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Original Article

Ectopic expression of AP-2α transcription factor suppresses glioma progression

Wenjing Su[1*], Juan Xia[1,2*], Xueqin Chen[1], Miao Xu[1], Ling Nie[1], Ni Chen[1], Jing Gong[1], Xinglan Li[1], Qiao Zhou[1]

[1]Laboratory of Pathology, State Key Laboratory of Biotherapy and Department of Pathology, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China; [2]Suining Central Hospital, Suining 629000, China. [*]Equal contributors.

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Abstract: The transcriptional factor AP-2α is a tumor suppressor gene and is downregulated in various neoplasms including glioma. Although the level of AP-2α is negatively associated with the grade of human glioma, the specific functions of AP-2α in glioma are still unknown. In this study, we experimentally showed that artificial overexpression of AP-2α in glioma T98G and U251 cells significantly downregulated the mRNA levels of Bcl-xl, Bcl-2, c-IAP2 and survivin, together with upregulation of the Hrk mRNA levels. Reintroduction of AP-2α also induced downregulation of the protein levels of survivin and VEGF in glioma cells. In biological assays with T98G and U251 cells, AP-2α reduced tumor cell growth, increased cell death, attenuated cell migration and endothelial tube formation. The AP-2α transcription factor may play an important role in suppressing glioma progression.

Keywords: AP-2 alpha, glioma, cell growth, apoptosis, invasion

Introduction

AP-2α, which is located in the short arm of chromosome 6 adjoining to the HLA gene, is a member of the transcription factor activator protein-2 (AP-2) family {Britto, 2007 #42} {Britto, 2007 #42}. AP-2α binds to CG-rich sequences such as 5'-GCCN$_3$GGC, 5'-GCCN$_4$GGC and 5'-GCCN$_{3/4}$GGG via a DNA-binding domain within the COOH-terminal half of the protein and plays key roles in transcriptional regulation [1].

In addition to its important function in physiological processes such as embryo development, AP-2α acts as a tumor suppressor gene, inhibiting the initiation and progression of a variety of malignancies. AP-2α expression was decreased in many neoplasms such as glioma [2-4], prostate cancer [5], breast cancer [6-9], colon cancer [10], melanoma [11, 12], ovarian cancer [13] and renal carcinoma [14].

AP-2α has a pivotal role in regulating the expression of key genes, the products of which are involved in tumor initiation and progression. For example, AP-2α regulates genes that are involved in proliferation (c-MYC), cell cycle regulation (HER-2 and p21WAF1), apoptosis (c-KIT, Bcl-2, and FAS/APO-1), cell adhesion(MCAM/MUC18 and E-cadherin), and tumor invasion/angiogenesis (MMP-9, PAI-I, and PAR-1) [15-19].

Decreased AP-2α level was associated with grade of glioma [20]. However, the specific effects of AP-2α on glioma cells were not clearly understand. In the present study, we artificially overexpressed AP-2α in glioma cells, and investigated its effects on glioma cell growth, apoptosis, migration and endothelial tube formation.

Materials and methods

Cell lines, tissue samples and general reagents

Human glioma cells T98G, U251, adenovirus-immortalized human embryonic kidney epithelial cell HEK-293, and human umbilical vein endothelial cells (HUVEC) were all from the American Type Culture Collection and were cultured in DMEM with 10% FCS (Life Technologies, TakaRa, Japan). Archived formalin-fixed, paraffin-embedded samples of glioma (n = 2) and

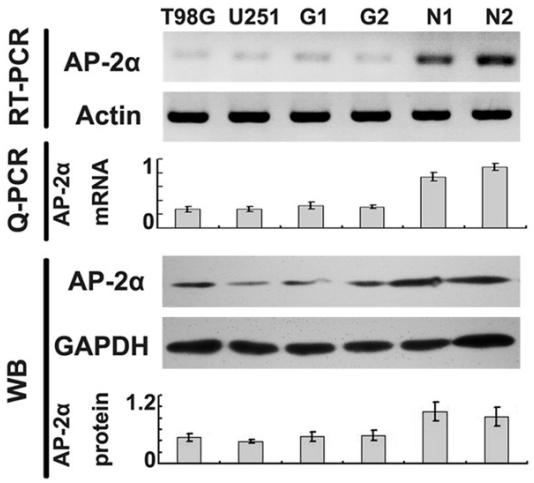


Figure 1. AP-2α expression in glioma. AP-2α expression in glioma cell lines (U251 and T98G), glioma tissues (G1 and G2) and normal brain tissues (N1 and N2). RT-PCR (upper), Q-PCR (middle) and western blot analysis (lower, with semiquantitative histograms) showed decreased AP-2α levels (relative to actin or GAPDH, mean ± SD of three independent experiments, $P < 0.05$) in glioma cell lines and tissues compared with that in normal brain tissues.

normal brain (n = 2), were also used. All tissue samples were from West China Hospital and were collected and used according to the ethical guidelines and procedures approved by the institutional supervisory committee. Tris base, EDTA, Tween 20 and dithiothreitol were from Amresco (Solon, OH). PMSF, aprotinin and pepstatin were from Roche Diagnostics (Mannheim, Germany).

RT-PCR and real-time qPCR

Total RNA was isolated using the TRIzol reagent (Invitrogen/Life Technologies, TakaRa, Japan). cDNA was synthesized from the isolated RNA by RT and amplified by PCR. PCR primer sequences and product lengths were as follows. AP-2α: 5'-ACT CCT TAC CTC ACG CCA TC-3', 5'-ATA GGG ATG GCG GAG ACG-3', 136 bp; actin: 5'-TGG AGA AAT CTG GCA CCA C-3', 5'-GAG GCG TAC AGG GAT AGC AC-3', 190 bp; Bcl-xl: 5'-CTG TGC GTG GAA AGC GTA G-3', 5'-CTCGGCTG-CTGCATTGTTC-3', 159 bp; Bcl-2: 5'-GTC ATG TGT GTG GAG AGC GTC-3', 5'-GAG TCT TCA GAC AGC CAG G-3', 193 bp; Hrk: 5'-CTA GGC GAC GAG CTG CAC CAG-3', 5'-GCA CAG CCA AGG CCA GTA GGT G-3', 102 bp; c-IAP2: 5'-AGG GAA GAG GAG AGA GAA AGA GC-3, 5'-CGG CAG TTA GTA GAC TAT CCA GG-3', 133 bp; Survivin: 5'-GCA GTT TGA AGA ATT AAC CCT TG-3', 5'-CAC TTT CTC CG CAG TTT CCT C-3', 121 bp. PCR products were separated by agarose gel electrophoresis and visualized by the fluorescent dye GoldView (Beijing SBS Genetech, Beijing, China) under UV light. Real time PCR was carried out as described [21].

Western blot

The primary antibodies used were as follows: AP-2α (rabbit monoclonal, 1:500, Santa Cruz Biotechnology, USA), VEGF (mouse Polyclonal, 1:500, Boster, China), Survivin (rabbit polyclonal, 1:500, R&D, USA), GAPDH (mouse monoclonal, 1:10000, Kangcheng, China). Horseradish peroxidase-labeled secondary antibodies were from Zymed Laboratories (South San Francisco, CA). Western blotting was carried out as previously described [22].

Recombinant adenoviral vectors for overexpression of AP-2α

The coding sequence of AP-2α (transcript variant 3, 1321 bp) was cloned from HEK-293 cells by RT-PCR with the following primers: 5'-CTC GAG CCG CGA TGT CCA TAC TTG C-3', 5'-AAG CTT GCC TCA CTT TCT GTG CTT CTC-3'. The adenovirus vector for AP-2α was constructed as described [21] and named AD-AP2α. The pAdTrack-CMV vector was used as control (AD-control). The titers and multiplicity of infection (MOI) were determined according to the manufacturer's protocols.

MTT assays

T98G and U251 cells transfected with AD-AP2α or AD-control were incubated in 96-well tissue culture plates for the indicated times. MTT (Sigma Aldrich, USA) was then added to each well and incubated for 4 hours. Then the supernatant was removed, replaced with 100 μl dimethylsulfoxide and incubated for 10 min in dark. The absorbance at 570 nm (A570) was measured by spectrophotometer (Bio-Tek, USA).

Migration assays

Transwell migration assays were used to examine the effect of AP-2α on glioma cell migration. T98G or U251 cell were transfected with AD-AP2α (AD-control as negative control). 24 h later, cells were isolated and 1×10^5 cells suspended in serum-free media were placed in the

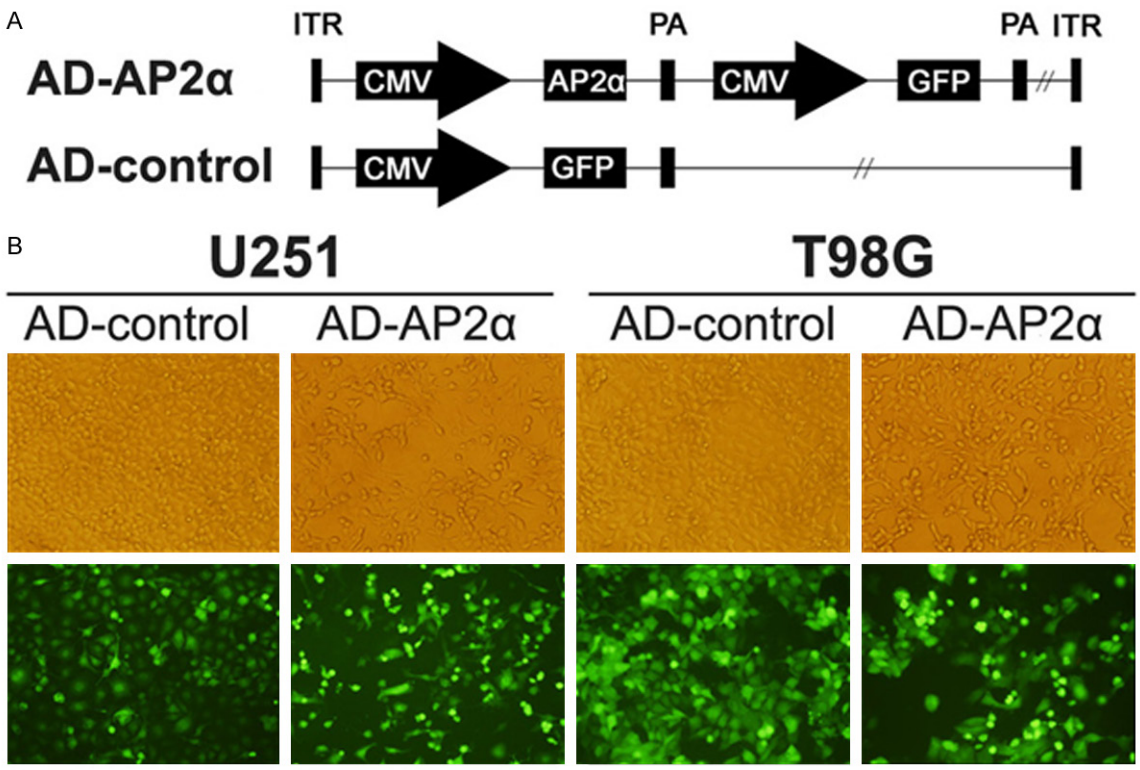


Figure 2. The AP-2α recombinant adenoviral vector was constructed. (A) Shows the recombinant adenoviral vector structure (ITR, inverted terminal repeat; CMV, cytomegalovirus; PA, poly A; GFP, green fluorescence protein.). The infection efficiency of AD-AP2α and AD-control of glioma cells was shown by homogenous green fluorescence protein expression of the infected cells (B).

upper compartment of 5 mm-pore transwells (Corning Costar, Lowell, MA). Cells were allowed to migrate for 24 h and the transwell slides were finally fixed with paraformaldehyde and stained with crystal violet.

Tube formation assay

Tube formation assay was performed as previously described [23]. Conditioned media were obtained by incubating the cells transfected with AD-AP2α or AD-control in DMEM without serum for 24 h. The 96-well plate was coated with 50 μl growth factor-reduced matrigel (BD Biosciences). A total of 4×10^5 HUVEC cells was resuspended in 100 μl of conditioned media with 1% FCS and seeded on matrigel-coated wells. HUVEC cells were incubated for 18 h to allow formation of tube-like structures. Total tube lengths formed were measured and compared from three different viewing fields at 10 × magnification using an inverted microscope (Nikon TMS). Tube formation assays were performed in triplicate.

Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed by using in situ cell death detection kit (Roche, USA) as previously described [22].

Statistical analysis

All experiments were repeated at least thrice. PASW Statistics 18.0 software package was used for statistical analysis. *P* value less than 0.05 was considered significant statistically.

Results

AP-2α expression was significantly decreased in human glioma cells

RT-PCR and western blot confirmed significant reduction of AP-2α level in human glioma cells (T98G, U251) and glioma tissues (G1, G2) as compared with normal human brain (N1, N2) at both the mRNA and protein levels (**Figure 1**).

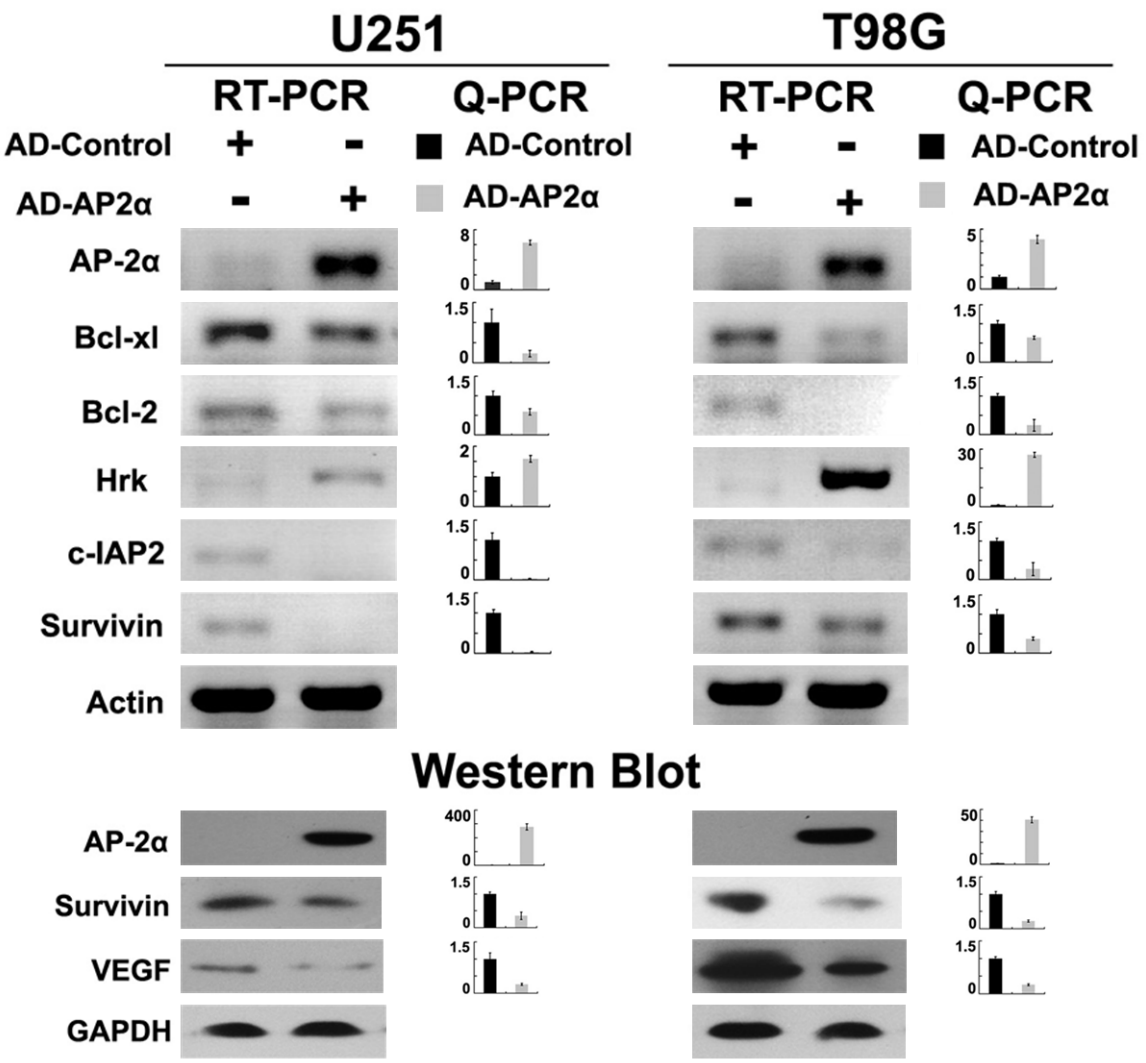


Figure 3. Overexpression of AP-2α was confirmed by PCR and WB. In glioma cells, ectopic expression of AD-AP2α resulted in significant downregulations of Bcl-2, Bcl-xl, c-IAP2 and Survivin mRNA levels, together with upregulation of Hrk mRNA level (upper) (relative to actin, means ± SD from three independent experiments, $P < 0.05$). Western blot showed decreased (relative to GAPDH, means ± SD from three independent experiments, $P < 0.05$) protein levels of VEGF and Survivin with AD-AP2α infection in both cells (lower).

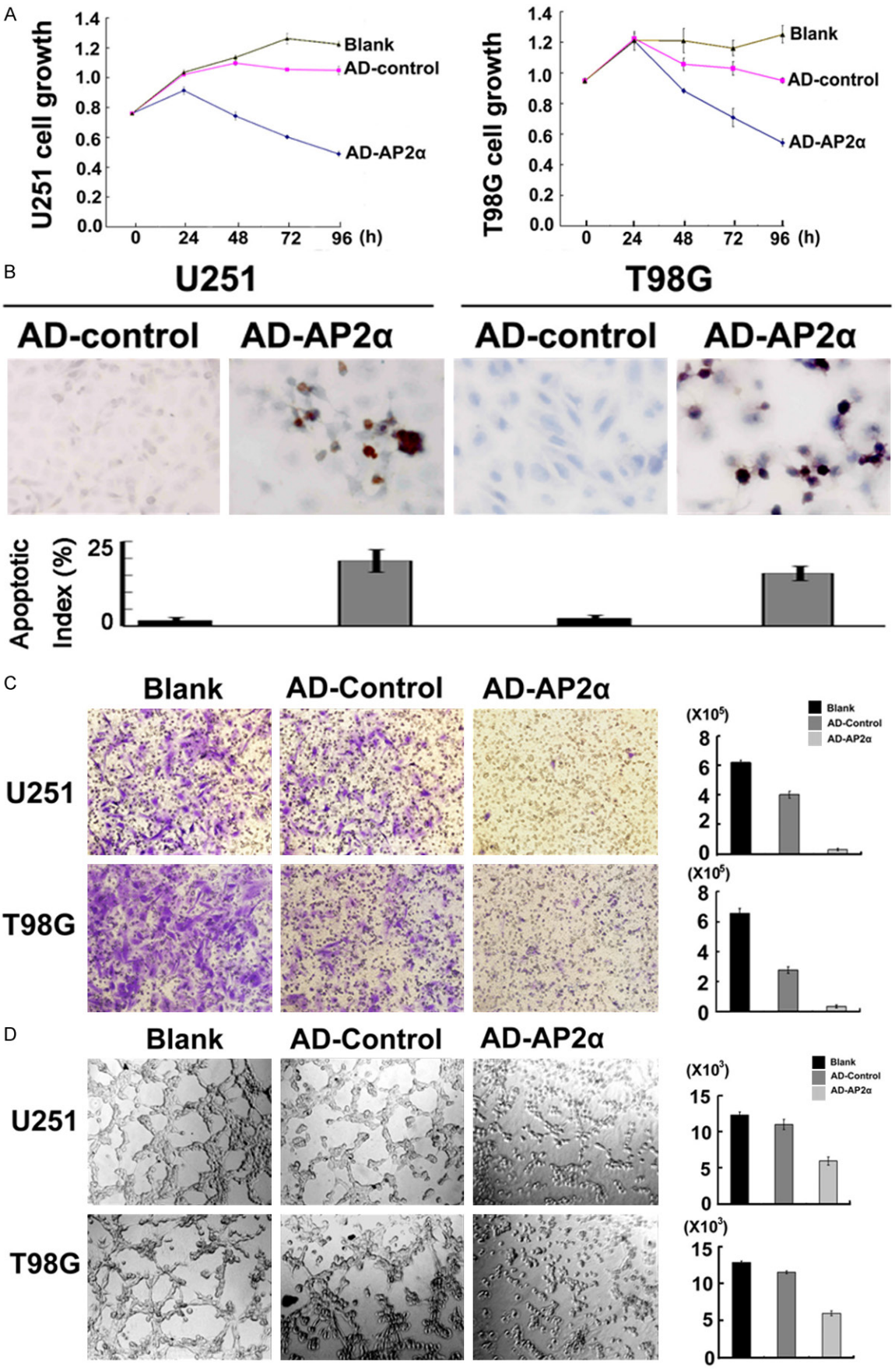
Artificial overexpression of AP-2α decreased mRNA levels of Bcl-xl, Bcl-2, c-IAP2 and survivin, increased mRNA levels of Hrk, and decreased protein levels of survivin and VEGF

To examine the effects of AP-2α on glioma cells, recombinant adenovirus for overexpression of AP-2α (AD-AP2α, MOI 50, 96 hours) was constructed (**Figure 2B**). RT-PCR, Q-PCR and western blot confirmed the efficient expression of AP-2α in AD-AP2α infected T98G and U251 cells (**Figure 3**).

Artificial overexpression of AP-2α in T98G and U251 resulted in significant decreased expression of Bcl-2, Bcl-xl, c-IAP2 and survivin, together with increased Hrk mRNA level, and western blot displayed repression of survivin and VEGF after AD-AP2α treatment (**Figure 3**). Inconspicuous alterations were detected in the levels of other molecules such as Bik, BNIP3, c-IAP1 and CASP8 in T98G and U251 overexpressing AD-AP2α (data not shown).

AP-2α inhibited cell growth, promoted apoptosis, inhibited cell migration and HUVEC tube formation ability in glioma

Having confirmed the contributions of AP-2α to those tumor-associated genes, we then turned


A
U251 cell growth
1.4
1.2
1.0
0.8
0.6
0.4
0.2
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24
48
72
96 (h)
Blank
AD-control
AD-AP2α
T98G cell growth
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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24
48
72
96 (h)
Blank
AD-control
AD-AP2α
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U251
T98G
AD-control
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AD-control
AD-AP2α
Apoptotic Index (%)
25
0
C
Blank
AD-Control
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U251
T98G
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U251
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(X10³)
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AD-AP2α
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Figure 4. Effects on cell behavior by artificial overexpression of AP-2α in U251 and T98G cells. MTT assay (A) showed that glioma cell growth was significantly inhibited concomitant with the reintroduction of AP-2α. TUNEL assay (B) demonstrated increased apoptosis (shown by brown-staining granules in apoptotic cells) in glioma cells with AP-2α overexpression. Migration assay (C) revealed that artificial re-expression of AP-2α inhibited tumor cell migration. Microtube formation assay (D) showed suppressed HUVEC microtubule formation induced by AP-2α over expression in U251 and T98G cell supernatant. Values are means ± SD from three independent experiments. $P < 0.05$ compared to AD-control.

to show the biological effects of artificial AP-2α overexpression in glioma cells. Concomitant with AD-AP2α mediated AP-2α overexpression, glioma cells showed significantly reduced cell growth (**Figure 4A**), increased cell apoptosis (**Figure 4B**) and decreased cell migration (**Figure 4C**). Microtubule formation of HUVEC cells was decreased when stimulated with supernatant from glioma cells re-expressing AP-2α (AD-AP2α) (**Figure 4C**).

Discussion

The AP-2 family consists of five highly homologous members, namely AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε [24], the expression of which is cell-type specific. The amine terminus of the AP-2α protein is responsible for transactivation activity, and a basic and helical region in the COOH terminus is responsible for dimerization and DNA binding [25, 26].

AP-2α mediates programmed gene expression in embryonic morphogenesis and cell differentiation [27]. AP-2α knockout mice die perinatally with severe multiple congenital defects involving face, skull, and sensory organs. In situ hybridization showed that mouse embryos specifically expressed AP-2α in ectoderm-derived tissues, including craniofacial, gonad, kidney, and skin [27, 28].

Also, AP-2α has been shown to be a tumor suppressor gene, the loss of which is linked to malignant transformation, tumor progression and elevated risk of metastasis in such tumors as melanoma [29-32], prostate cancer [33, 34], breast cancer [35, 36], pancreatic cancer [37, 38], colorectal cancer [39] and ovarian cancer [13]. Notably, loss of AP-2α was strongly correlated with higher grade in human glioma, and appeared to be the most frequent molecular change in astrocytic tumor progression from lower to higher grade, although no significant effect of AP-2α on survival was observed [20].

In this research, significant reductions of AP-2α were also detected in glioma cells and tissues, which is in consistence with studies before. However, the role of AP-2α in the development/progression of glioma has not been fully defined. It has been reported that there are multitudinous genes regulated by AP-2α such as those involved in cell proliferation (Hoxa, IGF receptor type I and IGF-binding proteins 3/5) [40-43], cell cycle regulation (p21WAF1/CIP17 and HER-2) [44, 45], apoptosis (c-KIT) [30, 46, 47], cell adhesion (E-cadherin and MCAM/MUC18) [48, 49], and cell migration/angiogenesis (MMP-2, and PAR-1) [48, 50, 51]. Our data showed that AP-2α inhibited the expression of the anti-apoptotic genes Bcl-xl, Bcl-2, c-IAP2 and surviving and the pro-angiogenic gene VEGF, whereas promoted the expression of the pro-apoptotic gene HRK, thus enhanced glioma cell apoptosis and inhibited HUVEC tube formation ability. These finding are compatible with our earlier works [47, 52, 53]. Except for this, we also detected the influences of AP-2α on a series of genes involving in cell proliferation and migration (data not shown) but failed to get meaningful results. Nonetheless, AP-2α induced attenuated glioma cell proliferation and migration in biological experiments, the mechanism of which has not been well understood yet.

In summary, the results of this study showed that AP-2α, which is dramatically down-regulated in glioma, suppresses the progression of glioma by regulating a series of tumor-associated genes. These findings may shed some new lights on the AP-2α dysfunction in glioma progression and suggest AP-2α a potential therapeutic target in glioma.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qiao Zhou, Laboratory of Pathology, State Key Laboratory of Biotherapy and Department of Pathology, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China. Tel: +86-28-8516-4027; Fax: +86-28-8516-4027; E-mail: zhouqiao@mcwcums.com

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