U251MG exposed to VM-26 could induce significant apoptosis (*66.8 ± 2.4% at 72h) compared with normal U251MG cells (46.035 ± 0.26%) and the cells treated with S/U251MG exposed to VM-26 cells (48.65± 0.08%).

tive-spliced isoform, Nop30, which is a hypothetical nucleolar protein. ARC and Nop30 share the same N-terminus [15]. Therefore, the antibody against the ARC C-terminus excludes the possibility that Nop30 will be recognized. We found that ARC was highly expressed to a variable extent in different glioma cell lines (**Figure 1A**).

Since the high levels of ARC in some glioma cell lines and previous studies also reported low levels of ARC protein were present in human, rat and mouse brain tissues [16, 17, 18], we further tested whether ARC was increasedly expressed in primary human glioma using immunoblotting. Quantitative densitometry and analysis of group data revealed that ARC expression level was 3–fold higher than that in the normal brain tissues ($P < 0.05$, **Figure 1B** and **C**), suggesting that ARC in glioma tissues is markedly increased in glioma cells. However, the expression of ARC did not correlate with stages of the tumor, or grades of the tumor. Meanwhile, we also investigate the presence of ARC in other types of human brain tumors: that are pituitary tumor and meningioma. Although ARC was expressed in the two types of tumors by western blotting, the expression levels of ARC in Pituitary adenomas was lower.

Knockdown of endogenous ARC by psilencer/ARC-siRNA increased the sensitivity of human glioma cell line U251MG to VM-26

To further investigate whether ARC contributes to resistance of GBM cells to chemotherapeutic agent VM-26, we made a construct targeting ARC by inserting the synthetic cDNA fragment encoding hairpin structure siRNA into the pSilence vector and established a U251MG cell line (A-/U251MG) transfected with the pSilencer/ARC-siRNA. The control cell line S/U251MG was also established by the same way. We verified that the expression level of ARC in cells treated with A-/U251MG was reduced nearly by 60% when compared with that in the cells treated with S/U251MG (**Figure 2A**).

By MTT assay, we found that the knockdown of endogenous ARC in U251MG cells exposed to Vm-26 decreased cell viability nearly by 50% when compared with that in untreated cells and in the cells treated with S/U251MGs (**Figure 2B**). Moreover, flow cytometry analysis showed that the cells treated with A-/U251MG exposed to VM-26 showed significantly increased apoptosis (66.8 ± 2.4% at 72h) when compared with

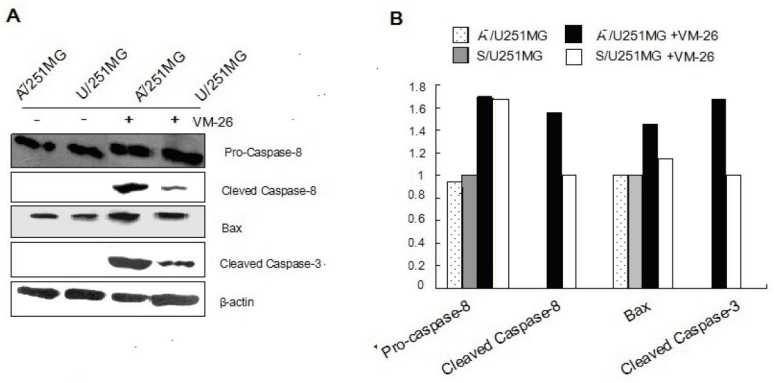

Figure 3. Increased sensitization to VM-26 by knockdown of ARC in U251MG. A. Enhancement of caspase-8, caspase-3 activity and Bax accumulation after endogenous ARC of U251MG were knocked down and were treated with indicated concentrations of VM-26 for 72h. B. A graph showing quantitative analysis of relative expression level of Caspase-8, Caspase-3 and Bax in different treatment groups.

that untreated U251MG cells (46.035 ± 0.26%) and the cells treated with S/U251MG (48.65 ± 0.08%) exposed to VM-26 cells (**Figure 2C**) ($P < 0.05$). However, knockdown of ARC alone did not show to induce apoptosis (Data were not shown).

Increased cell apoptosis via promoting activation of caspase-8, caspase-3 and Bax accumulation

Previous studies have shown that ARC can antagonize both extrinsic and intrinsic pathways by binding caspase-8 or Bax to prevent their activations [8]. Based on these findings, we speculated that the high sensitivity of A$^-$/U251MG to VM-26 is associated with increasing apoptosis. To explore the mechanism(s) by which knockdown of ARC could increase the sensitivity of GBM cells to VM-26, we examined the expression of procaspase-8, cleaved caspase-8, caspase-3 and Bax using western blotting. As indicated in **Figure 3**, knockdown of ARC promoted Caspase-8, Caspase-3 activation and Bax accumulation.

Discussion

In the present study, we demonstrated that ARC protein was highly expressed in primary human glioma compared with normal brain tissue. In order to further explore whether ARC plays a significant role in glioma cells, we knocked down its expression in U251MG with a plasmid expressing siRNA (hair-pin structure) that targets ARC, and we found that inhibition of ARC expression by siRNA contributed to the high sensitivity of U251MG cells to VM-26. Our current study also reveals that knockdown of ARC promoted Caspase-8, Caspase-3 activation and Bax accumulation in glioma cell line, further confirmed the anti-apoptosis function of ARC.

The human ARC protein with homology to the prodomains of caspases and Apaf-1 was identified by Koseki and his colleagues [7]. A year later, Stoss et al. reported the human ARC gene was located on chromosome 16q21-q23 and consists of four exons. ARC encodes a protein that is 208 aa in length and is initially thought to be primarily expressed in terminally differentiated cells, such as cardiac and skeletal myocytes [15]. ARC normally is not expression or low expression in many tissues, however, ARC is highly expressed in malignant tumors [14]. For example, it was found that ARC was expressed in hepatoma cell lines but not in normal liver cells [14]. Similarly, ARC protein is also increased in primary human ovarian and cervical carcinomas but not in normal tissue [19]. ARC was presented at high levels in 44 primary human colon adenocarcinomas compared with matching samples of adjacent benign colon tissue. Moreover, the subcellular localization of ARC differed between colon adenocarcinomas and benign colon tissue, human breast cancer and normal breast tissue [11, 19]. Our results indicate that ARC is expressed in normal brain tissue which is consistent with previous findings [16]. However, ARC protein was highly expressed in primary human glioma compared with normal brain tissue. These observations suggest that ARC may play a role in carcinogenesis.

The human ARC was identified as an endogenous inhibitor of apoptosis [8] . Apoptosis plays a very critical role in carcinogenesis [20]. Studies have revealed that ARC could block apoptosis through multiple mechanisms. ARC

can suppress death receptor-mediated apoptotic signal by binding to Fas, FADD, and procaspase-8, and the interaction of ARC with Bax inhibits Bax translocation to mitochondria, consequently preventing cytochrome c release [21]. It directly binds PUMA to inhibit Drpl accumulation in mitochondria and prevents consequent mitochondrial fission [22]. It also blocks Smac/DIABLO release and protects mitochondria against fission and maintains mitochondrial membrane potential [23, 24, 25]. Moreover, ARC can suppress an increase in intracellular Ca^{2+}, thereby blocking Ca^{2+}-mediated apoptosis [26]. Taken together, ARC is a protein that antagonizes both the extrinsic and intrinsic apoptotic pathways. The role of ARC implies that it may provide a growth advantage to cancer cells.

In the present study, we showed that inhibition of ARC expression by siRNA contributed to the high sensitivity of U251MG cells to VM-26, which could explain the reason why ARC contributes to chemotherapy or radiation resistance in some cancer cell lines as shown in previous studies [11, 12]. It has been shown that transfection of an ARC gene construct into Hs578T cell line that contains low levels of endogenous ARC suppresses apoptosis by doxorubicin (an anti-cancer drug). Whereas inhibition of ARC by small interfering RNA knockdown in Hela and SGC-7901 cells that express high level of endogenous ARC sensitized the cell lines to doxorubicin. Furthermore, knockdown of ARC promoted caspase-8, caspase-3 activation and Bax accumulation in glioma cell line. More studies are needed in order to understand the complex function of ARC in relation to cancer development and the biological significance of ARC to apoptosis resistance in cancer cells.

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