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Artemisinin inhibits neuroblastoma proliferation through activation of AHP-activated protein kinase (AMPK) signaling

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Recent population studies suggest that the use of artemisinin is associated with reduced incidence and improved prognosis of certain cancers. In the current study, we assessed the effect of artemisinin on neuroblastoma cells using SHSY5Y cells. We found that artemisinin inhibited growth and modulated expression of cell-cycle regulators in these cells. Treatment with artemisinin was also associated with activation of AMP kinase and inhibition of mTOR/p70S6K/pS6 signaling in SHSY5Y cells. In addition, inhibition of AMPK signaling reversed impact on the anti-proliferative roles of artemisinin. Taken together, these results provide evidence for a mechanism that may contribute to the antineoplastic effects of artemisinin suggested by recent population studies and justify further work to explore its potential roles in neuroblastoma prevention and treatment.

1. Introduction

Neuroblastoma is a tumor originating from from nerve tissue, and is the most frequent extracranial solid tumor in children (Pietras 2012; Tonini et al. 2012). A characteristic feature of neuroblastoma is its heterogeneity, ranging from spontaneous regression to fatal outcome. Its prognosis is very variable, with outcome related to age, stage and molecular pathology, which in turn has led to the development of targeted therapies (Pietras 2012). Extensive research projects have been focused on developing new chemotherapies either by exploring the anticancer ability of novel compounds or by assessing drugs conventionally used in other clinical diseases. Thus, there is always a constant need to develop alternative or synergistic anticancer drugs with minimal side-effects. One important strategy to develop effective anticancer agents is to study anticancer agents derived from natural sources. Natural products have been found to be a relevant source of novel and potent bioactive compounds, which have been proven to be effective for cancer prevention and therapey (McLeod 2013; Berkovich et al. 2012). Plant derivatives have been known to be effective against a range of diseases with broad antimicrobial activity, and some have also exhibited significant antitumor activity (Ali et al. 2012). One of the promising compounds is artemisinin, a naturally occurring antimalarial with anticancer properties (Efferth et al. 2001). Artemisinin and its derivatives, which are notably used in malaria therapy (Utzinger et al. 2007), have also potent anticancer activity in the nano- to micromolar range in sensitive and drug- or radiation-resistant cell lines (Efferth et al. 2003). Importantly, artemisinin is one of the very few drugs that have been widely used as antimalarials but has no significant side effects or clinical resistance, although tolerance has been reported (Gordi and Lepist 2004; Reungpatthanaphong and Mankhetkorn 2002). Here, we describe *in vitro* experiments carried out to investigate the hypothesis that artemisinin exhibits direct antiproliferative actions on neuroblastoma cells.

2. Investigations and results

2.1. Artemisinin treatment inhibited cell growth in a dose-dependent manner

To our knowledge, the effect of artemisinin on neuroblastoma cells remains unexplored. Thus, we selected SHSY5Y cells to investigate whether artemisinin has potential anti-proliferation roles. Cells were treated with artemisinin at several concentrations. After 30 h of treatment, growth was inhibited in a dose-dependent manner in SHSY5Y cells as determined by MTT and BrdU incorporation assays (Fig. 1A-1B). Moreover, these results suggested that the concentration of artemisinin at 20 μM was appropriate. Therefore, 20 μM of artemisinin was selected for the further analysis of genes expression in SHSY5Y cells.

2.2. Expression of cell-cycle regulators in artemisinin -treated cells

We speculate that growth inhibition in neuroblastomacells might be caused by cell-cycle arrest following artemisinin 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 Fig. 2A-2B, expression levels of p21 and p27 were significantly increased in artemisinin cells. Besides, the contents of Cyclin D1 and Cyclin

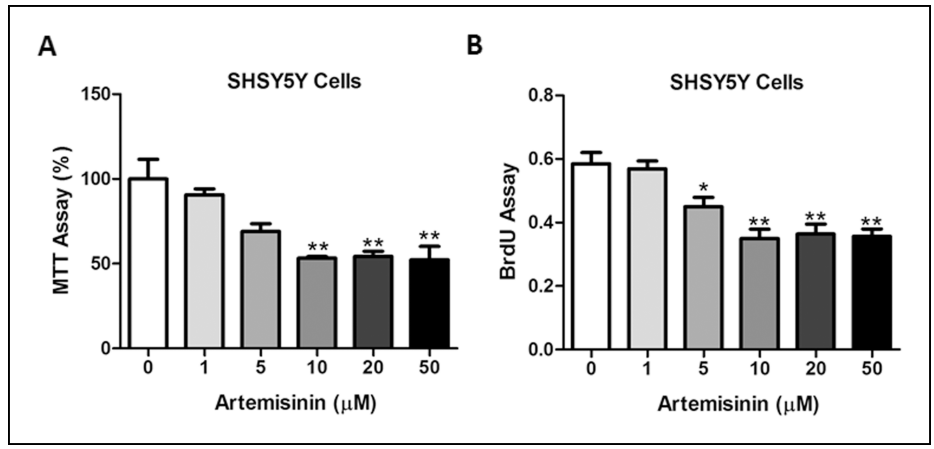

Fig. 1: Artemisinin inhibits cell proliferation in neuroblastomacells. (A) Cell viability was measured by MTT assays in SHSY5Y cells (A). Cells were treated with various concentrations of artemisinin as indicated. (B) Cell proliferation activity was measured by BrdU incorporation assays in SHSY5Ycells (B).

E were markedly down-regulated in artemisinin-treated cells (Fig. 2A-2B).

2.3. Artemisinin activates AMP kinase activity in neuroblastoma cells

Many studies have indicated that the antiproliferative effects of several compounds or drugs involve the AMP kinase pathway (Kim et al. 2012; Buzzai et al. 2007). Indeed, our western blot analysis indicated that artemisinin stimulated AMPK phosphorylation in SHSY5Y cells (Fig. 3A). Phosphorylated ACC, a downstream taget of AMPK, was also enhanced in cells treated with artemisinin (Fig. 3A). Because AMPK activation inhibits energy-consuming pathways and protein synthesis (Gong et al. 2013; Kim et al. 2012). We observed that AMPK activation is associated with a decreased phosphorylation of mTOR and S6 kinase (Fig. 3C).

2.4. Inhibition of AMPK pathway reversed the roles of artemisinin

We next test whether the inhibiting effect of artemisinin on cell proliferation is mediated by AMPK in neuroblastoma cells. As shown in Fig. 4A and 4B, pretreatment with the AMPK inhibitor (Compound C, CC) reverses the inhibitory effect of artemisinin on cell proliferation. Besides, expression levels of cell-cycle regulators were also inhibited by artemisinin in the presence of CC (Fig. 4C). To rule out any possible nonspecific effects of CC, siRNA oligos-mediated knockdown of AMPK 2 subunit was performed (Fig. 4D and 4E). As a result, we also

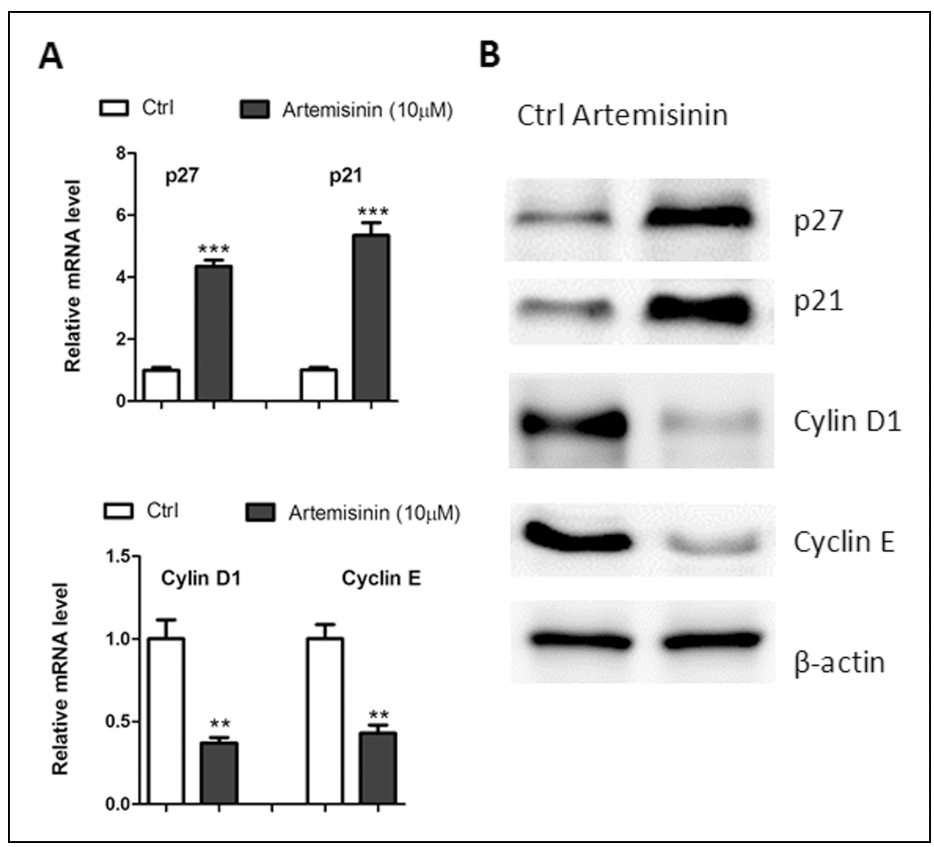

Fig. 2: Artemisinin regulates expression of cell-cycle regulators. (A-B) mRNA (A) and protein (B) levels of p21, p27, Cyclin D1 and Cyclin E were determined by real-time PCR and western blot in SHSY5Y cells treated with vehicle control (Ctrl) or artemisinin (20 μM).

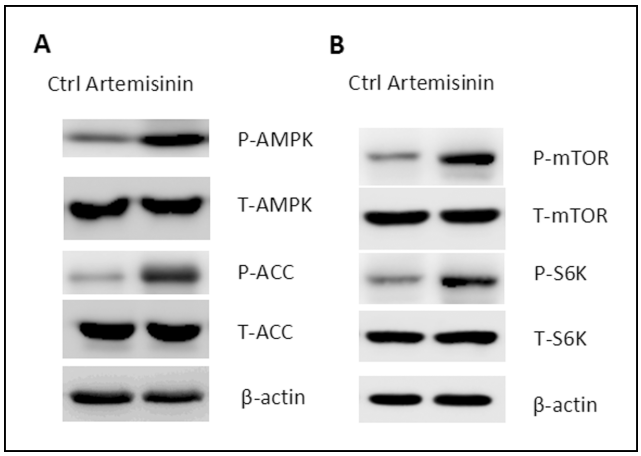

Fig. 3: Artemisinin induces AMPK activation in neuroblastoma cells. (A) Western blot analysis of phosphorylated AMPK and ACC in SHSY5Y cells (A). Contents of total AMPK, ACC and β-actin were used as loading controls. (B) Western blot analysis of phosphorylated mTOR and S6K in SHSY5Y cells (B). Contents of total mTOR, S6K and β-actin were used as loading controls.

oberved that artemisinin could not regulate cell proliferation and expression levels of cell-cycle regulators in cells with AMPK 2 subunit depletion (Fig. 4F-4H). Therefore, our results suggest that the antiproliferative effects of artemisinin in neuroblastoma is dependent of AMPK signaling.

3. Discussion

In the present study, we firstly explored the roles of artemisinin and its molecular mechanisms in neuroblastoma cells. Artemisinin was shown to inhibit cell proliferation in SHSY5Y cells as evidenced by MTT and BrdU incorporation assays. Moreover, artemisinin treatment induced p21 and p27 expression while repressed Cyclin D1 and Cyclin E expression. Significant antitumor activity of artemisinin and licensed semisynthetic artemisinin derivatives has been documented *in vitro* and in animal models (Riganti et al. 2009; Alcantara et al. 2013; Gong et al. 2013; Singh et al. 2011). Artemisinin was shown to induce doxorubicin resistance in human colon cancer cells *via* calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression (Riganti et al. 2009). In breast cancer cells, artemisinin repressed proliferation and induced a strong G1 cell cycle arrest in MCF-7 cells, an estrogen-responsive human breast cancer cell line that represents an early-stage cancer phenotype, and effectively inhibited the *in-vivo* growth of MCF-7 cell-derived tumors from xenografts in athymic nude mice (Tin et al. 2012). At the molecular level, artemisinin inhibited E2F1 interactions with the endogenous CDK2 and cyclin E promoters (Tin et al. 2012). Moreover, artesunate (ART), a semisynthetic derivative of artemisinin, induces apoptosis in human lung adenocarcinoma cell lines (ASTC-a-1 and A549). ART induces Bak-mediated caspase-independent intrinsic apoptosis in both ASTC-a-1 and A549 cell lines, suggesting a potential therapeutic effect of artemisinin on lung cancer (Zhou et al. 2012). Taken together, artemisinin has been reported by numerous studies to exest anti-cancer effects. At the molecular level, our results demonstrated that artemisinin activated AMP kinase activation as well as inhibition of mTOR signaling. Interestingly, inhibition of the AMPK pathway using antagonist or siRNA oligos largely abolished the anti-proliferative roles of artemisinin, suggesting that the function of artemisinin was AMPK-dependent. Because of its well-established roles in various aspects of metabolic physiology, AMPK has received great pharmaceutical interest as a target for insulin resistance and related metabolic syndrome (Hardie 2007). Besides, AMPK activation not only reprograms metabolism, but also enforces a metabolic checkpoint on the cell cycle through effects on p53 and mTOR signaling, indicating that AMPK activated drugs may be useful as cancer therapeutics (Shackelford and Shaw 2009; Buzzai et al. 2007b; Swinnen et al. 2005). In SHSY5Y cells, pharmacological activator of AMPK significantly protected cells against cytotoxicity imposed by tunicamycin and homocysteine (Park et al. 2013), suggesting that AMPK signaling could also be a therapeutic target in neuroblastoma cells.

Our results revealed that artemisinin exhibits direct antiproliferative actions on neuroblastoma cells. Due to its broad therapeutic activity, clinicians should take into account to further investigate the efficacy of artemisinin in patients.

4. Experimental

4.1. Cell cultures

The neuroblastoma cell line SHSY5Y cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, USA).

4.2. 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). After preculture, cells were treated with medium containing different doses of artemisinin (Sigma) and/or different agents as described. MTT assay was performed by incubating the cells with 0.5 mg/ml MTT for 8 hours. The formazan product was dissolved in dimethyl sulfoxide, and absorbance was read at 470 nm. A cell proliferationenzyme-linked immunosorbent assay kit (Beyotime, Shanghai) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were repeated at least four times.

4.3. RNA isolation and real-time PCR

Total RNAs were isolated from cells by TRIzol reagent (Invitrogen, 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 7900 machine.

4.4. Western blot analysis

Cells after different treatments were lysed with RIPA 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 h at 22∼25 °C. The immunoblots were incubated overnight at 4 °C with anbidodies. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 2 h at 22∼25 °C. The immunoreactive bands were detected with a chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA) under the Fluor Chem FC2 system. Antidodies were purchased from Abcam (anti-β actin, anti-AMPK, anti-ACC, anti-mTOR and anti-S6K) or Cellsignaling Company (anti-p21, anti-p27, anti-Cyclin D1 and anti-Cyclin E).

4.5. Small interfering RNA (siRNA)

Cells were transfected with siRNA targeting the AMPKα2 subunit or a negative control (all siRNA oligos from QIAGEN, Valencia, CA) using Lipofectamine 2000 (Invitrogen, USA) as described by the manufacturer's instructions. Cell cultures were incubated for 18 hours with 100 nM siRNA before artemisinin treatment.

4.6. Statistical analysis

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

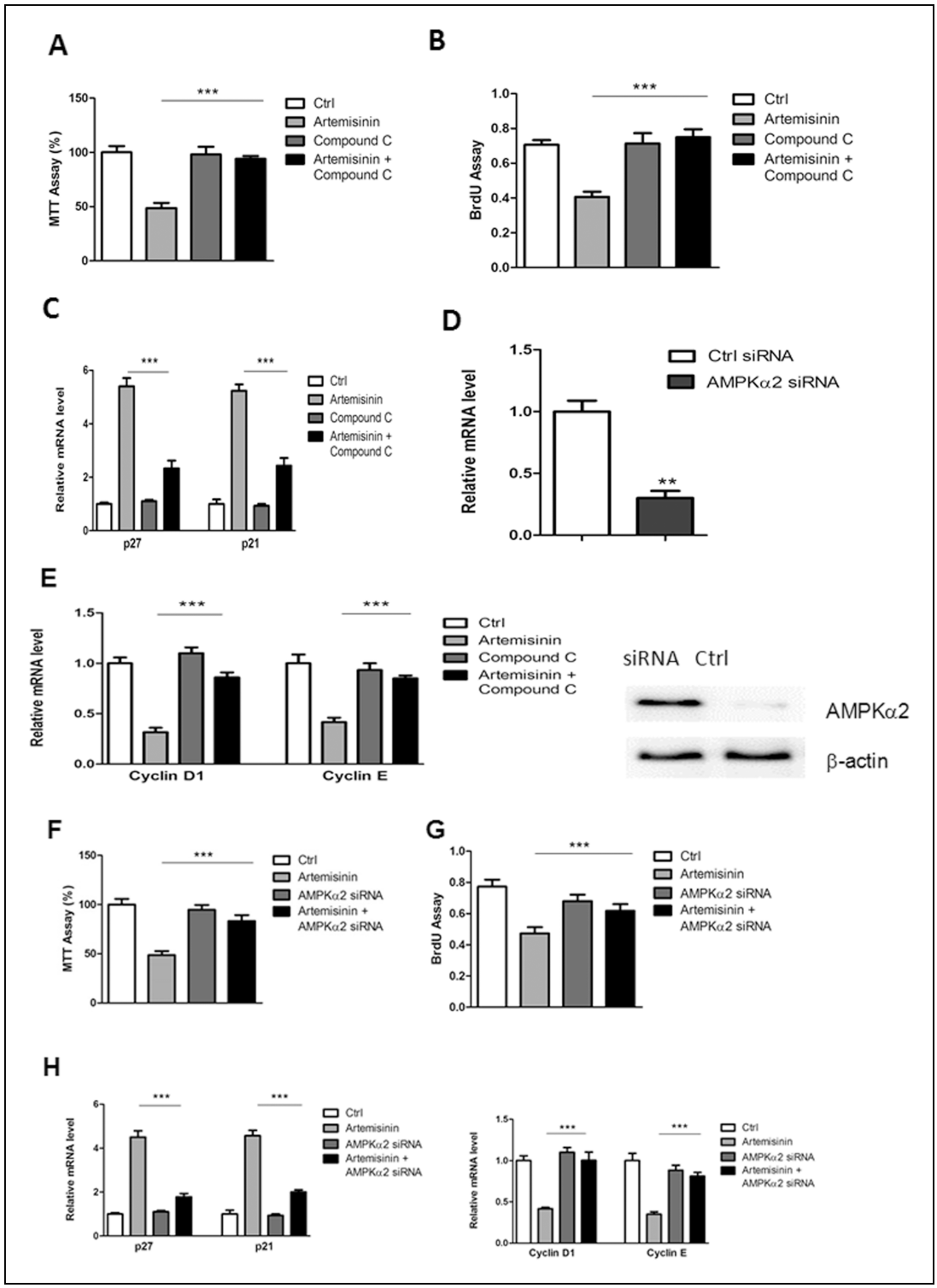

Fig. 4: The anti-proliferative action of artemisinin is dependent on AMPK signaling activation. (A-B) Cell proliferation activity was measured by MTT or BrdU incorporation assays in SHSY5Y cells. Cells were pretreated with vehicle control (DMSO) or Compound C (CC) for 12 h. (C) mRNA levels of p21, p27, Cyclin D1 and Cyclin E were determined by real-time PCR in SHSY5Y cells. (D-E) Real-time PCR and Western blot analysis of AMPKα2 in SHSY5Y cells transfected with siRNA oligos against AMPKα2 or scramble siRNA (Ctrl). (F-G) Cell proliferation activity was measured by MTT or BrdU incorporation assays in SHSY5Y cells. Cells were pre-transfected with siRNA oligos against AMPK □□ or scramble siRNA (Ctrl). (H) mRNA levels of p21, p27 and Cyclin D1 were determined by real-time PCR in SHSY5Y cells.

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