p38 MAPK and ERK1/2 pathways are involved in the pro-apoptotic effect of notoginsenoside Ft1 on human neuroblastoma SH-SY5Y cells

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Aims: This study aims to investigate the effect and the mechanisms of notoginsenoside Ft1, a natural compound exclusively found in P. notoginseng, on the proliferation and apoptosis of human neuroblastoma SH-SY5Y cells.

Main methods: CCK-8 assay was used to assess the cell proliferation. Flow cytometry was performed to measure the cell cycle distribution and cell apoptosis. Hoechst 33258 staining was conducted to confirm the morphological changes of apoptotic cells. Protein expression was detected by western blot analysis and caspase 3 activity was measured by colorimetric assay kit.

Key findings: Among the saponins examined, Ft1 showed the best inhibitory effect on cell proliferation of SH-SY5Y cells with IC50 of 45 μM. Ft1 not only arrested the cell cycle at S, G2/M stages, but also promoted cell apoptosis, which was confirmed by Hoechst 33258 staining. Further studies demonstrated that Ft1 up-regulated the protein expressions of cleaved caspase 3, phospho-p53, p21, and cyclin B1, but down-regulated that of Bcl-2. Moreover, Ft1 enhanced the phosphorylation of ERK1/2, JNK and p38 MAPK. However, the phosphorylation of Jak2 and p85 PI3K was reduced by Ft1. Inhibitors of p38 MAPK and ERK1/2 but not JNK abrogated the up-regulated protein expressions of cleaved caspase 3, p21 and down-regulated protein expression of Bcl-2 as well as elevated caspase 3 activity induced by Ft1.

Significance: Ft1 arrested the proliferation and elicited the apoptosis of SH-SY5Y cells possibility via p38 MAPK and ERK1/2 pathways, which indicates the potential therapeutic effect of it on human neuroblastoma.

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Introduction

Radix Notoginseng (san qi), the root of Panax notoginseng (Burk.) F.H. Chen, has been used worldwide as a medicine or a dietary supplement for removing blood stasis, improving blood circulation and alleviating pain (Jia et al., 2010; Ruan et al., 2010). P. notoginseng derived products such as notoginseng tea, capsules and even its extracts have already been marketed in European countries and the United States as dietary supplements or functional foods (Ruan et al., 2010; Sun et al., 2010; He et al., 2012). Studies revealed that the crude extracts of P. notoginseng can inhibit the growth of many human cancer cells including hepatocarcinoma SMMC-7721, prostate cancer PC-3 and breast cancer MCF-7 (Chuang et al., 2004; Shang et al., 2006; Ng, 2006) through interfering cell cycle and eliciting apoptosis. Saponins containing ginsenosides and notoginsenosides are thought to be the major components from P. notoginseng responsible for the anticancer effect (He et al., 2012).

Neuroblastoma, predominantly a peripheral nervous solid tumor, is the most common extracranial neoplasm of early childhood that accounts for 7%–10% of all childhood cancers (Kim et al., 2007; Brard et al., 2009). Despite multimodal therapies including surgery, irradiation, autologous stem-cell transplantation and chemotherapy have been applied, however, the cure rate of neuroblastoma has not been elevated compared with other childhood malignancies. The majority of the neuroblastomas in children over one year of age are aggressive metastatic tumors with poor clinical outcome (Matthay et al., 1999; Kim et al., 2007). Moreover, more than 50% of children have high recurrence rates because of drug-resistant residual disease (Kim et al., 2007; Brard et al., 2009). Thus, new drug development targeting neuroblastoma with higher efficacy is still urgently needed.
Notoginsenoside F1 (Ft1), one of notoginsenosides found exclusively in *P. notoginseng*, has been reported by our group to promote blood vessel formation, thus contributing to wound healing (Shen et al., 2012) in addition to enhancing platelet aggregation (Gao et al., 2014). However, whether the natural compound possesses anti-cancer effect has not been investigated yet. In the current study, we discovered that Ft1 can effectively prevent the proliferation of human neuroblastoma SH-SY5Y cells and promote cell apoptosis. Thereafter, possible underlying mechanisms were investigated, which may contribute to the clinical application of Ft1 or its derivatives in therapy of neuroplastomas.

### Materials and methods

#### Materials

Saponins including notoginsenoside Ft1, notoginsenoside Fe, notoginsenoside Fc, ginsenoside Rh2, vietnamginsenoside R7, ginsenoside F2, gypenoside IX, ginsenoside Rb3, ginsenoside Re, notoginsenoside R1, ginsenoside Rd, ginsenoside Rg2, pseudoginsenoside F11, and ginsenoside Rg1 were isolated, purified and identified using mass spectrometry by Shanghai R&D centre for standardization of Chinese medicines (Shanghai, China). The purity of all of the saponins was greater than 98%. Cell Counting Kit-8 (CCK-8) was from Dojindo (Kumamoto, Japan). Caspase 3/PP 32 colorimetric assay kit was purchased from BioVision (California, USA). PD98059 and SB202190 were obtained from Selleckchem (Houston, USA). BI 78D3 was purchased from Tocris (Bristol, United Kingdom). PI and DMSO were purchased from Sigma-Aldrich Co. (MO, USA). Hoechst 33258 was obtained from AnaSpec Inc. (CA, USA). Trypsin and annexin V were purchased from Life Technologies (NY, USA). RNase A was from Beijing Jingkehongda Biotechology Co. (Beijing, China).

#### Cell culture

Human neuroblastoma SH-SY5Y, human gliomas U87-MG and U251, rat glioma C6 were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). SH-SY5Y was routinely maintained in a DMEM/F-12 medium, while all the other glioma cell lines were cultured in a DMEM medium (Thermo Fisher, IL, USA). All the cells were grown in the medium supplemented with 10% FBS (Gibco, NY, USA) at 37 °C with 5% CO2.

#### Proliferation assay

Cells were seeded in 100 μl of medium at 2.5 × 10^5 cells/ml in 96-well culture plates and grown overnight. After treated by drug for 24 h, 10 μl of CCK-8 solution was added to each well and incubated at 37 °C for another 1 h. The absorbance of the solutions was detected at 450 nm by a microplate reader (ThermoFisher, USA). The cell viability rate was calculated as the percentage of CCK-8 absorption as follows: (absorbance of drug-treated sample / absorbance of control sample) × 100.

#### Cell cycle distribution analysis

Cells were seeded in 6 well plates at 2.5 × 10^5 cells/well in 3 mL medium and cultured overnight. After serum starvation for 24 h, cells were incubated with Ft1 for 24 h. The cells were harvested by trypsinization, washed twice with phosphate buffered saline (PBS), fixed with cold 70% ethanol overnight followed by staining with PI solution containing 50 μg/mL RNase A and 0.1% Triton X-100. The distribution of cell cycle was examined using FACScan flow cytometer (Becton Dickinson, USA), and the data were analyzed by ModFitLT V3.0 software.

#### Annexin V/PI double-staining

Cells were plated in 6-well culture plates at a density of 2.5 × 10^5 cells/ml in 3 mL of medium and allowed to adhere to the plates overnight. The cells were incubated with different concentrations of Ft1 (45 and 67.5 μM) or vehicle solution (0.1% DMSO) in a medium containing 10% FBS for another 24 h. Then, the cells were harvested by trypsinization, washed twice with 1 × annexin V binding buffer, and double-stained with annexin V and PI. Cell apoptosis was examined using the Guava flow cytometer (Millipore, USA).

#### Hoechst 33258 staining

Cells were seeded in 6-well culture plates at a density of 2.5 × 10^5 cells/ml in 3 mL of medium and allowed to adhere to the plates overnight. The cells were incubated with different concentrations of Ft1 (45 and 67.5 μM) or vehicle solution (0.1% DMSO) in a medium containing 10% FBS for 24 h. After the treatment, the cells were fixed with 4% PFA for 10 min, followed by incubation with Hoechst 33258 staining solution (10 μg/mL) for 5 min and finally analyzed for morphological characteristics of cell apoptosis under a fluorescence microscope (Olympus CKX41, Japan).

#### Western blot analysis

Cells were plated in 6-well culture plates at a density of 2.5 × 10^5 cells/ml in 3 mL of medium and allowed to adhere to the plates overnight. The cells were incubated with different concentrations of Ft1 (22.5, 45 and 67.5 μM) or 0.1% DMSO in a medium containing 10% FBS for 48 h. For the investigation of involvement of MAPK pathways in Ft1 induced apoptosis, the cells were pre-incubated with different inhibitors for 1 h, then co-treated with Ft1 at 45 μg/ml for 24 h. Afterwards, the cells were collected, lysed with cell lysis buffer and sonicated three times each for 15 s. The cell lysates were centrifuged at 14,000 g for 15 min at 4 °C, and the supernatants were collected. Protein samples were separated by SDS-PAGE (15% or 8%) and transferred onto Hybond-NC membranes. Subsequently, the NC membranes were blocked with 5% non-fat milk solution and incubated with the primary antibodies against cleaved caspase 3 (Asp175), cyclin B1, Bax, Bcl-2, phospho-p53 (Ser15), p21, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p38 MAPK, p-p38 MAPK (Thr180/Tyr182), SAPK/JNK, p-SAPK/JNK (Thr183/Tyr185), Jak2, p-Jak2 (Tyr1007/1008), p-p85 Jak3 (Tyr458), p85 PI3K (Cell Signaling Technology, MA, USA) and GAPDH (Epitomics, CA, USA) overnight at 4 °C. After thoroughly washed with 1 × TBST, the NC membranes were incubated with respective secondary antibodies. The protein bands were visualized with the ECL prime kit (GE Healthcare, NA, UK) and scanned with SmartView software (Furi, China).

#### Caspase 3 activity analysis

The activity of caspase 3 was determined by caspase colorimetric assay kit (BioVisionn Milipats, CA, USA). Briefly, after pre-incubation with SB202190 (inhibitor of p38 MAPK, 20 μM), BI 78D3 (inhibitor of JNK, 2 μM) and PD98059 (inhibitor of ERK1/2, 20 μM) for 1 h, the cells were then co-treated with Ft1 at 45 μM for 24 h. Afterwards, cells were harvested and lysed with cell lysis buffer for 10 min on ice followed by centrifugation at 10,000 g for 1 min at 4 °C. The supernatant was collected and the protein concentration was determined. Then, 50 μg protein diluted in 50 μl cell lysis buffer was incubated with 50 μl of 2 × Reaction Buffer containing 400 μM substrate (DEVD-pNA) and 10 mM DTT at 37 °C for 2 h. The absorbance of samples was measured at 405 nm by the microplate reader.
Statistical analysis

The data were represented by the mean ± standard deviation (SD). Differences among groups were analyzed by one-way ANOVA with Dunnett or Bonferroni or Newman–Keuls multiple comparison test using PrismDemo 4 software (GraphPad Software Inc., USA). \( P < 0.05 \) was considered to be statistically significant.

Results

Ft1 showed the best antiproliferative effect on SH-SY5Y cells among fifteen saponins from P. notoginseng

Fifteen saponins isolated from P. notoginseng were screened for their antiproliferative effect on SH-SY5Y cells. As shown in Fig. 1A, when used at 50 μM, both Ft1 and Fe significantly inhibited the viability of SH-SY5Y cells in 24 h. By contrast, other saponins did not affect the growth of the cells. However, Ft1 exhibited better inhibitory effect than Fe did. Therefore, further studies were focused on this compound. As displayed in Fig. 1B, Ft1 dose-dependently prevented the proliferation of SH-SY5Y cells and the IC\(_{50}\) of it was 45 μM. Consistently, Ft1 induced remarkable morphologic changes of the cells in a dose-dependent manner (Fig. 1C). Generally, normal growing SH-SY5Y cells form clumps surrounded by outspread individual cells (Fig. 1Ca). After Ft1 treatment, the size of the cell clumps became smaller. Cells surrounding the clumps detached from the culture dishes and floated in the medium (Fig. 1Cb–c). When
the concentration of Ft1 was incremental to 67.5 μM, only a few cell clumps still attached to the dishes were accompanied with more cell debris formation (Fig. 1Cd).

By contrast, as illustrated in Fig. 2, Ft1 suppressed glioma proliferation only at high concentrations (50 and 100 μM). Although Ft1 (100 μM) inhibited 60% of C6 proliferation, it prevented relatively lower percentage of U87-MG and U251 cells from proliferation and the inhibitory rates of them were about 20% and 30%, respectively.

**Ft1 induced S, G2/M phase arrest of SH-SY5Y cells**

To examine the effect of Ft1 on cell cycle distribution, SH-SY5Y cells were treated with Ft1 (22.5, 45 and 67.5 μM) for 24 h followed by PI staining. As shown in Fig. 3, flow cytometric assay exposed that Ft1 significantly arrested SH-SY5Y cells at both S and G2/M phases. However, Ft1 triggered only G2/M phase arrest when used at 67.5 μM.

**Ft1 promoted apoptosis of SH-SY5Y cells**

To analyze the effect of Ft1 on cell apoptosis, SH-SY5Y cells were subjected to Hoechst 33258 and Annexin/PI staining, respectively, after Ft1 treatment. As displayed in Fig. 4A, the fluorescence of Hoechst 33258 was dim in control cells. However, the fluorescence became brilliant and aggregative in Ft1-treated cells accompanied with chromatin condensation and nuclear shrinkage or fragmentation, indicating the initiation of apoptosis.

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**Fig. 3.** Effect of Ft1 on cell cycle distribution after treated for 24 h: A: Statistical analysis of the cell cycle phase distributions after treated for 24 h; B: Flow cytometric analysis of the cell cycle phase distributions induced by Ft1 for 24 h detected by cell cycle distribution analysis with FACScan flow cytometer as described in the Materials and methods. Data represent mean ± SD, \(^*\) \(P < 0.01\), \(^{***}\) \(P < 0.001\) vs control.
After treated for 24 h, Ft1 induced significant cell apoptosis as the early cell apoptosis rate was increased from 13.64% (in control cells) to 48.85% (in 67.5 μM Ft1-treated cells) (Fig. 4B).

**MAPK pathway was involved in the anti-proliferative and pro-apoptotic effects of Ft1 on SH-SY5Y cells**

As revealed in Fig. 5, Ft1 treatment for 48 h led to prominent elevation of cyclin B1. Similarly, the cleaved caspase 3, phosphorylated p53 and p21 were increased significantly by Ft1. By contrast, Bcl-2, the anti-apoptotic protein, was decreased. However, Ft1 did not affect the expression of Bax protein.

When treated for 48 h, Ft1 dose-dependently activated ERK1/2, SAPK/JNK and p38 MAPK. As demonstrated in Fig. 6A, phosphorylated ERK1/2, SAPK/JNK and p38 MAPK were up-regulated dramatically by Ft1 treatment, while the total protein levels of ERK1/2, SAPK/JNK and p38 MAPK were not influenced. However, activation of both Jak2 and PI3K was suppressed. As shown in Fig. 6B, Ft1 dose-dependently counteracted the phosphorylation of p85 PI3K and Jak2 without influencing the total p85 PI3K and Jak2.

In order to further clarify the role of MAPKs on cell apoptosis induced by Ft1, the inhibitors of MAPKs were used. As shown in Fig. 7A, the inhibitors of MAPKs attenuated the antiproliferative effect of Ft1 on SH-SY5Y cells. After treated with Ft1 at 45 μM (Fig. 7B), the caspase 3 activity in SH-SY5Y cells was significantly increased. However, the elevated caspase 3 activity was suppressed remarkably by the inhibitors of p38 MAPK (SB202190) and ERK1/2 (PD98059). BI 78D3 only had a tendency to inhibit the elevated caspase 3 activity induced by Ft1 but without significant difference. The results from western blot analysis showed that the inhibitors of p38 MAPK and ERK1/2 could decrease
the effect of Ft1 on the protein expression of p21, cleaved caspase 3 and Bcl-2. However, BI 78D3 had no obvious effect on the protein expression of p21 and cleaved caspase 3 induced by Ft1, but could significantly inhibit the effect of Ft1 on protein expression of Bcl-2 (Fig. 7C).

Discussion

In the present study, we evaluated the effects of fifteen types of saponins found in P. notoginseng on the proliferation of SH-SYSY cells. Previous researches have already shown the bioactivities of P. notoginseng saponins (PNS) on the nervous system. For instances, PNS promote proliferation and differentiation of neural stem cells (Si et al., 2008; Zhang et al., 2010), attenuate hypoxia/reoxygenation induced oxidative stress in cortical neurons (Yan et al., 2012) and relieve Pertussis bacilli-induced brain edema (Li et al., 2003). And PNS have displayed anti-tumor activities in many cancer cells (He et al., 2012). However, whether PNS suppress the proliferation of neuroblastoma has not been reported yet. Our study found that both Ft1 and Fe could prevent the proliferation of SH-SYSY cells when used at 50 μM. As Ft1 showed better inhibitory effect than Fe, therefore, further studies were focused on Ft1 to uncover the possible underlying mechanisms of it in anti-neuroblastoma.

The imbalance between cell proliferation and apoptosis contributes to the progression of tumor cell growth. Thus, to redress the disturbed balance of cell proliferation and apoptosis is an important therapeutic strategy for treating human cancers. In our investigation, Ft1 dose-dependently inhibited cell proliferation, induced cell cycle arrest on S and/or G2/M phase, and enhanced cell apoptosis of SH-SYSY, which indicated therapeutic effect of Ft1 on neuroblastoma.

In normal cells, cyclin B1 regulates the transition of cell cycles from the G2 phase to the mitotic M phase. When cyclin B1 is inhibited, cells will be arrested in the G2 phase. On the contrary, cells will enter and proceed through mitosis, if the activity of cyclin B1 is increased. However, in tumor cells, the up-regulation of cyclin B1 mediates the induction of mitotic prometaphase arrest (Morgan, 1995; Choi and Zhu, 2012). In our study, the increased expression of cyclin B1 concomitant with the S, G2/M arrest was elicited by Ft1. This result implicated that Ft1 induced the prometaphase arrest. JNK has been suggested to be actively involved in the up-regulation of cyclin B1 (Choi and Zhu, 2012). Consistent with it, the elevated phosphorylation of JNK was observed in SH-SYSY cells after Ft1 treatment. But whether it directs the elevation of cyclin B1 has not been elucidated yet.

Cell apoptosis is mediated by caspase-dependent and caspase-independent pathways. Caspase-3 is one of the key players in cell apoptosis. At least 42 of the 58 known caspase substrates are specifically cleaved by caspase 3. Once caspase 3 is activated, it will increase cytochrome c release from mitochondria by cleaving Bcl-2 and converting it from an anti-apoptotic to a pro-apoptotic protein (Cheng et al., 1997; Nicholson and Thornberry, 1997; Porter and Jänicke, 1999). Moreover, it can process pro-caspases 2, 6, 7 and 9, therefore, speed up the cascade of apoptosis. Mitochondria-dependent pathway is the classical apoptotic pathway. Bax and Bcl-2 are the predominant regulators in controlling the release of cytochrome c in mitochondria-dependent apoptosis. When Bax inserts into the outer membrane of mitochondria, cytochrome c will be released from the organelle. However, when Bcl-2 instead of Bax binds to the outer mitochondrial membrane, release of cytochrome c will be blocked (Ow et al., 2008). Many anticancer agents can induce the release of cytochrome c through either up-regulating Bax expression and/or down-regulating Bcl-2 expression (El-Mahdy et al., 2005; Das et al., 2006; Reyes-Zurita et al., 2009). In our study, Ft1 dose-dependently elicited the generation of cleaved caspase-3 but suppressed that of Bcl-2 without interfering the expression of Bax. Therefore, both mitochondria-dependent and caspase-3-dependent pathways were involved in inducing cell apoptosis triggered by Ft1.
Many anticancer compounds induce apoptosis via up-regulation of p53 protein expression (Choi et al., 2008). However, curcumin induces apoptosis in vascular smooth muscle cells with a tendency to decrease gene expression of p53 (Chen and Huang, 1998). p21 is the downstream molecule of p53 which is activated by phosphorylated p53 (Persons et al., 2000; Yeh et al., 2001; Lu and Xu, 2006). In this study, the phosphorylated p53 in SH-SY5Y cells was up-regulated by Ft1 dose-dependently and accompanied by the elevation of p21. Therefore, Ft1 induced the apoptosis of SH-SY5Y in a p53 dependent pathway.

Mitogen-activated protein kinase (MAPK) pathway is actively involved in drug-induced cell apoptosis of tremendous cancer cells including SH-SY5Y (Frasca et al., 2004; Kim et al., 2005; Uehara et al., 2012). When the activation of MAPKs is inhibited, the apoptosis and caspase-3 cleavage in tumor cells are abrogated (Jo et al., 2005; Kim et al., 2005; Uehara et al., 2012). In our study, Ft1 could enhance the phosphorylation of all of the three major MAPKs in SH-SY5Y. Activation of MAPKs results in inactivation of Bcl-2, enhanced phosphorylation of p53 and thus activation of downstream p21 (Persons et al., 2000; Yeh et al., 2001; Shimada et al., 2003; Lu and Xu, 2006). In agreement with the reports, we also observed increased cleavage of caspase 3, enhanced phosphorylation of p53 and p21 but reduced Bcl-2 in the study, which provided strong evidence for the involvement of MAPK pathways in the apoptosis induced by Ft1.

Inhibition of janus kinase 2 (Jak2) promotes cell apoptosis (Chen et al., 2010; Parada et al., 2010; Will et al., 2010). Activation of the phosphatidylinositol 3-kinase (PI3K) pathway shows pro-survival effect. In SH-SY5Y cells, activation of the PI3K/Akt pathway has already been found to prevent the apoptosis of cells (Pettifer et al., 2004; Shi et al., 2010). In the current study, Ft1 could inhibit the activation of both Jak2 and p85 PI3K shown by reduced phosphorylation of Jak2 and p85 PI3K, which indicated that these two pathways were also involved in Ft1 elicited apoptosis.

Because of the critical role of the MAPK pathway in cell survival and apoptosis, in our further investigation, the inhibitors of p38 MAPK, JNK and ERK1/2 were used to confirm the effect of the MAPK pathway on Ft1 induced apoptosis. The results showed that the increased caspase 3, p21 but decreased Bcl-2 induced by Ft1 could be blocked by p38 MAPK and ERK1/2 pathway inhibitors, which indicated the involvement of p38 MAPK and ERK1/2 pathways in Ft1 induced cell apoptosis.

**Conclusion**

Among the fifteen saponins screened, Ft1 showed the best anti-proliferative effect on SH-SY5Y cells. Ft1 treatment resulted in S and/or G2/M phase arrest through up-regulating protein expression of cyclin B1. Further studies disclosed that Ft1 might act through activation of p38 MAPK and ERK1/2 but suppression of Jak2 and PI3K pathways, therefore, regulate downstream protein expressions of caspase 3, p53, p21 and Bcl-2 to induce the apoptosis of the neuroblastoma SH-SY5Y cells. This study provides evidences in vitro for supporting the potential clinical usage of Ft1 in therapy of human neuroblastoma.

**Conflict of interest statement**

The authors declare that they have no competing interests.

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