Solamargine inhibits proliferation and promotes apoptosis of CM-319 human chordoma cells through suppression of notch pathway

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Background: Solamargine (SM), which represents a natural steroid alkaloid glycoside compound and a cytotoxic agent, has been proved to enhance the sensitivity of lung cancer cells to tumor necrosis factors (TNFs). In this study, we aimed to investigate the roles and mechanisms of SM in chordoma.

Methods: Cell viability, proliferation, apoptosis and cell cycle were measured by cell counting Kit-8 (CCK-8) assay, 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and flow cytometry (FCM), respectively. Western blot and quantitative real-time reverse transcription PCR (qRT-PCR) assays were performed to detect the expressions of related mRNAs and proteins.

Results: The results revealed that SM distinctly suppressed the proliferation of CM-319 cells. SM significantly induced the CM-319 cells apoptosis through up-regulating the expression levels of Caspase-3/8/9. The cell cycle of CM-319 cells was blocked by SM in G1 phase. Moreover, SM could significantly suppress the Notch pathway in CM-319 cells.

Conclusions: In conclusion, SM suppressed the proliferation and enhanced the apoptosis ability of CM-319 cells via suppressing the Notch pathway. The results suggested that SM might be a novel therapeutic agent and supported the utilization of SM in chordoma.

Keywords: Solamargine (SM); proliferation; apoptosis; CM-319; chordoma; Notch pathway

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Notch gene was first reported by Morgan in 1917 and has been successfully cloned in the mid-1980s through investigating the mutant type of fruit flies (11). There are four mature single transmembrane receptors including Notch1, Notch2, Notch3 and Notch 4 (12). Overexpression of Notch1 has been demonstrated to induce the rat mammary tumor generation, while studies also found that Notch1 strongly inhibited the growth of human cervical cancer cells and mice skin tumor cells (13,14). Notch signaling pathway is widely presented in embryos and adult individual tissues, and it plays an important role in the embryonic development and steady-state maintenance of adult tissues (15). It has been proved that the activation of Notch pathway could suppress the proliferation of tumor cells including prostate cancer, lung cancer, brain tumor and hepatocellular carcinoma (16-18). However, there are few reports considering the effects of Notch pathway in chordoma cells.

Solamargine (SM), which serves as a natural steroid alkaloid glycoside compound and cytotoxic agent, is isolated from the Chinese herb Solanum species. Several studies reported that SM had significant in vitro toxicity effects on cancer cells (19-21). For example, Munari et al. reported that SM had pronounced anti-proliferative activities with IC_{50} values ranging from 4.58 to 18.23 μg/mL. Especially, the IC_{50} of HepG2 was 4.58 μg/mL treated by SM (22). It has been reported that SM strengthened the human epidermal growth factor receptor 2 (HER2) expression, regulated the expression levels of factor associated suicide (Fas) and enhanced the sensitivity of lung cancer cells to trastuzumab and epirubicin (23,24). Moreover, the inhibitory effects of SM on lung cancer cells were possibly realized through affecting signal transducer and activator of transcription stat 3 (stat3) pathway (25). In the leukemia cells, SM was found to activate the lysosome-mitochondria pathway (26). However, the pharmacological activity and mechanism of SM in the proliferation and apoptosis of chordoma cells have not been studied yet.

In the present study, we investigated whether SM could serve as a novel therapeutic agent affected the growth and apoptosis of CM-319 cells. Furthermore, it was also fascinating to explore the crucial role of Notch pathway in the pathomechanism of chordoma.

Methods

Reagents and cells

The products utilized in cell culture were purchased from Gibco (Carlsbad, CA, USA). Antibodies were obtained from Abcam (Camb, UK). The CM-319 human chordoma cell line was obtained from the Cell bank of Chinese Academy of Sciences. The cells were cultured at 37 °C with 5% CO_2 in RPMI-1640 medium (GENOM, Hangzhou, China) with 0.3 μL 2-mercaptoethanol and 10% fetal bovine serum (Thermo Fisher, USA) (27). CM-319 cell was a polygonal cell and expressed CD24, which was traditionally used as diagnostic markers of chordoma (28). The cell line was first derived from sacral chordoma (27).

Cell viability analysis

Cell counting kit-8 (CCK-8; Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) assay was carried out to measure the cell viability. About 6×10^5 cells/ml of CM-319 cells in the logarithmic phase were seeded in the wells of 96-well plates and maintained in an incubator (at 37 °C, 5% CO_2) for 12 h. After that, CM-319 cells were treated with different concentrations of SM (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM) for 12, 24 and 48 h. Cells were then maintained in the incubator (37 °C, 5% CO_2) for 12 h. Next, the cells were supplemented with 10 μL CCK reagent in each well, followed by being put in an incubator with 5% CO_2 at 37 °C for 3 h. The absorbance at 450 nm was read by an enzyme labeling instrument (Bio-Rad Laboratories, Inc., USA). Cell viability was determined in terms of the proportion of cell survival, compared with control.

Apoptosis assay

Flow cytometry (FCM) was performed to determine cell apoptosis. CM-319 cells were harvested 48 hours after transfection. Then the cells were suspended in an incubation buffer at a density of 1×10^6 cells/mL. After that, the cells were incubated with Annexin V-FITC and propidium iodide (PI) at room temperature in the dark for 15 min. The proportion of apoptosis cell number was quantified by FACSCalibur (BD Biosciences, San Diego, CA, USA).

5(6)-carboxyfluorescein diacetate succinimidy l ester (CFSE) labeling

CM-319 cells were suspended in phosphate buffered solution (PBS) at a final concentration of 1×10^6 cells/ml and 5 μM CFSE (Thermofisher, New York, USA) was
supplemented into the cells. The cells were treated with 10 volumes of ice-cold dulbecco’s modified eagle medium (DMEM; Gibco) containing 10% fetal calf serum (FCS; Gibco) after incubation at 37 °C for 10 min. Next, the cells were maintained on ice for 5 min and then centrifuged for 1 min. After centrifugation, the cells were washed twice by culture medium and sowed into 24-well plates (about 1×10^5 cells per well). The cells were harvested and then treated with 0, 25, 50 and 100 μM SM, respectively.

**Cell cycle analysis**

After being digested by 0.25% trypsin (Beyotime, Shanghai, China), cells were seeded in the wells of 6-well plates at a final density of 1×10^7 cells/well and maintained for 24 h in an incubator (at 37 °C, 5% CO_2). Four treatment groups were performed on the cells in 6-well plates and the cells were then incubated for 48 h. The cells were re-suspended after being washed by PBS and then centrifuged for 5 min at 1,000 rpm/min and the supernate was discarded. Pre-cooling 70% ethanol (1 mL) was added into the cells, which were then blown gently. After that, the cells were saved in a refrigerator at 4 °C overnight. After centrifugation, the ethanol was discarded and the cells were washed thrice by PBS. 500 μL PBS containing PI (50 μg/mL), RNase A (100 μg/mL) and Triton X-100 (0.2%) was added into the cells, which were then dyed at dark for 30 min. Cell cycle were measured by FCM.

**Quantitative real-time reverse transcription PCR (qRT-PCR) analysis**

The total RNA was extracted from CM-319 cells by TRIzol (Thermo Fisher Scientific Inc, New York, USA) following the instructions of manufacturers. Afterwards, two microliters of RNA were used for the cDNA synthesis with a first strand cDNA kit (Sigma, Munich, Germany) following the specification. SYBR Green Premix Reagent (TakaraBio Inc., Shiga, Japan) was utilized. qRT-PCR assay was carried out using ABI 7500 Thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR cycles were under the following conditions: 10 min pretreatment at 94 °C, 95 °C for 5 s, 65 °C for 30 s (35 cycles), 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, a final extension at 72 °C for 10 min and held at 4 °C. The primers were designed by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) and were as follows: Caspase-3, forward: 5’-GGCGCTCTGTTTTCGTATAA-3’ and reverse: 5’-GCTGCATCGACATCTGTACC-3’ (product: 246 bp); Caspase-8, forward: 5’-CTGGTCTGAAGGCTGTGTTG-3’ and reverse: 5’-CGAGGCTCGAATCCGGG-3’ (product: 275 bp); Caspase-9, forward: 5’-CTGGCTTTCTTCTGGCACC-3’ and reverse: 5’-GCACAGGGACCCACGTAAA-3’ (product: 253 bp); Ki67, forward: 5’-CTGACCGCTATGAGTGAGGA-3’ and reverse: 5’-ACTCTGTAAGGTCGACGAGG-3’ (product: 736 bp); Cyclin D1, forward: 5’-TGAGGGACGCTTTTGCTGTC-3’ and reverse: 5’-CTTCTGCTGAAACATGCG-3’ (product: 223 bp); DLL1, forward: 5’-CAACCTGATGAGTGAGGA-3’ and reverse: 5’-TGACGGCTTTTGCTGTC-3’ (product: 284 bp); DLL3, forward: 5’-CAACCTGATGAGTGAGGA-3’ and reverse: 5’-CAGGACGAATCGAGGAG-3’ (product: 140 bp); HES1, forward: 5’-GGCTAAGGTGTTTGGAGCT-3’ and reverse: 5’-GGTGGGTTGGGGAGTTTAGG-3’ (product: 250 bp); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5’-AATGGGCAGCCGTTAGGAAA-3’ and reverse: 5’-GCCCAATAGCAACATG-3’ (product: 168 bp). GAPDH was used as the control for the input RNA level.

**Western blot analysis**

Cell lysates were lysed by RIPA buffer (Sigma-Aldrich, USA) and moved into tubes for later use. After being washed with PBS twice, the proteins from cells were extracted. Proteins were partitioned on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Next, the cells were moved to a PVDF membrane (Millipore, Billerica, MA, USA). Blots were carried out with anti-Caspase-3, dilution, 1:500, Abcam, ab13585; anti-Caspase-8, dilution, 1:1,000, Abcam, ab32397; anti-Caspase-9, dilution, 1:2,000, Abcam, ab202068; anti-Ki67, dilution, 1:5,000, Abcam, ab92742; anti-Cyclin D1, dilution, 1:10,000, Abcam, ab134175; anti-DLL1, dilution, 1:500, Abcam, ab10554; anti-DLL3, dilution, 1:500, Abcam, ab63707; anti-Notch1, dilution, 1:500, Abcam, ab65297; anti-HES1, dilution, 1:500, Abcam, ab108937; anti-GAPDH, dilution, 1:10,000, Abcam, ab18602. After that, horseradish peroxidase-conjugated secondary antibodies
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Statistical analysis

The results in our study were shown as mean ± SEM of at least three independent experiments. Data were analyzed by Student’s t-test, or one-way analysis of variance (ANOVA). The statistical significance was defined as P<0.05.

Results

SM distinctly suppressed the cell viability and proliferation of CM-319 cells

Previous study has already used CM-319 cell lines to carry out several experiments on the resistance to chemotherapy and radiotherapy in chordoma research. Therefore, CM-319 cell lines were also selected to perform this experiment (27,29). CCK-8 assay results revealed that the cell activity of CM-319 cells treated with 60, 70, 80, 90 and 100 μM SM was distinctly lower than that in control (Figure 1; P<0.05). Based on the processing time, we found that the trends of cell viability of the cells with 48 h of treatment were more obvious than those with 12 and 24 h. Moreover, as shown in CFSE results, the cell proliferation assessment of CM-319 cells treated with different concentrations of SM was obviously lower than that in control, especially with the treatment of 100 μM (Figure 2; P<0.01). These outcomes revealed that SM could reduce the cell activity and proliferation capacity of CM-319 cells. All together, we confirmed that SM led to a significant low cell viability and proliferation capacity of CM-319 cells in a dose-dependent manner.

SM significantly induced the CM-319 cells apoptosis

As FCM data shown in Figure 3, the proportion of apoptosis cell number in control was 3.13%. However, after being treated with SM in 25, 50 and 100 μM, the apoptosis rate of CM-319 cells was enhanced to 6.09%, 12.78% and 17.37%, respectively. The percentages of apoptosis cell number in SM treatment groups were significantly higher than those in control (25 μM, P<0.05; 50 μM, P<0.01; 100 μM, P<0.001), indicating that SM enhanced the apoptosis capacity of CM-319 cells.

The cell cycle of CM-319 cells was blocked by SM in G1 phase

We evaluated the cell cycle of CM-319 treated with different concentrations of SM, and the results were revealed in Figure 4. We found that the G1 phase of CM-319 cells in control group was 40.78%. After treating with different concentrations of SM, the G1 phase of CM-319 cells increased from 47.89% to 60.75%. However, the G2 and S phases of CM-319 cells in four groups had no significant difference. Altogether, it was indicated that SM markedly increased the G1 phase of CM-319 cells in a dose-dependent manner (P<0.05). Therefore, we could draw a conclusion that the cell cycle of CM-319 cells was blocked by SM in G1 phase.

The expression levels of proliferation-, apoptosis-, and cell cycle-associated genes and proteins in CM-319 cells treated by SM

To investigate the mechanism of SM affecting the proliferation, apoptosis and cell cycle of CM-319 cells, we therefore measured the expression levels of Caspase-3/8/9, Ki67 and Cyclin D1 in CM-319 cells from each group. According to the qRT-PCR results, we found that the expression levels of Caspase-3, Caspase-8 and Caspase-9 in CM-319 cells were significantly enhanced by treating the cells with different concentrations of SM (Figure 5A; 50 μM, P<0.01). However, compared with control, a sharp
Figure 2 SM suppressed the proliferation of CM-319 cells. CFSE labeling was carried out to determine the proliferation of CM-319 cells treated with different concentrations of SM (0, 25, 50 and 100 μM). *, P<0.05; **, P<0.01 versus control. SM, solamargine; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester.

decrease in Ki67 expression in CM-319 cells treated with different concentrations of SM was observed (Figure 5A; 25 μM, P<0.05; 50 and 100 μM, P<0.01). The expression level of Cyclin D1 in CM-319 cells was found to be distinctly strengthened by treating the cells with SM (Figure 5A; 25 and 50 μM, P<0.05; 100 μM, P<0.01). Moreover, western blot data also revealed the similar outcomes. It was indicated that SM markedly up-regulated the expression levels of Caspase-3, Caspase-8, Caspase-9 and Cyclin D1, while down-regulating the Ki67 expression in CM-319 cells (Figure 5B; 50 μM, P<0.01). These conclusions suggested that SM affected the proliferation, apoptosis and cell cycle of CM-319 cells through regulating the expression levels of Caspase-3/8/9, Ki67 and Cyclin D1 in CM-319 cells.

SM down-regulated the Notch pathway in CM-319 cells

We assessed the level of Notch pathway in CM-319 cells treated with different concentrations of SM, and the expression levels of delta-like 1 (DLL1), DLL3, Notch1 and hairy and enhancer of split-1 (HES1) were measured in our study. The qRT-PCR data revealed that the expression levels of DLL1 (50 μM, P<0.05; 100 μM, P<0.01), DLL3 (P<0.05), Notch1 (50 μM, P<0.05; 100 μM, P<0.01) and HES1 (25 μM, P<0.05; 50 and 100 μM, P<0.01) in CM-319 cells treated with different concentrations of SM were significantly lower than those in control (Figure 6A). These results indicated that SM could reduce the DLL1 (50 μM, P<0.01; 100 μM, P<0.001), DLL3 (100 μM, P<0.05), Notch1 (25 μM, P<0.05; 50 and 100 μM, P<0.01) and HES1 (50 and 100 μM, P<0.001) expressions in CM-319 cells. Additionally, compared with control, the protein expressions of DLL1, DLL3, Notch1 and HES1 in CM-319 cells were markedly reduced by treating the cells with different concentrations of SM (Figure 6B). Therefore, we confirmed that SM could significantly down-regulate the expression...
Figure 3  SM induced the apoptosis of CM-319 cells. FCM was performed to measure the proportion of apoptosis cell number of CM-319 cells treated with 0, 25, 50 and 100 μM SM, respectively. *, P<0.05; **, P<0.01; ***, P<0.001 versus control. SM, solamargine; FCM, flow cytometry.

Discussion

On account of the low sensitivity of chordoma in radiotherapy and chemotherapy, surgical resection is still the preferred method for treating chordoma (30). However, the local recurrence of chordoma remains a major problem in the treatment of chordoma (31). Chondrosarcoma and malignant fibrous histiocytoma structure can often be found in the tissues from frequentative recurrence of chordoma, indicating that the recurrence of chordoma caused enhanced malignancy and poor prognosis (2). Due to the high recurrence rate of chordoma and its insensitivity to conventional radiotherapy and chemotherapy, drug and molecular targeted therapies are particularly important in treating chordoma.

SM is a steroidal glycoalkaloid separated from the Chinese herb Solanum. As a main active component or a preparation, SM is helpful for treating several diseases including keratosis, basal cell carcinomas and squamous cell carcinomas (32,33). Yang et al. reported that Solanum incanum extract (SR-T100) may be effectively and safely treated for keratosis through a phase III randomized double-blind vehicle-controlled parallel trial (34). Cisplatin can be toxic to a wide variety of cancers by inducing apoptosis via lethal DNA damage, however, it is associated with several mechanisms including intrinsic and acquired resistance. Moreover, high concentration of Cisplatin produces resistance to breast and lung cancer cells, and SM could enhance the cell death of several cell lines of breast and lung cancers in the presence of Cisplatin (35,36). In addition, SM not only displayed a significant cytotoxicity in the
multidrug resistance (MDR) sublines, but also could inhibit the expressions of MDR-related genes including P-gp (37,38). It has been demonstrated that SM possessed the function of inhibiting the migration and invasion of human hepatocellular carcinoma cells (39). Furthermore, studies have reported that SM suppressed the growth of human tumor cells, involving colon cancer cells, prostate cancer cells, breast cancer cells and hepatoma cells (32,40-42). Nevertheless, the pharmacological activity and mechanism of SM in the growth and apoptosis of chordoma cells have not been investigated yet. Thus, we set out to explore whether SM positively affected chordoma cell proliferation and apoptosis. Firstly, we assessed the cell viability of CM-319 human chordoma cells treated with different concentrations of SM. It was confirmed that SM significantly reduced the cell activity of CM-319 cells, indicating that SM might possess the antitumor effects on chordoma. Secondly, we evaluated the anti-proliferation capacity of SM on CM-319 cells, and the results showed that SM distinctly suppressed the proliferation of CM-319 cells in a dose-dependent manner. In addition, we found that SM could significantly enhance the apoptosis ability of CM-319 cells, and it was revealed that SM arrested the cell cycle of CM-319 cells in the G1 phase. These results suggested that SM might serve as a novel therapeutic agent targeting the CM-319 cells. Furthermore, we investigated the mechanisms of SM affecting the proliferation, apoptosis and cell cycle of CM-319 cells. As shown in the results, SM significantly enhanced the expression levels of Caspase-3, Caspase-8, Caspase-9 and Cyclin D1, while down-regulating the Ki67 expression in CM-319 cells. These results further confirmed that SM suppressed the proliferation, induced the apoptosis, and affected the cell cycle of CM-319 through regulating the apoptosis- and cell cycle-associated genes and proteins.

Notch pathway, which is a signaling pathway conserved in evolution, participates in various physiological and pathological processes and plays an important role in cell...
proliferation, differentiation and apoptosis (43). In the development and progression of tumors, Notch pathway also exerts important effects (44). Nevertheless, Notch pathway plays different roles in different tumors. On one hand, Notch pathway acts as cancer suppressor gene, on the other hand, Notch pathway represents as oncogene (45). Therefore, it is necessary to determine the exact role of Notch pathway in the tumor before blocking or activating. In the current study, we envisioned whether SM affected the proliferation and apoptosis of chordoma cells via interposing the Notch signaling pathway. DLL1 and DLL3 acted as ligands of Notch (46), and HES1 was reported as a down-stream target of Notch (47). Previous researches have suggested that regulating the expression levels of these factors in Notch pathway is related to the proliferation or apoptosis of tumor cells (48-50). Nevertheless, few studies were conducted on the regulation of Notch pathway by SM in chordoma cells. In our study, we found that SM markedly reduced the expression levels of DLL1, DLL3, Notch1 and HES1 in CM-319 cells. It was indicated that SM might regulate the Notch pathway in CM-319, thereby affecting the proliferation and apoptosis of CM-319 cells.

Recently, Cornelius et al. reported photodynamic adjuvant therapy of chordoma using in vitro experiments (51). We believe that SM in combination with photodynamic therapy could be more useful in treatment of chordoma. Base on the results of in vitro experiments, the prospect of clinical application of SM is possible. However, human body is a complex environment, in vivo model is required to further study the effects of SM on chordoma and we are
planning to take a related in vivo experiment.

Altogether, it was demonstrated that SM reduced the cell viability of CM-319 cells. SM inhibited the proliferation and induced the apoptosis of CM-319 cells through blocking the cell cycle of CM-319 cells in G1 phase and regulating the expression levels of Caspase-3/8/9 and Ki67. Furthermore, SM suppressed the Notch pathway in CM-319 cells.

Conclusions

In summary, we confirmed that SM suppressed the proliferation and enhanced the apoptosis ability of CM-319 cells via down-regulating the Notch pathway. These results suggested that SM might be a novel therapeutic agent and supported the utilization of SM in chordoma.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.03.07). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).
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