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Cucurbitacin-B inhibits neuroblastoma cell proliferation through up-regulation of PTEN

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Abstract. – OBJECTIVE: Cucurbitacins belong to a class of highly oxidized tetracyclic triterpenoids. Recent studies suggest that the use of Cucurbitacin could repress cancer cell progression. However, the biological effect of Cucurbitacin-B in neuroblastoma cells remains unexplored.

MATERIALS AND METHODS: MTT and BrdU (bromodeoxyuridine) incorporation assays were used to determine the anti-proliferation roles of Cucurbitacin-B. Real-time PCR and Western blot assays were used to detect the expression of cell cycle regulators. Small interfering RNAs (siRNAs) were used to silence the expression of PTEN (phosphatase and tensin homolog gene).

RESULTS: We found that Cucurbitacin-B inhibited growth and modulated expression of cell-cycle regulators in SHSY5Y cells. At the molecular level, we found that Cucurbitacin-B inhibited AKT signaling activation through up-regulation of PTEN. Indeed, PTEN deficiency using siRNA oligos attenuated the anti-proliferative roles of Cucurbitacin-B.

CONCLUSIONS: These results provide evidence for a mechanism that may contribute to the antineoplastic effects of Cucurbitacin-B in neuroblastoma.

Key Words:
Homocysteine (Hcy), Atherosclerosis (AS), Oxidative stress, Lutein.

Abbreviations

NFkB = Nuclear Factor k B; AKt: Protein Kinase B; src = tyrosine-protein kinase CSK; RAC1 = Rasreleted C3 botulinum toxin substrate 1; MMP = Matrix metalloproteinase; PTP1B = non receptor phospho-tyrosine protein phosphatase; FAK = Focal Adhesion Kinase; AMPK = AMP-activated protein kinase; p38 = mitogen activated protein 38; myr-AKt = myristoilated active form of AKt; TRIB 3 = Tribbles holog 3.

Introduction

Neuroblastoma is a tumor originating from nerve tissues, and is the most frequent extracranial solid tumor in children[1,2]. A characteristic feature of neuroblastoma is its heterogeneity, ranging from spontaneous regression to fatal outcome[3]. Its prognosis is very variable, with outcome related to age, stage and molecular pathology, which in turn led to the development of targeted therapies[3].

Cucurbitacins belong to a class of highly oxidized tetracyclic triterpenoids, which are widely distributed in the plant[4]. Recent studies have shown their promising anticancer activities including anti-proliferation, induction of apoptosis and cell-cycle arrest[5,6]. For instance, Cucurbitacin-D induces growth inhibition, cell cycle arrest, and apoptosis in human endometrial and ovarian cancer cells[7]. Cucurbitacin-E inhibits breast tumor metastasis by suppressing cell migration and invasion[8]. At the molecular level, Cucurbitacin E impaired actin-related protein (Arp)2/3-dependent actin polymerization and suppressed Src/FAK/Rac1/MMP involved pathway[8]. In addition, Cucurbitacin-B regulates immature myeloid cell differentiation and enhances antitumor immunity in patients with lung cancer[9,10]. However, the biological effect of Cucurbitacin-B in neuroblastoma cells remains unexplored. In the present study, we investigated the anti-proliferative roles of Cucrubitacin-B in SHSY5Y cells.

Materials and Methods

Cell Cultures

SHSY5Y cells were purchased from The Cell Bank of Type Culture Collection of Chinese

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Academy of Sciences (CAS, Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA).

Cell Viability and BrdU Incorporation Assays

Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Beyotime, Shanghai, China). After preculture, cells were treated with medium containing different doses of Cucurbitacin-B (Sigma, St Louis, MO, USA) and/or different agents as described in Results and figure legends. MTT assay was performed b4y incubating the cells with 0.5 mg/ml MTT for 8 hours. The formazan product was dissolved in dimethyl sulfoxide (DMSO), and absorbance was read at 470 nm. A cell proliferationenzyme-linked immunosorbent assay kit (Beyotime, Shanghai, China) was used to analyze the incorporation of BrdU (bromodeoxyuridine) during DNA synthesis following the manufacturer's protocols. All experiments were repeated at least four times in quadruplicate.

siRNA Oligo, RNA Isolation and Real-time PCR

Small interfering RNA (siRNA) oligos targeting PTEN (phosphatase and tensin homolog gene) or GFP (green fluorescent protein) gene were obtained from Genepharm Company (Shanghai, China). GFP siRNA was used as a negative control. Total RNAs were isolated from cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China), following the manufacturer's instructions. In order to determine the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Dalian, China) on an ABI 7500 machine.

Western Blot Analysis

Cells after different treatments were lysed with RIPA (radioimmunoprecipitation assay) buffers. An equal amount of protein was subjected to 12% SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in 10% milk for 2 hour at 22-25°C. The immunoblots were incubated overnight at 4 with anbidodies. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hour at 22-25°C. The immunoreactive bands were detected with chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA, USa) under the Fluor Chem FC2 system. Antibodies were purchased from Abcam (anti-β actin, anti-AKT and anti-PTEN) (Cambridge, UK) or Cellsignaling Company (anti-p21, anti-p27, anti-Cyclin D1 and anti-Cyclin E) (Beverly, MA, USA).

Statistical Analysis

Statistical analysis was performed with SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Numerical data are expressed as mean±SEM. Statistical significance is shown as *($p < .05$), **($p < .01$), or ***($p < .001$).

Results

Cucurbitacin-B Treatment Inhibited Proliferation in SHSY5Y Cells

To our knowledge, the effect of Cucurbitacin-B on neuroblastoma cells remains to be defined. Thus, we selected SHSY5Y cells to investigate whether Cucurbitacin-B has potential anti-proliferation roles. Cells were treated with Cucurbitacin-B at several concentrations. As a result, growth was inhibited in a dose-dependent manner in SHSY5Y cells as determined by MTT and BrdU incorporation assays (Figure 1A-B). Moreover, these results suggested that the concentration of Cucurbitacin-B at 5 μM was appropriate. Therefore, 5 μM of Cucurbitacin-B was selected for the further analysis of genes expression in SHSY5Y cells.

Expression of Cell-cycle Regulators in Cucurbitacin-B Treated Cells

We speculate that growth inhibition in neuroblastoma cells might be caused by cell-cycle arrest following Cucurbitacin-B treatment. To confirm this hypothesis, we analyzed the expression contents of p21, p27, Cyclin D1 and Cyclin E, which are known as key molecules involved in cell-cycle arrest. As shown in Figure 2A-2B, expression levels of p21 and p27 were significantly increased in Cucurbitacin-B treated cells. Whereas, the contents of Cyclin D1 and Cyclin E were markedly reduced (Figure 2A-B).

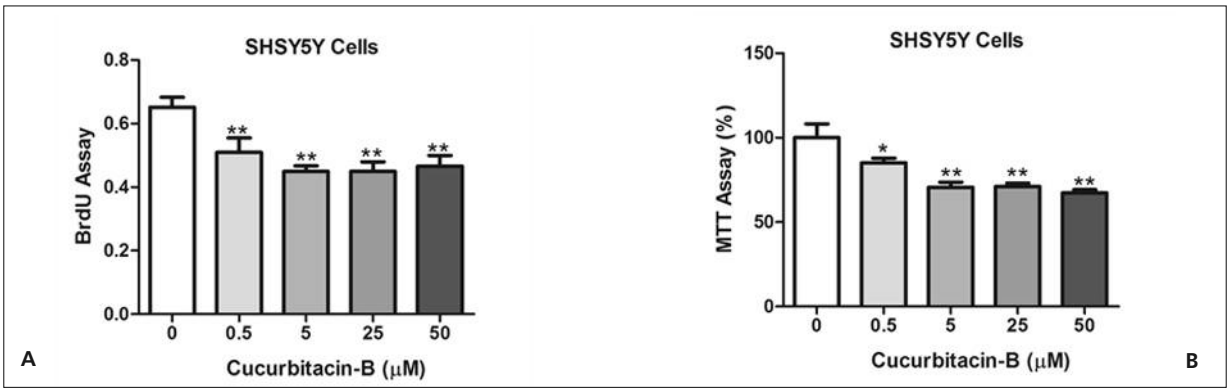

Figure 1. Cucurbitacin-B inhibits cell proliferation in neuroblastoma cells. Cell viability was measured by BrdU assays in SHSY5Y cells ***(A)***. Cells were treated with various concentrations of Cucurbitacin-B as indicated. ***(B)*** Cell proliferation activity was measured by MTT assays in SHSY5Ycells (B). $*p < 0.05$, $**p < 0.01$.

Cucurbitacin-B Inhibits Akt Signaling Pathway in Neuroblastoma Cells

Many compounds such as Metformin and Berberine were shown to regulate cancer cell proliferation through regulation of signaling pathways such as NF-κB, AKT, AMPK and p38[11-12]. Therefore, we determined whether these pathways were affected by Curcubitacin-B treatment in SHSY5Y cells. Indeed, our western blot analysis indicated that Cucurbitacin-B treatment inhibited AKT signaling, as evidenced by its phosphorylation at Serine 473 (Figure 3A). However, phosphorylation of NF-κB, AMPK and p38 were not changed (Figure 3A). To confirm the role of the AKT in proliferation inhibited by Curcubitacin-B, we transfected SHSY5Y cells with a plasmid vector carrying myr-AKT, a constitutively activated AKT (Figure 3B). As a result, Myr-AKT overexpression reversed Curcubitacin-B-mediated suppression of neuroblastoma cell proliferation *in vitro* (Figure 3C-D), further suggesting that Cucurbitacin-B inhibits cell proliferation through repression of AKT signaling.

Cucurbitacin-B up-Regulates PTEN Expression in SHSY5Y Cells

Next, we sought to determine the mechanisms of AKT inhibition by Cucurbitacin-B in neuroblastoma cells. AKT phosphorylation is tightly regulated by several phosphatase including PTEN, PTP1B and TRB3[13-15]. As shown in Figure 4A, Cucurbitacin-B treatment resulted in a robust up-regula-

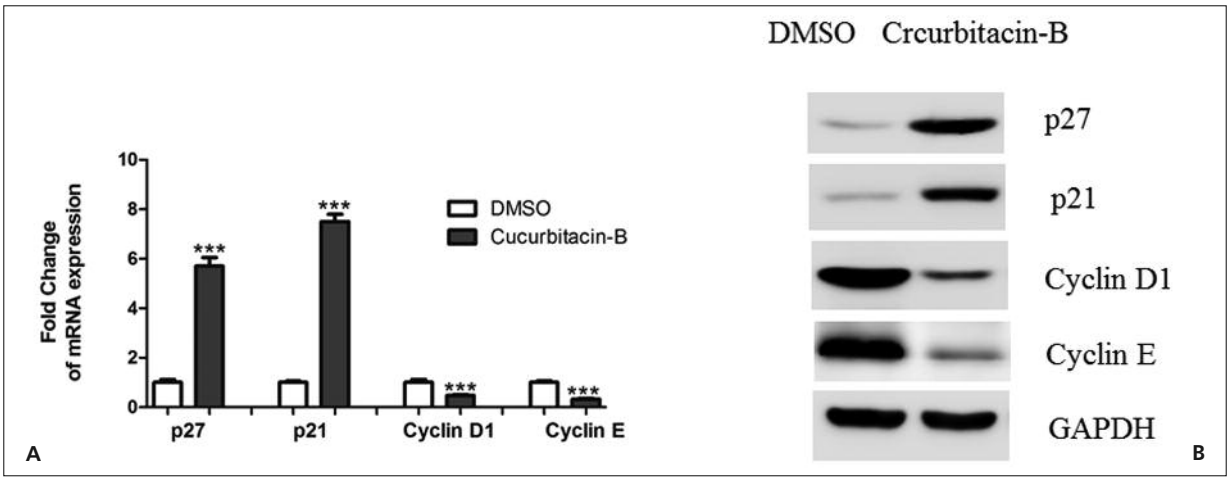

Figure 2. Cucurbitacin-B regulates expression of cell-cycle regulators. ***(A-B)*** mRNA ***(A)*** and protein ***(B)*** levels of p21, p27, Cyclin D1 and Cyclin E were determined by real-time PCR and western blot in SHSY5Y cells treated with vehicle control (Ctrl) or Cucurbitacin-B (5 μM). $***p < 0.001$.

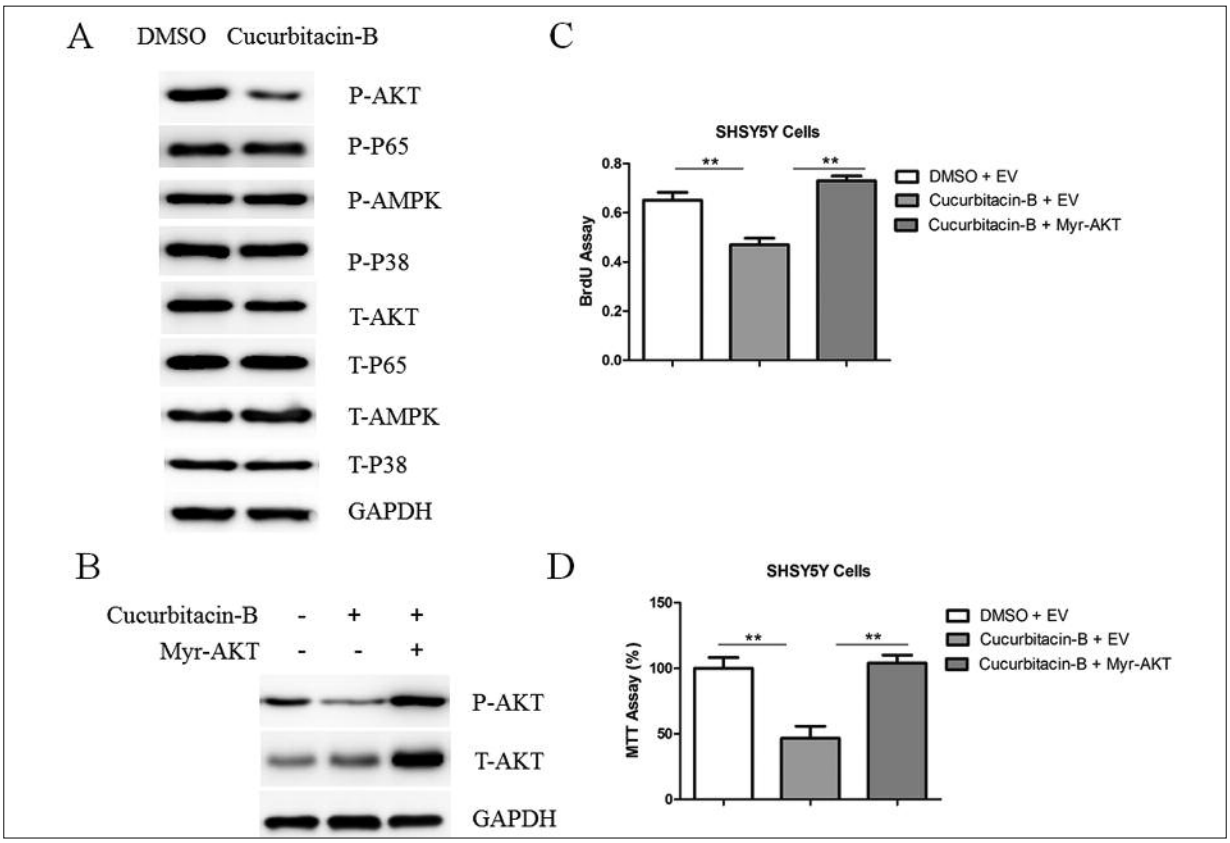

Figure 3. Cucurbitacin-B inhibits AKT activation in neuroblastoma cells. ***(A)*** Western blot analysis of phosphorylated (P) AKT, P65, AMPK and P38 in SHSY5Y cells. Contents of total (T) AKT, P65, AMPK, P38 and GAPDH were used as loading controls. ***(B)*** Western blot analysis of phosphorylated AKT in SHSY5Y cells. Cells were pre-transfected with Myr-AKT or empty vector (EV) for 24 hr and then treated with Cucurbitacin-B or vehicle control (DMSO). ***(C-D)*** Cell viability and proliferation activity were measured by BrdU ***(C)*** and MTT ***(D)*** assays in SHSY5Y cells. $^{**}p < 0.01$.

tion of PTEN mRNA in SHSY5Y cells, while expression of PTP1B and TRB3 remained unchanged. Besides, the induction of PTEN was also confirmed by western blot analysis (Figure 4B).

PTEN is Essential for the Anti-Proliferative Roles of Cucurbitacin-B

Finally, we examined whether the anti-proliferative effect of Cucurbitacin-B relies on PTEN

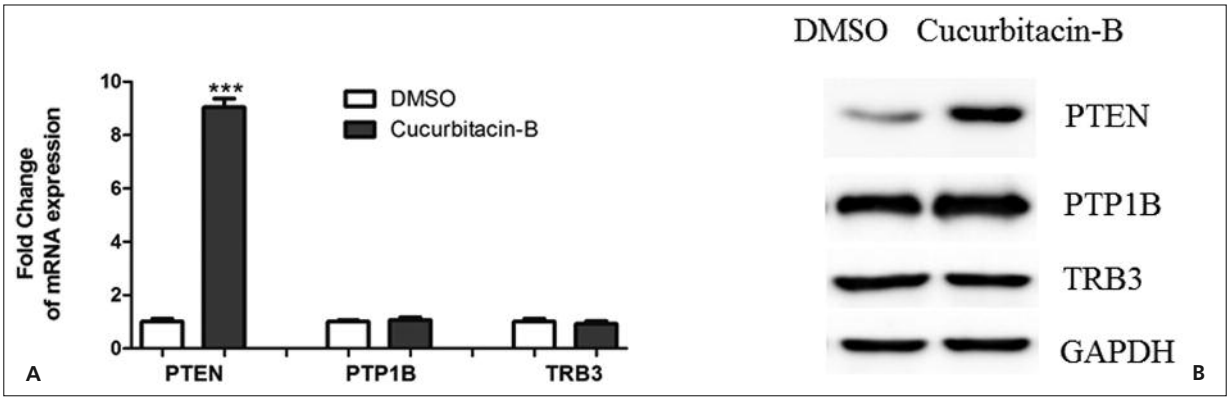

Figure 4. Cucurbitacin-B up-regulates PTEN expression in neuroblastoma cells. ***(A-B)*** mRNA and protein levels of PTEN, PTP1B and TRB3 were determined by real-time PCR ***(A)*** and western blot ***(B)*** in SHSY5Y cells treated with Cucurbitacin-B or vehicle control (DMSO). $^{***}p < 0.001$.

expression. SHSY5Y cells were pre-transfected with siRNA oligos targeting PTEN gene, which successfully led to a deficiency of PTEN and activation of AKT signaling (Figure 5A-5B). As a result, PTEN deficiency significantly reversed the inhibitory effect of Cucurbitacin-B on cell proliferation (Figure 5C and D). Besides, expression levels of cell-cycle regulators by Cucur-

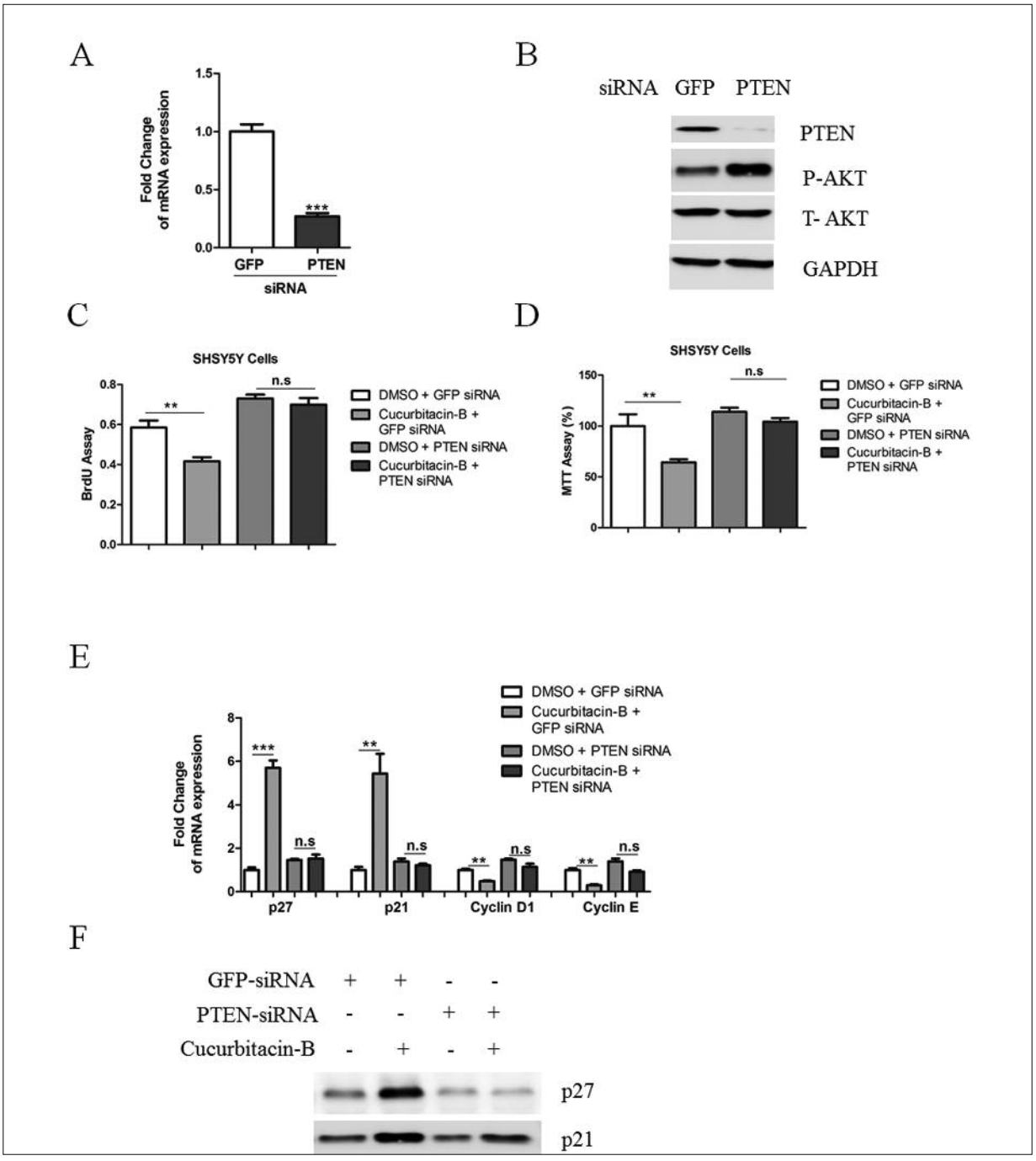

Figure 5. The anti-proliferative action of Cucurbitacin-B is dependent on PTEN. ***(A-B)*** Real-time PCR (A) and western blot ***(B)*** analysis of PTEN and phosphorylated (P) AKT in SHSY5Y cells. Cells were transfected with siRNA oligos targeting PTEN or GFP gene. GFP siRNA was used as a negative control. ***(C-D)*** Cell proliferation activity was measured by BrdU ***(C)*** or MTT ***(D)*** assays in SHSY5Y cells. Cells were pre-transfected with siRNA oligos for 24 hr and then treated with Cucurbitacin-B or vehicle control (DMSO). ***(E-F)*** mRNA and protein levels of p21, p27, Cyclin D1 and Cyclin E were determined by real-time PCR ***(E)*** and western blot ***(F)*** in SHSY5Y cells.

bitacin-B were also attenuated by PTEN knockdown (Figure 5E-F). Therefore, our results suggest that the anti-proliferative effect of Cucurbitacin-B in neuroblastoma is dependent on PTEN expression.

Discussion

In the present study, we firstly explored the roles of Cucurbitacin-B and its molecular mechanisms in neuroblastoma cells. Cucurbitacin-B was shown to inhibit cell proliferation in SHSY5Y cells as evidenced by MTT and BrdU incorporation assays. Moreover, Cucurbitacin-B treatment induced p21 and p27 expression while repressed Cyclin D1 and Cyclin E expression. At the molecular level, our results demonstrated that Cucurbitacin-B inhibited AKT signaling pathway through up-regulation of PTEN. Interestingly, knockdown of PTEN expression using siRNA oligos largely abolished the anti-proliferative roles of Cucurbitacin-B, suggesting that the function of Cucurbitacin-B was PTEN-dependent. Previous studies have revealed that Cucurbitacins are considered to be selective modulators of the JAK/STAT and MAPK pathways[16]. Besides, other mechanisms may also be implicated in its anti-tumor effects, including PARP cleavage, expression of active caspase-3, all of which are implicated in cell proliferation and the cell-cycle regulation[5]. Therefore, together with these studies, our data revealed that the anti-proliferative mechanisms of Cucurbitacin-B might be tissue or cell-specific.

PTEN was considered as a key tumor suppressor gene frequently lost on chromosome 10q23[17]. Heterozygous loss of PTEN in the mouse resulted in the development of cancer of multiple origins, as well as in a lethal lymphoproliferative disease[18]. In humans, germline loss and mutation of PTEN is observed in a group of autosomal dominant syndromes [PTEN hamartoma tumor syndromes (PHTS)], which are characterized by neurologic disorders, multiple hamartomas, and cancer susceptibility[19]. Importantly, aberrant methylation of PTEN gene was observed in neuroblastoma tissues, which resulted in its inactivation and AKT hyper-activation[20]. Indeed, compounds with a direct or indirect inhibitory effect on the AKT pathway, used alone or in combination with other drugs, seem to hold great promise as a new therapeutic modality in neuroblastoma[21].

Conclusions

Our data demonstrate that Cucurbitacin-B could exert direct anti-proliferative actions on neuroblastoma cells. Although the mechanisms of up-regulation of PTEN by Cucurbitacin-B remain to be defined, and *in vivo* studies are still needed to fully understand its anti-tumor roles, our present study suggests that Cucurbitacin-B might be a potential therapeutic target for neuroblastoma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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