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AKT1 and AKT2 Promote Malignant Transformation in Human Brain Glioma LN229 Cells

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OBJECTIVE To confirm the role played by AKT1 and AKT2 in the β-catenin/Tcf-4 signaling pathway in promoting malignant transformation of glioma cells.
METHODS LN229 cells were divided into five groups: a control group, acetone (ACE)group, acetylsalicylic acid (ASA; aspirin) group, ASA+AKT1 plasmid group and ASA+AKT2 plasmid group. Western blot and PCR were used to detect the expression of AKT1 and AKT2 after dealing with ASA and transferring AKT1/2 genes into LN229 cells. Cell proliferation was determined by flow cytometry, cell invasion was evaluated by transwell assay and cell apoptosis was detected with annexin V staining. The molecules regulating proliferation and invasion were examined by western blot analysis.
RESULTS Aspirin down-regulates AKT1 and AKT2 expression by modulating β-catenin/Tcf-4 activity. AKT1 and AKT2 can enhance cell proliferation and invasion by up-regulating the expression of cyclin-D and matrix metalloprotein-9 (MMP-9) in LN229 glioma cells.
CONCLUSION AKT1 and AKT2 play an important role in the β-catenin/Tcf-4 signaling pathway promoting malignant transformation; AKT1 is more effective than AKT2. AKT1 and AKT2 may be potential targets for brain glioma therapy and an effective way to prevent metastasis of gliomas.

KEY WORDS: AKT1, AKT2, brain glioma, malignant transformation.

Introduction

Gliomas, are the most common primary brain tumors. Glioblastoma multiforme (grade IV) is the most malignant form of glioma and one of the most aggressive human cancers, with a median patient survival of 1 year[1]. Despite recent advances in cancer treatment, this statistic has not changed significantly over recent years[2,3]. Therefore, this study aimed at exploring the molecular mechanism of glioma proliferation and metastasis to find new methods of treatment to achieve longer survival rates in glioma patients.

The Wnt/β-catenin/Tcf signaling pathway has been shown to be a crucial factor in the development of many cancers[4,5]. β-Catenin and Tcf-4 are core components of the canonical Wnt/β-catenin/Tcf pathway[6]. The β-catenin/Lef/Tcf complex regulates transcription of multiple genes involved in cellular proliferation, differentiation, survival and apoptosis, including Fra-1, c-myc and cyclin D[7,8]. The EGFR-AKT-PI3K pathway connects with the Wnt/β-catenin/Tcf signaling pathway since AKT can regulate β-catenin directly by inducing phosphorylation at Ser552, resulting in β-catenin translocation from the cytosol into the nucleus, increasing Tcf-4 transcriptional activity[9]. Recently, several reports have

shown that dysregulation of the canonical Wnt/β-catenin pathway and EGFR-AKT-PI3K pathway leads to tumorigenesis[10,11].

AKT1 is regulated by the β-catenin/Tcf-4 signaling pathway in colorectal cancer[12]. AKT2 may play a critical role in the development of gliomas and presents a potential therapeutic target for malignant gliomas[13]. Reduction of AKT2 inhibits migration and invasion of glioma cells[14]. However, little evidence exists to show that AKT1 or AKT2 participate in the β-catenin/Tcf-4 signaling pathway promoting malignant transformation in human brain glioma cells.

The purpose of this study was to confirm that AKT1 and AKT2 do participate in the β-catenin/Tcf-4 signaling pathway promoting malignant transformation of glioma cells.

Materials and Methods

Cell culture, transfection and aspirin treatment

Human glioblastoma cells LN229 were obtained from the China Academia Sinica Cell Repository, Shanghai, China. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma, USA), 100 μg/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma), and incubated at 37°C with 5% CO_2. Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection, according to the manufacturer's instructions. Aspirin (acetylsalicylic acid [ASA]) was purchased from Sigma and a 1 M stock solution was prepared in acetone. Cells were treated in the absence of ASA or in the presence of increasing concentrations (0.5, 1, 5, 10 or 20 mM) of ASA for 1, 2, 6, 12 or 24 h. ASA (10 μM) can inhibit the activity of β-catenin/Tcf-4 signal pathway effectively after 24 h.

Plasmids transfection

Full-length human AKT1 and AKT2 constructs in pLXSN were kindly provided by Dr Cheng JQ, H Lee Moffitt Cancer Center & Research Institute. After treating with ASA for 24 h, Lipofectamine 2000 was used to transfer the plasmids, into the glioma cells according to the manufacturer's instructions. Before transfection, cells were cultured in medium without serum or antibiotics for at least 4 h. Then, a mixture of vector and Lipofectamine 2000 was added to the plates. Cells were cultured for 6 h, then incubated with fresh medium for the desired time period. Culture of the cells was continued for 48 h.

RNA extraction and RT-PCR analysis

RT-PCR

Total RNA was extracted by the guanidinium thiocyanate–phenol chloroform extraction method, using Trizol (Invitrogen), according to the manufacturer's instructions. The quality of the isolated RNA was determined by electrophoresis in a 1.0% agarose-formaldehyde gel. RNA (2.0 μg) was converted to cDNA by reverse transcription. The PCR protocol consisted of initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 57°C and extension for 30 s at 72°C; and a final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis on a 1.8% agarose gel containing ethidium bromide and photographed under UV light. The semiquantitative value of each PCR band, equal to the ratio of the CAR value to the β-actin value, was analyzed by Quantity One 1-D Analysis software, version 4.6.0.

Western blot analysis

Parental and transfected cells were washed with precooled phosphate-buffered saline (PBS) three times. The cells were then solubilized in 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate and a protease inhibitor mixture). Total protein lysates were separated by SDS-PAGE. The separated proteins were transferred to PVDF membranes. The blot was incubated with primary antibody AKT1 (Cell Signaling, USA), AKT2 (Cell Signaling), p-AKT (Santa Cruz, USA), GAPDH (Santa Cruz), cyclin-D (Santa Cruz) and matrix metalloproteinase-9 (MMP-9; Santa Cruz) followed by incubation with horseradish peroxidase-conjugated secondary antibody. The specific protein was detected using a super signal protein detection kit (Pierce, USA). After washing with buffer, the PVDF membrane was reprobed with antibody against GAPDH (Santa Cruz).

Luciferase reporter assay

To evaluate the T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcriptional activity, we used a pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate Biotechnology, USA). TOP-FLASH contains three copies of the Tcf/Lef binding site (AAGATCAAAGGGGGT) upstream from the thymidine kinase minimal promoter, and FOP-FLASH contains a mutated Tcf/Lef binding site (AAGGCCAAAGGGGGT). Cells were transiently transfected by one of these luciferase reporters and pRL-TK (Promega, USA) using L (Invitrogen catalogue No 11668-027), as instructed by the suppliers. Luciferase activity was measured with the dual-luciferase reporter assay system (Promega), with Renilla luciferase activity as an internal control, 48 h after transfection.

Cell cycle analysis

Cells were harvested, washed with PBS, fixed with 75% ethanol overnight at 4°C and then incubated with RNase at 37°C for 30 min. Nuclei of cells were stained with propidium iodide for 30 min. A total of 10^4 nuclei were examined in a FACS Calibur flow cytometer (Becton-Dickinson, USA), and DNA histograms were analyzed

by Modifit software. Results are presented as the percentage of cells in each phase.

Transwell invasion assay

Transwell membranes coated with Matrigel (Becton-Dickinson) were used to assay invasion of glioma cells in vitro. Cells were plated at 5×10^4 cells/well in the upper chamber in serum-free medium. Fetal bovine serum (20%) was added to the medium in the lower chamber. After incubating for 24 h, non-invading cells were removed from the top well with a cotton swab while the bottom cells were fixed in 95% ethanol and stained with hematoxylin and photographed in three separate fields for each well. Three independent experiments were done and used to calculate the fold migration relative to a blank control.

Apoptosis

The annexin V-FITC Apoptosis Detection Kit I (Abcam, USA) was used to detect and quantify apoptosis by flow cytometry. In brief, transfected and control cells in the log phase of growth were harvested in cold PBS and collected by centrifugation for 10 min at 500 *g*. Cells were resuspended at a density of 1×10^6 cells/mL in $1 \times$ binding buffer, stained with FITC-labeled annexin V for 5 min and immediately analyzed by FACScan Flow Cytometer (Becton Dickinson). Data were analyzed by Cell Quest software (USA).

Statistical analysis

Data were analyzed with SPSS 10.0. Analysis of variance, *t*-test, χ^2-test and Pearson correlation were used to analyze the significance between groups. Statistical significance was assigned to P values < 0.05.

Results

ASA inhibits the expression of AKT1 and AKT2 by blocking the activity of the β-catenin/Tcf-4 complex in human glioma LN229 cells

After treating with ASA, the expression of AKT1 and AKT2 was decreased (Fig.1A,B). A luciferase reporter assay showed that the activity of the β-catenin/Tcf-4 complex was reduced by ASA (Fig.1C).

Re-instating the expression of AKT1 and AKT2 decreased by ASA promotes malignant transformation of human glioma LN229 cells

The expression of AKT1 and AKT2 was up-regulated after blocking β-catenin/Tcf-4 transcriptional activity with ASA. Flow cytometry was used to check cell proliferation, transwell assay to evaluate cell invasion and annexin V staining to detect cell apoptosis. The percentage of cells in the G_0/G_1 phase in the ASA group was significantly increased, while cells in ASA+AKT1, ASA+AKT2 and ASA+AKT1+AKT2 groups were significantly increased in the S phase (Fig.2A). Thus AKT1 and AKT2, especially the AKT1 gene, promote the cell cycle to the S phase. Furthermore, the transwell invasion assay showed that the cells in the ASA group had reduced ability to migrate through matrigel compared with cells in the control groups, but cells in the ASA+AKT1, ASA+AKT2 and ASA+AKT1+AKT2 groups

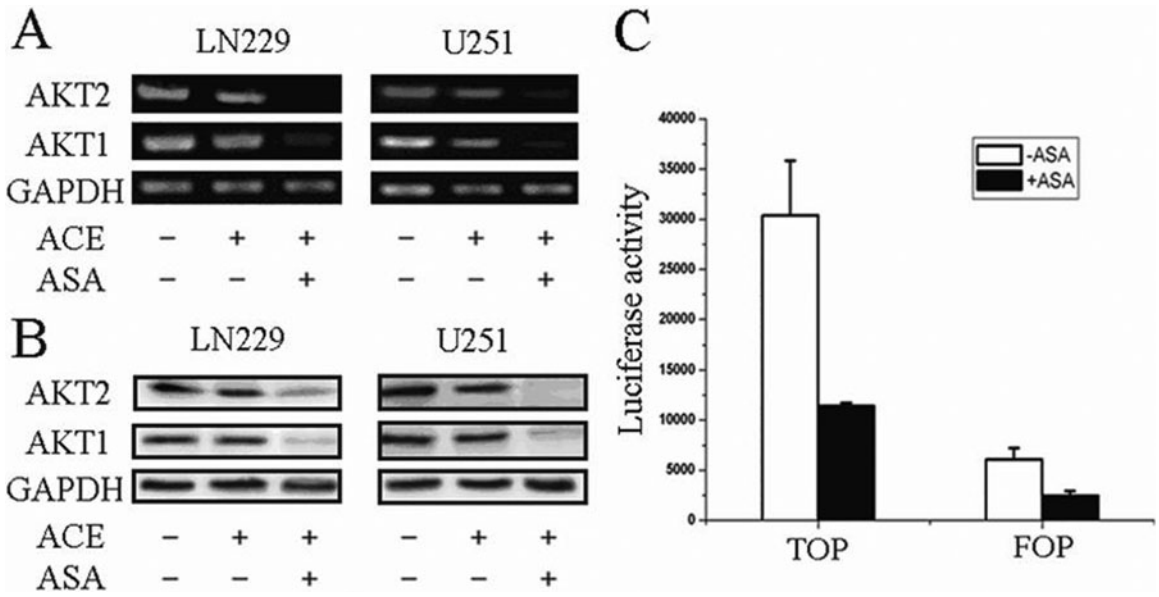

Fig.1. Acetylsalicylic acid (ASA, aspirin) decreases the transcriptional activity of Tcf-4 to down-regulate the expression of AKT1 and AKT2. (A,B) The expression of AKT1 and AKT2 is down-regulated after dealing with ASA on both the mRNA level and protein level. (C) ASA decreases the luciferase activity of TOP-FLASH, so ASA can decrease the transcriptional activity of Tcf-4.

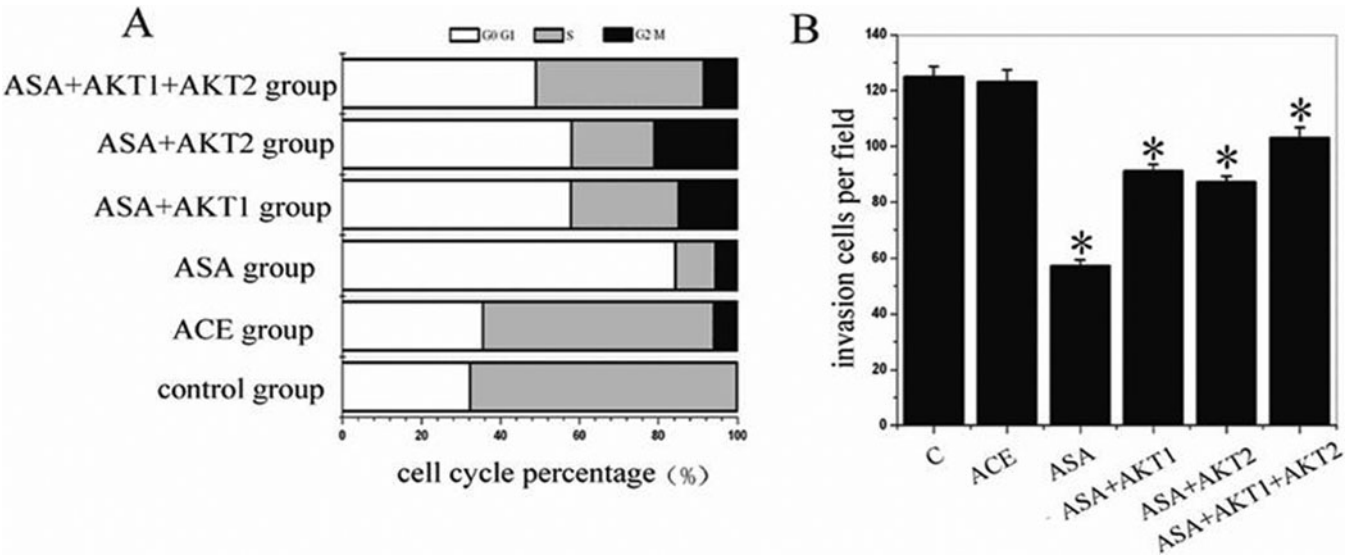

Fig.2. Re-instating the expression of AKT1 and AKT2 increases glioma cell proliferation and invasion. (A) Acetylsalicylic acid (ASA, aspirin) blocks the cell cycle at the G_0/G_1 phase, while AKT1 and AKT2 promote the S phase of the cell cycle. (B) ASA alone decreases, and AKT1 and AKT2 increase, cellular invasion ability.

had greater ability to migrate through matrigel than cells in the ASA only group (Fig.2B). Apoptosis in all groups did not change. The results suggest that AKT1 and AKT2 can enhance the LN229 glioma cell proliferation and invasion, promoting malignant transformation of glioma cells.

Reinstating the expression of AKT1 and AKT2 decreased by ASA enhances the expression of cyclin-D and MMP-9

The expression of cyclin-D and MMP-9 is decreased in the ASA group, but increased in the ASA+AKT1, ASA+AKT2 and ASA+AKT1+AKT2 groups (Fig.3). AKT1 and AKT2 may affect cellular biological behavior by up-regulating the expression of cyclin-D and MMP-9.

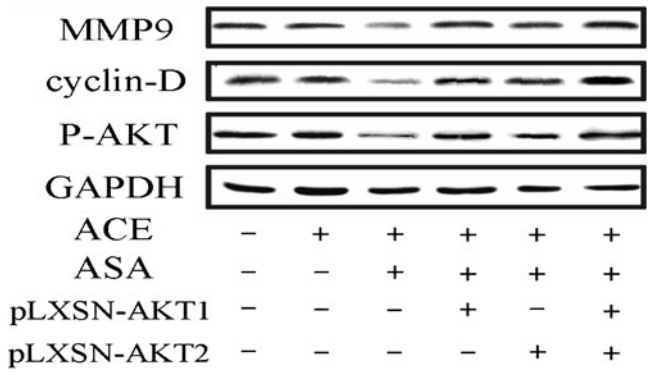

Fig.3. Acetylsalicylic acid (ASA, aspirin) decreases the expression of cyclin-D and matrix metalloproteinase-9 (MMP-9) while re-instating AKT1 and AKT2 up-regulates their expression.

Discussion

The WNT-β-catenin/Tcf-4 and EGFR-PI3K-AKT-MMP signaling pathways play important roles in tumorigenesis. β-Catenin plays a pivotal role in the WNT-β-catenin/Tcf-4 signaling pathway. AKT, a serine/threonine kinase, also known as PKB, is a key mediator of the PI3K/AKT pathway. Activated AKT is phosphorylated at Thr308 and Ser473, and subsequently regulates β-catenin by inducing phosphorylation of GSK3β at Ser9[15,16]. Additionally, AKT can also regulate β-catenin directly by inducing phosphorylation at Ser552, resulting in β-catenin translocation from the cytosol into the nucleus, increasing Tcf-4 transcriptional activity[9]. In turn, AKT1, an isoform of AKT, is regulated by the β-catenin/Tcf-4 complex at the transcriptional level. ASA induces a decrease of AKT1 expression, which is regulated by the β-catenin/Tcf-4 complex as shown by reporter assay[12]. AKT2 may play a critical role in the development of gliomas and is a potential target for treatment of malignant gliomas[13], since reduction of AKT2 inhibits migration and invasion of glioma cells[14].

It is reported that β-catenin activity can be reduced by ASA in colorectal cell lines by phosphorylation of β-catenin but not degradation. To determine whether this effect occurs in gliomas, parallel samples of the LN229 cells used for western blot analysis were transiently transfected with a β-catenin/Tcf-responsive luciferase reporter construct. In LN229 cells, ASA reduced TOP-FLASH activity in a time-dependent manner at a concentration of 10 mmol/L, with a reduction of 20% and 60% in 6 and 24 h, respectively, compared with untreated cells. Western blot showed that the total amount of β-catenin was not reduced, thus showing that β-catenin activity can be reduced by ASA in human glioma LN229 cells.

Recent reports have shown that AKT1 and AKT2 expression is associated with more advanced and particularly aggressive gliomas[17–19]. Additionally, AKT1 and AKT2 contribute to glioma cell migration and invasion by regulating the formation of the cytoskeleton, influencing adhesion and increasing expression of MMP-9[20]. In our study, down-regulation of AKT1 and AKT2 expression by ASA induced cell cycle G0/G1 phase arrest and inhibited cell LN229 glioma cell invasion. These results suggest that AKT1 and AKT2 genes can affect LN229 glioma cell proliferation and invasion.

The results of our study indicate that ASA down-regulates AKT1 and AKT2 expression by modulation of β-catenin/Tcf-4 activity in a time and concentration dependent manner. AKT1 and AKT2 can enhance LN229 glioma cell proliferation and invasion, promoting malignant transformation of glioma cells by up-regulating the expression of cyclin-D and MMP-9.

Conclusion

AKT1 and AKT2 can enhance LN229 glioma cell proliferation and invasion by up-regulating the expression of cyclin-D and MMP-9. AKT1 and AKT2 play important roles in promoting malignant transformation of glioma cells and may be targets for the treatment of glioma in the future.

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Conflict of interest statement

No potential conflicts of interest were disclosed.

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