Salidroside inhibits migration and invasion of poorly differentiated thyroid cancer cells

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Abstract
Background: No effective treatment is currently available for poorly differentiated thyroid cancer which is resistant to radioiodine, especially with migration and invasion. A great number of researches have revealed the anticancer effects of salidroside, but none have studied the effects of salidroside on thyroid cancer. This study aimed to investigate the effect of salidroside on migration and invasion of poorly differentiated thyroid cancer cells.

Methods: The effects of salidroside on migration, invasion and apoptosis of poorly differentiated thyroid cancer WRO cells and normal thyroid follicular epithelial Nthy-ori 3-1 cells were measured by wound-healing assay, transwell migration/invasion assay and flow cytometry, respectively. The expression levels of MMP2 and MMP9 at RNA and protein levels in WRO cells were detected by qRT-PCR and western blot. The phosphorylation levels of Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3) and the apoptosis-related protein levels of Bax, cleaved caspase 3 and Bcl-2 were assessed by western blot.

Results: Salidroside significantly suppressed migration/invasion and induced apoptosis in poorly differentiated thyroid cancer WRO cells. We further illustrated that salidroside significantly inhibited expressions of MMP2 and MMP9 at mRNA and protein levels and the phosphorylation activation of JAK2/STAT3 in WRO cells. In addition, salidroside increased expressions of pro-apoptotic factors (Bax and cleaved caspase 3) and decreased expression of anti-apoptotic factor (Bcl-2) significantly in WRO cells.

Conclusion: The present study demonstrates that salidroside inhibits migration and invasion of WRO cells (a kind of poorly differentiated cancer cell line) significantly, which might be via suppressing JAK2-STAT3 signaling pathway.

Introduction
Poorly differentiated thyroid carcinoma (PDTC) displays intermediate biological features and clinical behaviors between well-differentiated thyroid cancer (WDTC) and anaplastic thyroid cancer (ATC), which has been added to the World Health Organization classification as a distinctive entity.1-3 Although a wide variety of treatments including surgery, radioiodide therapy, radiotherapy, and chemotherapy have been designed to treat patients with thyroid cancer, the success to date is limited in PDTC, due to resistance to conventional therapy, so PDTC shows aggressive behaviors and poor prognosis due to high probability of recurrences and metastases, and may even be fatal within a few months of diagnosis. Therefore, searching for new effective therapeutic agents with minimal side effects remains the top priority in PDTC research.

It has been indicated that some plant-derived drugs are more effective and have minimal side effects compared with synthetic drugs in cancers.4 Salidroside (%hydroxyphenethyl-%D-glucoside), purified from Rhodiolarosea...
Salidroside inhibits migration and invasion

H. Shang et al.

Salidroside, an extract of *Rhodiola rosea* L., exerts varieties of pharmacological properties, such as antioxidant, anti-aging, anti-fatigue, anti-inflammatory, neuroprotective and cardiovascular protective effects, as well as anticancer effects. Various anticancer investigations have showed the effects of salidroside on cell proliferation, apoptosis, migration and invasion in breast cancer, colorectal cancer, lung cancer, bladder cancer and malignant glioma. However, to date, no research has been undertaken to study the effects of salidroside on thyroid cancer. In our previous study, we found the expression of sodium iodide symporter (NIS), specific thyroid gene associated with differentiation, especially iodide uptake, was increased by salidroside, but the rate of iodide uptake was not significantly improved.

The purpose of the present study was to explore the effects of salidroside on cell migration and invasion in PDTC cells.

**Methods**

**Reagents and antibodies**

PRIM1640 medium, penicillin-streptomycin solution and trypsin-EDTA were purchased from Gibco (California, USA). Fetal bovine serum (FBS) was purchased from Lonsera (Montevideo, Uruguay). Salidroside (pure ≥98%) was purchased from Tauto Biotech Co., Ltd. (Shanghai, China) and its product ID is E-0069. Salidroside was dissolved in phosphate buffer solution (PBS) and filtered through a 0.22-μm filter before use. Annexin V apoptosis detection kit was purchased from NeoBioscience (Shenzhen, China). Antibodies were obtained from the following sources: rabbit anti-MMP9 (#ab38898), anti-pSTAT3 (Tyr705) (#ab76315), anti-STAT3 (#ab68153), anti-JAK2 (#ab108596) and anti-pJAK2 (Y1007 + Y1008) (#ab32101) from Abcam, rabbit anti-MMP2 (#AF0577) from Affinity, mouse anti-Bcl-2 (C-2) (#sc-7382) from Santa Cruz Biotechnology, rabbit anti-Bax (#GB11007) from Servicebio, rabbit anti-cleaved caspase 3 (#9664) from Cell Signaling Technology, mouse anti-β-Actin (#LocusID60) from OriGene, rabbit anti-GAPDH (#10494-1-AP) and HRP-conjugated secondary anti-rabbit IgG antibody (#SA00001-2) from Proteintech.

**Cell culture**

Human poorly differentiated thyroid cancer cell line (WRO) and human normal thyroid follicular epithelial cell line (Nthy-ori 3-1) were respectively cultured in PRIM1640 medium supplemented with 10% FBS and 100 U/mL penicillin and streptomycin at 37°C in an incubator containing 5% CO2. The medium was replaced with fresh medium every 2–3 days. WRO and Nthy-ori 3-1 cells were harvested with trypsin-EDTA.

**RNA extraction and quantitative RT-PCR**

Total RNA was isolated from WRO cells using TriZol reagent (TaKaRa, Japan) according to the manufacturer’s protocol. cDNA was then synthesized using the First Strand cDNA Synthesis Kit (TaKaRa, Japan). The SYBR green-based qPCR master mixes were obtained from GenStar. Quantitative RT-PCR was performed with SYBR with an ABI PRISM 7500 Real-time PCR System (Applied Biosystems, USA). The primer sequences are as follows: MMP-2 forward 5′- CTG GGA GCA TGG CGA TGG ATA-3′, reverse 5′- GGA AGC GGA ATG GAA ACT TG-3′; MMP-9 forward 5′- GCC ATG TCT GCT GTT TTC TAG AGG-3′, reverse 5′- CAC ACT CCA GGC TCT GTC CTC TTT-3′; and GAPDH forward 5′-CAG AAC ATC ATC CCT GCC TCT AC-3′, reverse 5′- TTG AAG TCA GAG GAG ACC ACC ACC TG-3′. The expression of target genes was calculated using ΔΔCt and comparative methods after being normalized to the expression of GAPDH.

**Protein extraction and Western blot analysis**

WRO cells were lysed with RIPA buffer containing protease inhibitors cocktail 1× protease inhibitor cocktail (100:1), scraped, centrifuged at 12 000 rpm for 20 minutes and the supernatant was diluted with 5× loading buffer (4:1) and denatured at 95°C for five minutes, then stored at −80°C. Protein concentrations were estimated by BCA assay. Thirty micrograms of total protein lysate were subjected to SDS-PAGE. The membranes were treated with 5% non-fat dry milk for one hour at room temperature. The membranes were then incubated overnight with primary antibody against MMP2 (1:5000), MMP9 (1:1000), pSTAT3 (Tyr705) (1:1000), STAT3 (1:2000), anti-pJAK2 (Y1007 + Y1008) (1:1000), anti-JAK2 (1:1000), Bax (1:1000), cleaved caspase 3 (1:1000), Bcl-2 (1:500), β-actin (1:2000) and GAPDH (1:5000). The next day, the membranes were washed and incubated with HRP-conjugated secondary anti-rabbit IgG antibody (1:10 000) or anti-mouse IgG antibody (1:10 000) for one hour at room temperature. After washing again, detection of blots was performed using an enhanced ECL chemiluminescence system (Millipore, USA).

**Wound-healing assay**

WRO and Nthy-ori 3-1 cells were respectively seeded on six-well plates at densities of 1 × 105 cells per well. Cells were grown for 24 hours and thereafter the wounds were created with a wound maker tool. Cells were then washed twice with PRIM 1640 medium. Thereafter, the medium
wash was changed to PRIM 1640 and supplemented with 100 U/mL penicillin-streptomycin containing either the control solution (PBS) or salidroside for 24, 48 and 72 hours. Imaging was conducted with an optical microscope (Leica, Germany). The wound area analysis was conducted with ImageJ software. The rate of cell wound closure was measured and calculated at different time points according to the equation: wound closure (%) = (gap area at zero hour - the remaining gap area) / gap area at zero hour × 100%. The rate of wound closure at zero hour in each group was considered as 0.

Transwell migration and invasion assays
To assess the effect of salidroside on cell migration and invasion, the migration and invasion assays were performed using 24-well cell culture inserts chambers (Corning, USA) with a polyethylene terephthalate membrane (8 μm pores). For the migration assay, WRO and Nthy-ori 3-1 cells were respectively incubated with different concentrations of salidroside (0, 10, 20 and 40 μg/mL) for 72 hours. After treatment, 200 μL of cells (2.5 × 10⁴/mL in serum-free PRIM 1640 medium) were seeded into the upper chamber and 750 μL medium with 10% FBS serving as a chemoattractant were added to lower chamber. After being cultured for 24 hours, cells migrated to the lower surface of the filter were washed twice in PBS, fixed with 4% formaldehyde for 15 minutes, permeabilized by 100% methanol for 20 minutes, and then stained with 0.25% crystal violet for 20 minutes at room temperature. The non-migrated cells were scrapped off with a cotton swab. Cells that had migrated through the pores were counted in five randomly selected fields per filter under a light microscope (Leica, Germany) at 20x objective magnification. For the invasion assay, the membranes of each upper chamber were coated with Matrigel (100 μg/cm², BD) at 37°C overnight for gelling and reconstituted with serum-free medium for one hour at 37°C prior to the experiment. Unlike the migration assay, cells seeded into the upper chamber were incubated for 48 hour instead of 24 hour. Other experimental procedures were the same as the migration experiment.

Cell apoptosis analysis
WRO and Nthy-ori 3-1 cells in six-well plates were respectively treated with 0, 10, 20 and 40 μg/mL salidroside for 72 hour. An Annexin V apoptosis detection kit was used to detect cell apoptosis. Briefly, 1 × 10⁵ cells were mixed with Annexin V-FITC and PI, and incubated for 15 minutes at room temperature in the dark. The rates of apoptosis were obtained by a flow cytometer.

Statistical analysis
All numerical data were presented as the mean ± SD for at least three independent measurements and analyzed by one-way ANOVA test or two-tailed Student’s t-test. *P < 0.05 was considered statistically significant.

Results
Salidroside inhibits migration of poorly differentiated thyroid cancer cells
Wound-healing assay and transwell migration experiment were performed (Fig 1) to determine whether salidroside could suppress migration of poorly differentiated thyroid cancer cells. As presented in Figure 1a, salidroside had no effect on the migration of normal thyroid follicular epithelial Nthy-ori 3-1 cells, but it inhibited poorly differentiated thyroid cancer WRO cell migration in a dose-dependent manner. Notably, in the group treated with 40 μg/mL salidroside, hardly any WRO cells had migrated into the wound area during 72 hours. As shown in Figure 1b,c, salidroside did not have any effect on the migration of Nthy-ori 3-1 cells, but numbers of migrated WRO cells with 10, 20 and 40 μg/mL salidroside for 72 hours were significantly decreased in a dose-dependent manner (P < 0.05). These data showed that salidroside inhibited cell migration in poorly differentiated thyroid cells.

Salidroside inhibits invasion of poorly differentiated thyroid cancer cells
A Matrigel-coated filter was used to evaluate the effect of salidroside on capacity of cell invasion. As shown in Figure 2, Nthy-ori 3-1 cells with, or without, salidroside scarcely invaded into lower chambers, but numbers of invasive cells in WRO cells with 10, 20 and 40 μg/mL salidroside for 72 hours were significantly reduced compared with that in untreated cells (P < 0.05). The result demonstrated that the ability of traversing Matrigel-coated layer in salidroside-treated WRO cells was decreased significantly compared with that in control cells, namely salidroside inhibited invasion of WRO cells significantly.

Salidroside suppresses expression of MMP2 and MMP9 at mRNA and protein levels in poorly differentiated thyroid cancer cells
To further investigate the mechanisms of how salidroside inhibits the migration and invasion of poorly differentiated
thyroid cancer, the mRNAs and proteins related to cell migration and invasion were evaluated by Real-time PCR and western blot analysis (Fig 3). As shown in Figure 3a, salidroside downregulated the expression of MMP2 and MMP9 mRNA in WRO cells, in particular 40 μg/mL salidroside significantly inhibited the expression of MMP2 and MMP9 mRNA by 91.7% and 74.9%, respectively (P < 0.05). As presented in Figure 3b, salidroside also significantly decreased the expression of MMP2 and MMP9 at protein levels. Similarly, maximum inhibition of proteins was achieved at 40 μg/mL salidroside, which downregulated expression of MMP2 and MMP9 protein by 87.7% and 57.9% compared with the control group.

Salidroside inhibits phosphorylation activation of JAK2/STAT3 signaling in poorly differentiated thyroid cancer cells

Studies have demonstrated that salidroside inhibited JAK2/STAT3 signaling pathway in many cancers and downregulated MMP-2 and MMP-9 proteins. It has also been demonstrated that MMP-2 and MMP-9 are associated with the JAK2/STAT3 signaling pathway.

**Figure 1** Salidroside inhibited the migration of poorly differentiated thyroid cancer WRO cells. Cell migration of normal thyroid follicular epithelial Nthy-ori 3-1 cells and poorly differentiated thyroid cancer WRO cells was detected by wound-healing assay (a) and transwell migration assay (b). (c) The histogram shows the results of the quantitative analysis of transwell migration assay. Data are shown as the mean ± SD of three experiments. *P < 0.05 versus control.
and MMP9 are important effector molecules in JAK2/STAT3 pathway,17,27 and activation of JAK2/STAT3 can increase expression of MMP2 and MMP9, resulting in degradation of extracellular matrix.27,28 Western blot experiments were performed in order to explore whether salidroside inhibits JAK2/STAT3 expression or phosphorylation activation (Fig 4). As expected, phosphorylation levels of JAK2 (Y1007 + Y1008) and STAT3 (Y705) were decreased by salidroside for 72 hours in a dose-dependent manner compared with the control group; specifically, 40 μg/mL salidroside decreased expression of pJAK2/JAK2 and pSTAT3/STAT3 by 52.5% and 69.3%, respectively (P < 0.05). Although phosphorylation of STAT3 (Y705) was slightly increased by 10 μg/mL salidroside, there was no statistical difference when compared with the control group (P > 0.05). The results indicated that salidroside may suppress migration and invasion of poorly differentiated thyroid cancer WRO cells by inhibiting the JAK2/STAT3 signaling pathway.

Salidroside induces apoptosis of poorly differentiated thyroid cancer cells

In our previous work, we demonstrated that salidroside could inhibit cell viability in WRO poorly differentiated thyroid cancer cells (Supplementary Fig. 1). However, whether salidroside also plays a crucial role on apoptosis of poorly differentiated thyroid cancer cells is still to be determined. To evaluate whether salidroside plays a role in regulating apoptosis in WRO cells, an Annexin V-fluorescein isothiocyanate and propidium iodide detection kit was used to show the apoptotic rate and western blot was used.
to display expression changes of apoptosis-related proteins, Bax, cleaved caspase 3 and Bcl-2. As shown in Figure 5a, the apoptotic rate in the control group was 4.37 ± 0.86%, after treatment with 10, 20 and 40 μg/mL salidroside, 4.47 ± 0.80%, 4.63 ± 0.95%, 4.9 ± 0.62% induction of apoptosis were observed in Nthy-ori 3-1 cells, respectively \((P > 0.05)\). The results showed that salidroside had no toxicity to normal thyroid cells as to poorly differentiated thyroid cancer WRO cells. The apoptotic rates of WRO cells were significantly increased compared to that in the control group \((P < 0.05, \text{Fig 5a})\). The apoptotic rate in the control group was 5.90 ± 0.60%, after treatment with 10, 20 and 40 μg/mL salidroside, 7.97 ± 0.74%, 10.53 ± 0.32%, 11.73 ± 0.40% induction of apoptosis were observed in WRO cells, respectively \((P < 0.05)\). As shown in Figure 5c, salidroside significantly increased expressions of the pro-apoptotic proteins Bax and cleaved caspase 3 and decreased expression of the anti-apoptotic protein Bcl-2 at protein levels. Similarly, maximum inhibition of proteins was achieved at 40 μg/mL salidroside, which upregulated expression of Bax and cleaved caspase 3 protein by 0.99-fold and 1.06-fold compared with the control group, and downregulated expression of Bcl-2 protein by 59.71% compared with the control group \((P < 0.05)\).

**Discussion**

The effects of salidroside on cancer or non-cancer cells have been extensively studied in previous researches, including studies regarding inhibition of cell viability and cell cycle, promotion of apoptosis, inhibition of invasion and metastasis, etc.\(^{17,19,25,30}\) Although there are many...
studies on other cancers, there have been no studies on the effects of salidroside on thyroid cancer. Radioactive iodine treatment is the preferred therapy for postoperative patients with thyroid cancer, but the treatment of poorly differentiated thyroid cancer patients resistant to radioactive iodine is currently in a bottleneck. In order to secure better treatment for patients with poorly differentiated thyroid cancer, discovering new drugs/agents is imperative. In our previous study, salidroside inhibited cell proliferation and elevated both mRNA and protein expressions of NIS in WRO cells, but failed to cause any significant increase in iodide uptake. The present study aimed to elucidate the ability of salidroside to inhibit poorly differentiated thyroid cancer progression.

The basement membrane and extracellular matrix are protective barriers for cancer cells to metastasize. MMPs, the family of metal matrix enzymes function to digest collagen, which makes cancer cells move to other sites, and are often found upregulated during cancer progression. MMP9 primarily degrades collagen type IV, the main component of the basement membrane. MMP2 is associated with cancer invasion. Figure 2 indicates that intracellular expression of MMP2 and MMP9 at mRNA and protein levels was repressed by salidroside. The present results are consistent with salidroside significantly suppressing expressions of MMP2 and MMP9 mRNA and protein in breast cancer cells. Further, the results of wound-healing assays suggest that salidroside inhibited WRO cell migration. In order to confirm this effect, transwell migration assay and Matrigel invasion assay were performed, and the results clearly indicated that salidroside had a crucial role in the migration and invasion process.

It is well known that the JAK2-STAT3 signaling pathway plays an important role in cancer progression, including cell migration and invasion. For example, the overexpression of STAT3 and its phosphorylation promote cancer development and are associated with poor prognosis in gastric cancer. In addition, the JAK2-STAT3 pathway is activated and implicated in colon cancer, breast cancer, hepatocellular carcinoma, osteosarcoma, melanoma, and so on. It has also been demonstrated that MMP2 and MMP9 are linked to the STAT3 pathway. The present study also observed phosphorylation activation of the JAK2-STAT3 signaling pathway in WRO cells and
Salidroside decreased expression of pJAK2 (Y1007+Y1008) and pSTAT3 (Tyr705). The above results suggested that salidroside may exert anti-tumor effects by inhibition of the JAK2-STAT3 pathway.

Salidroside also promoted apoptosis of poorly differentiated thyroid cancer cells. In particular, 40 μg/mL salidroside killed almost 14% of WRO cells. These results strongly suggested that salidroside may be considered a good candidate as an anticancer drug for poorly differentiated thyroid cancer.

In summary, the present data indicated that salidroside inhibited poorly differentiated thyroid cancer cell migration and invasion, which may be associated with inhibition of JAK2-STAT3 signaling. Our data suggest that salidroside may provide a promising therapeutic for patients with poorly differentiated thyroid cancer.

**Figure 5** Salidroside induced apoptosis in poorly differentiated thyroid cancer WRO cells. (a) The apoptotic cells were detected by Annexin V-FITC and PI staining using flow cytometry with normal thyroid follicular epithelial Nthy-ori 3-1 cells and poorly differentiated thyroid cancer WRO cells. The percentage of apoptotic cells is shown as the mean ± SD above the panels. (b) The histogram below shows the results of the quantitative analysis. Data are shown as the mean ± SD of three experiments. 
*P < 0.05 versus control. (c) The protein expression of Bax, cleaved caspase 3 and Bcl-2 was detected by western blot. (d) The histogram shows the results of the quantitative analysis of changes in Bax, cleaved caspase 3 and Bcl-2 protein expression. Data are shown as the mean ± SD of three experiments. 
*P < 0.05 versus control.

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**Disclosure**

The authors declare no potential conflicts of interest.

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Salidroside inhibits migration and invasion

H. Shang et al.

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