Sinomenine Can Inhibit the Growth and Invasion Ability of Retinoblastoma Cell through Regulating PI3K/AKT Signaling Pathway

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Received April 30, 2020; accepted July 21, 2020; advance publication released online August 4, 2020

Sinomenine was found to play anti-cancer functions in different type of cancers, while the mechanisms underlying the anticancer effects of sinomenine in retinoblastoma (RB) remains unclear. The present study was designed to explore the impacts of sinomenine on cell proliferation and invasion ability of RB cells and the related mechanism. Human retinoblastoma cell line WERI-RB-1 and Y79 cells were cultured and treated by different concentration of sinomenine, and then the proliferation ability of the cells was determined via performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. The cell apoptosis was examined via performing the flow cytometry assay. Then scratch wound healing analysis as well as transwell invasion analysis have been performed to determine the effect of sinomenine on cell migration ability as well as invasion ability. The proteins level of phosphatidylinositol 3-kinase (PI3K)/AKT signaling molecules were determined with Western blot assay. We found that sinomenine was able to decrease the proliferation and promote the apoptosis of RB cells in a dose-dependent manner; moreover, sinomenine also significantly suppressed the migration as well as invasion ability of WERI-RB-1 and Y79 cells in vitro. Furthermore, sinomenine also de-activated PI3K/AKT signaling in WERI-RB-1 cells via inhibited the phosphorylation of PI3K and AKT proteins. Sinomenine can exert anti-tumor function on RB cells in vitro, therefore sinomenine might be a potential alternative medication for the treatment for RB.

Key words sinomenine; retinoblastoma; proliferation; migration

INTRODUCTION

Retinoblastoma (RB) is a malignancy originates from the retinal cells that compose the light-sensing tissue. According to the previous statistics, RB represents over 4% of all pediatric malignancies, and it has been identified as one of the most common primary intraocular cancers among children. More than 70% of children with RB were diagnosed at the age of 3 years old, and the prevalence of RB is significantly higher in developing countries than developed countries. Although some children may survive from this type of cancer, most of them will lose their vision. Therefore, the management of RB needs a multidisciplinary approach to increase the therapeutical effects.

In recent years, virus reports have shown the roles of traditional herbal medicine for the treatment of different type of cancers. Sinomenine is one of the main components of the traditional Chinese medicine (TCM) Sinomenium acutum. In clinical field, sinomenine has been proved to play protective roles in cardiovascular system, immune system, as well as other tissues and organs. So far, sinomenine has been regarded as an anti-tumor reagent for the treatment of many types of cancers, for example, gastric cancer, breast cancer and so on. However, the roles as well as underlying mechanisms of sinomenine in regulating the progression of RB have not been fully elucidated.

Therefore, the present work was designed as a preliminary to explore the effects of sinomenine on the behavior of RB cells, and elucidate the possible underlying mechanisms. We hypothesized that sinomenine was function as an anti-tumor reagent in RB as in other type of cancers, which might provide novel evidence for the potential clinical use sinomenine on RB treatment.

MATERIALS AND METHODS

Cell Source and Treatment Human retinoblastoma cell line WERI-RB-1 and Y79 cells were purchased from Cell Bank of of Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured by the RPMI-1640 culture medium supplied with the fetal bovine serum (10%, Gibco, Rockville, U.S.A.) as well as streptomycin (100 µg/mL) and penicillin (100U/mL). Cells were maintained at a 37°C incubated and supplied with 5% CO₂. At confluence, WERI-RB-1 and Y79 cells were passaged and then treated by the 0, 25, 50, and 100 µmol/L of sinomenine for the further analysis.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Proliferation Assay The cell proliferation was measured by MTT assay. The WERI-RB-1 and Y79 cells were placed onto the 96-well cell culture plates and subsequently, MTT Cell Viability Assay Kit (Beyotime, Shanghai, China) was performed based on the manufacturer’s instructions. Optical density (OD) value at the wavelength of 570 nm has been recorded to assess the proliferation ability of the cells at the 0, 24 and 48th time point.

Flow Cytometry Cell Apoptosis Assay At 48h after treatment, WERI-RB-1 and Y79 cells of different treatment were collected, re-suspended and then incubated by the Annexin-V-fluorescein isothiocyanate (FITC) reagent (5 µL) as well as propidium iodide (PI) reagent (2.5 µL, kit was purchased from Beyotime). The apoptosis of the cells was then determined with FACS Calibur (BD Biosciences, San Jose, CA, U.S.A.). The apoptosis of the cells was evaluated as the sum of the ratio of cells in the Q3 + Q2 regions of the scatter
diagram.

Scratch Wound Healing Assay To determine the effects of sinomenine on the migration ability of the WERI-RB-1 cells, scratch assay was performed. Briefly, The WERI-RB-1 and Y79 cells were seeded on to the 24-well plates and a monolayer of the cells has been obtained. Then, a new 1-mL pipette tip was used to generate a scratch across the center of the wells and the cell were imaged, and after 12 and 24h, the cells in each well was imaged again and the wound healing ability of the cells with different treatments was analyzed.

Transwell Assay Furthermore, the invasion ability of the cells was measured by performing the transwell analysis. Briefly, cells were placed on to the upper chamber of the transwell that pre-coated with Matrigel (BD Biosciences), and the lower chamber was filled with 500 µL cell culture medium supplied with 10% FBS. After 24h incubation, cells invaded to the bottom of the chamber were stained by crystal violet (0.1%) and imaged by a microscope.

Western Blot Protein Assay The Western blot protein assay was applied for the quantification of the expression levels of the proteins extracted from the WERI-RB-1 cells treated with different concentrations of sinomenine. To begin with, WERI-RB-1 cells of different treatment were collected and then lysed by RIPA reagent (Thermo Fisher Scientific, U.S.A.) and then the concentration of the total proteins was quantified with the BCA kit (Beyotime). Twenty micrograms of proteins were separated with gel electrophoresis (8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used), and the proteins were then transferred from the gels to the polyvinylidene difluoride (PVDF) membranes (Millipore, the Netherlands). After that, membranes were blocked with skimmed milk (non-fat, 5%) for 2h. Subsequently, the primary antibodies were incubated for overnight at 4°C in a refrigerator. Furthermore, in the following day, the membranes have been washed and then treated by with secondary antibodies (HRP-conjugated, Beyotime) for 1h at room temperature. Finally, an enhanced chemiluminescence (ECL) kit (Beyotime) was used for the membrane stain and the membranes were photographed by Tannon 6100 imaging system (Tannon, Shanghai, China).

Statistical Methods The data of the present work were expressed as the mean ± standard deviation (S.D.). Data was compared by the GraphPad Prism software (version 6.0, GraphPad Software, U.S.A.). One-way ANOVA was employed for data comparison. *-Value that less than 0.05 has been regarded as the significant difference.

RESULTS

Effect of Sinomenine Treatment on the Proliferation Ability of the WERI-RB-1 and Y79 Cells First, WERI-RB-1 and Y79 cells were randomly divided into 4 groups and treated by 0, 25, 50, and 100 µmol/L of sinomenine as discussed above, and then the effects of sinomenine on the growth of the WERI-RB-1 cells were examined. As Fig. 1 shows, 25, 50, and 100 µmol/L of sinomenine significantly decreased in the proliferation of WERI-RB-1 and Y79 cells in a dose-dependent manner, and 100 µmol/L of sinomenine has shown the best inhibitory effects (*p < 0.01).

Effect of Sinomenine on the Apoptosis of the WERI-RB-1 and Y79 Cells Furthermore, WERI-RB-1 and Y79 cells have been treated by 0, 25, 50, and 100 µmol/L of sinomenine, and cell apoptosis was analyzed by using flow cytometry methods. We observed that 25, 50, and 100 µmol/L of sinomenine markedly increased the apoptosis of the WERI-RB-1 and Y79 cells in a dose-dependent manner (Fig. 2).

Effect of Sinomenine Treatment on the Migration as well as Invasion Ability of the WERI-RB-1 and Y79 Cells Furthermore, the effects of sinomenine on migration as well as invasion ability of WERI-RB-1 cells were evaluated with the wound healing scratch assay and transwell analysis. In Figs. 3 and 4, the results indicated that 25, 50, and 100 µmol/L of sinomenine treatment lead to reduction of migration (Fig. 3) as well as invasion (Fig. 4) ability of the WERI-RB-1 and Y79 cells in a dose dependent manner.

Sinomenine Can Inhibit the Growth and Invasion Ability of WERI-RB-1 Cells through Down-Regulating PI3K/AKT Signaling Finally, the underlying mechanism of the anti-tumor effects of sinomenine on RB cells were evaluated. The effects of sinomenine on the activation of the PI3K/AKT signaling pathway in WERI-RB-1 cells were determined by quantifying the expression levels of p-PI3K, p-AKT, Bcl-2 and Bax in 0, 25, 50, and 100 µmol/L of sinomenine treated WERI-RB-1 cells for 24h. We found that sinomenine inhibited the PI3K/AKT signaling pathway in WERI-RB-1 cells by decreasing the expression of p-PI3K and AKT, the downstream anti-apoptotic factor Bcl-2, and increasing the expression of apoptotic factor Bax in a dose dependent manner (Fig. 5).
Fig. 2. Sinomenine Promotes Apoptosis of WERI-RB-1 and Y79 Cells in Vitro
The cells apoptosis was determined by flow cytometry assay after 48 h with different treatment. Cells were treated with 0, 25, 50, and 100 µmol/L sinomenine. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0 µmol/L sinomenine.

Fig. 3. Inhibited Cell Migration by Sinomenine
The cell migration ability was determined by scratch assay after 12 and 24 h. Cells were treated with 0, 25, 50, and 100 µmol/L sinomenine. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0 µmol/L sinomenine.
DISCUSSION

The present study explored the roles and functions of sinomenine in the proliferation and migration of RB cells and the underlying mechanism. We proved that sinomenine can regulate the proliferation and migration of RB cells via regulating the PI3K/AKT signaling pathway, suggesting that sinomenine has the potential to become an alternative medication for the treatment of RB.

In recent years, with the exploration of traditional medicine research, there are numerous reports about natural compounds in the treatment of RB. For example, Cui et al. suggested that ginsenoside-Rg5 can inhibit the growth, and on the other hand, increase apoptosis of the RB cells via down-regulating the expression of BCL2); Su et al. reported that shikonin can increase the expression of microRNA-34a as well as microRNA-202 in RB and inhibit the growth of retinoblastoma cells); Li et al. suggested that curcumin can inhibit the carcinogenic behaviors of RB cells lines via regulating the JAK/STAT signaling pathway. However, investigations on the roles of sinomenine in RB were limited. In this study, RB cells were treated by different concentrations of sinomenine in vitro, and the cell proliferation, apoptosis, migration as well as invasion ability were measured. As expected, sinomenine can affect the growth and apoptosis of RB cells in vitro; moreover, sinomenine also lead to decreased migration as well as invasion ability of the RB cells, and the effects of sinomenine in vitro were in a dose-dependent manner. These results were consistent with its anti-tumor behaviors in other cancers. Like any other type of cancer cell, RB cells were characterized
by the uncontrolled growth and increased migration and invasion ability of the tumor cells, the above observations suggested that sinomenine can inhibit the growth and metastasis of WERI-RB-1 and Y79 cells in vitro, suggesting that sinomenine can exert anti-tumor effects in RB.

PI3K/AKT signaling pathway has been demonstrated as an important signaling pathway in the process of carcinogenesis in different types of cancers,\(^{19–21}\) including RB.\(^{7,12,22}\) Thus, to determine whether sinomenine treatment can inhibit the activation of the PI3K/AKT signaling pathway in RB, the expressions of the active form of PI3K and AKT, p-PI3K and p-AKT, in WERI-RB-1 cells with different treatment of sinomenine were also examined. We have observed a significant decrease in the expressions of p-PI3K, p-AKT in sinomenine treated WERI-RB-1 cells, and the expression of the downstream anti-apoptotic factor Bcl-2\(^ {14}\) were decreased, and apoptotic factor Bax\(^ {12}\) were increased. Therefore, the data of the present work provided novel evidence that sinomenine can inhibit the growth and migration of RB cells through regulating the PI3K/AKT signaling.

To sum up, results of the present work proved for the first time that sinomenine may exert tumor suppressive effects in the pathogenesis of RB through regulating PI3K/AKT signaling pathway. Although further clinical study and animal study is needed in the future, our study has provided theoretic bases and novel evidence for the potential application of sinomenine as a therapeutic method for the treatment of RB.

**Conflict of Interest** The authors declare no conflict of interest.

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