Biotherapeutic Effects of Salidroside on Gastric Carcinoma Cells

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Abstract. Salidroside is verified to have tumor inhibitory effects. Nevertheless, the mechanisms are not clarified. The research aims to explore the tumor inhibitory mechanisms of salidroside on gastric carcinoma. The tumor inhibitory effects were examined by the cell viability assay. Flow cytometric analysis was adopted to estimate the cancer cells apoptosis. The expressions of differentiated embryo-chondrocyte expressed gene 1 (DEC1), hypoxia-inducible factor-1α (HIF-1α) and signal transducer and activator of transcription 3 (STAT3) in carcinoma cells were measured by immunohistochemistry assay. Salidroside displayed definite cytotoxicity effects on gastric carcinoma cells in a dose and time dependent manner. Apoptotic percent in carcinoma cells treated with salidroside were significantly increased (P < 0.05). The migrations of cancer cells were suppressed with the increasing doses of salidroside. The expressions of HIF-1α, DEC1 and STAT3 in cancer cells with salidroside treatment were down-regulated. The findings suggested that salidroside suppressed the growth of gastric cancer cells by eliciting the cancer cells apoptosis and lowering the expressions of HIF-1α, DEC1 and STAT3 signal molecules.

1. Introduction
Gastric carcinoma is the general gastrointestinal cancers around the global [1,2] The traditional therapeutic strategies for gastric cancer contains surgical therapy, drug therapy and radiotherapy, nevertheless it still has a poor prognosis [1-3]. So it is essential to pursuit more effective agents against gastrocarcinoma cells. A large number of food derivatives presented specific tumor inhibitory characteristics and many studies verified that food derivatives have been supposed to be effective agents against tumors [4,5]. So, inhibiting the proliferation of cancer cells with herb derivatives has increasingly turned into an important area in recent years [4-7].

Salidroside, is generally known as a traditional herb around the world, which was verified to own certain biological effects, for example, anti-inflammatory and tumor inhibitory activities [8,9]. HIF-1α was confirmed to take part in transcripting some targeted genes, such as erythropoietin (EPO), glucose transporter 1 (GLUT-1) gene, vascular endothelial growth factor (VEGF) and stimulate some signaling molecules that result in cancer cell growth and progression [10]. Researches showed that breast cancer, renal cell carcinoma, colon cancer and gastric cancer highly expressed HIF-1α [11]. Studies indicated that over-expression of HIF-1α was correlated with the development of some cancers [12,13].
DEC1 is referred as an essential transcription factor, which could promote cell proliferation through binding the DNA encoding sites [14]. Studies suggested that the over-expression of HIF-1α and DEC1 in cancers could promote the proliferation, which is beneficial to the development of carcinoma [15]. The over-expression of DEC1 in gastric cancers is linked with worse prognosis [14-16]. Janus kinase/signal transducer and activator of transcription pathways (JAK/STATs) are significantly correlated with cell apoptosis, proliferation and differentiation, which could lead to dysplasia and malignant transformation [17]. Research proved that over-expression of STAT3 in carcinoma was intimately correlated with metastasis and tumor grades [18]. Tumor vascularization is the foundation of invasion and spread.

In view of the researches, it is indicated that over-expression of HIF-1α in gastric cancer was probably related with DEC1 and STAT3. The three molecules constituted the HIF-1α/DEC1/STAT3 signal pathways, which exerted important roles in the progression of gastric cancer.

In this research, we explored the mechanisms of salidroside on the growth of gastric carcinoma and determined whether salidroside can intervene the HIF-1α/DEC1/STAT3 molecules, and bring about the apoptosis, further affirming the multiple tumor inhibitory effects of salidroside.

2. Materials and Methods

2.1. Reagents
Gastric carcinomas cells (BGC-823) were gifted from Laboratory of Molecular Center, Xi’an Jiaotong University. Cells were cultivated in RPMI 1640 medium (Gibco-BRL, USA) with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% newborn bovine serum (Thermo Fisher Scientific Inc., USA).

Rabbit anti-human HIF-1α, DEC1 and STAT3 antibodies were provided by Santa Cruz Company (CA, USA). Secondary EnVisionTM kit was provided by Dako Company (Carpinteria, CA, USA).

2.2. Salidroside Solution
Salidroside was provided by Sigma Company (St. Louis, Mo, USA) and dissolved in normal saline (NS), kept at -20°C. It is the storage solution and may be diluted in normal saline.

2.3. Cell Viability Examination
Gastric carcinoma cells (4x10⁶) were cultivated in 96-well plates. After cultured for overnight, cancer cells were cultured with different doses of salidroside for 6h, 12h, 24h, 48h and 72h. The cytotoxicity of salidroside on cancer cells was analyzed in the concentrations of 10μmol, 20μmol, 30μmol, 40μmol, 50μmol, 60μmol and 80μmol by the LDH release assay kit (Promega, USA). Lysis rate was summarized based on the formula: lysis % = (experimental OD value − effector OD value − target spontaneous OD value)/(target maximum OD value − target spontaneous OD value) × 100%. The results repeated three times are representative.

2.4. Apoptosis Detection
Gastric carcinoma cells were detached and the cells was diluted to 1×10⁶ /ml, cultured with salidroside in the concentrations of 20μmol, 40μmol and 60μmol for 48h. Then the cells were obtained to evaluate the apoptotic rates.

To detect the apoptosis percentage, apoptotic cells were analyzed via flow cytometry. Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining were applied to examine the apoptosis. 1x10⁶ cells from the samples were pretreated with ribonuclease A (RNase), then stained with annexin V-FITC and PI. The apoptotic cells were examined and the collected data were analyzed with CELLQUEST and ModFIT LT software (Becton Dickinson, USA).

2.5. Immunohistochemistry
The slides of cancer cells were fixed with 70% alcohol for 30 min, then rinsed with PBS. Then 3mL/L H₂O₂ was used to block endogenous peroxidase at room temperature for 30 min. The slides were co-cultured with STAT3, HIF-1α or DEC1 polyclonal antibody (1:100) respectively at 4°C for 12h. After rinsing, the slides were cultivated with HRP labeled goat anti-rabbit antibody (1:100) at 37°C for 30 min. Then the slides were stained with fresh 0.5g/L 3,3’-diaminobenzidine solution (DAB) for 5 min. Normal human albumin as a negative control was used to block endogenous peroxidase. After rinsing, the slides were incubated with diaminobenzidine solution (H₂O₂: DAB=1:1) for 3 min. Then the slides were counterstained with hematoxylin for 30 s. The slides were rinsed in tap water for 5 min, dehydrated in 100% alcohol for 3 min, then immersed in 100% alcohol for 1 min, air dried for 5 min, and placed on glass slides.

The slides were dehydrated in 3-methyl-butanol and xylool for 3 min each. The slides were then immersed in paraffin for 1 h at 56°C. Then the slides were cut into 3 μm sections and mounted on gelatin-coated glass slides. The sections were allowed to cool to room temperature. After drying in room temperature for 24 h, the sections were deparaffinized and rehydrated in a graded series of alcohol (100%, 95%, 75% and 50% for 5 min each) followed by water (two times for 5 min each).

2.6. Statistical Analysis
The statistical analysis were carried out with the SPSS 13.0 statistical software. The Student’s t test was applied to analyze the quantitative data and the chi-square test was used for the qualitative data in different groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Salidroside Inhibited the Proliferation of Gastric Carcinoma Cells
To confirm the proliferation inhibition of salidroside on gastric cancer, we examined the cell viability. Cancer cell proliferation was significantly inhibited after different concentrations of salidroside treatment. The inhibitory rates of gastric cancer cells were elevated with the increased concentrations of salidroside and the cultivating time extension (the inhibitory rates in the concentrations of 10μmol, 20μmol, 30μmol, 40μmol, 50μmol 60μmol and 80μmol salidroside treatment groups for 24h, 4.73±2.35, 25.46±3.12, 32.77±2.34, 36.28±2.43, 41.84±3.12, 56.81±2.36, 63.47±2.65, P < 0.05). Our results indicated that the proliferation suppressing effect of salidroside on cancer cells was dose and time-dependent, and the differences between the groups treated with salidroside and the control group were significant.

3.2. Salidroside Elicited the Cancer Cells Apoptosis
Compared with the control group, after treated with different dosages of salidroside, carcinoma cells exhibited increased apoptotic percentage in the concentrations of 20μmol, 40μmol and 60μmol salidroside treatment groups, from 18.3%, 25.6%, 87.9% vs 1.3%, (P < 0.05, Table 1).

| Treatment          | Apoptotic percentage (%) |
|--------------------|--------------------------|
| 60μmol salidroside | 87.9a                    |
| 40μmol salidroside | 25.6b                    |
| 20μmol salidroside | 18.3c                    |
| Saline             | 1.3                      |

*P<0.05, *P<0.05, *P<0.05 vs control group.

3.3. Salidroside Suppressed the Expressions of HIF-1α, DEC1 and STAT3
To verify whether the expressions of HIF-1α, DEC1 and STAT3 signaling pathways in cancer cells were influenced by salidroside treatment, we detected and analyzed the expressions of the signal pathways via immunostaining. Most of molecules were located in the cell nucleus. The OD values of the three molecules in the salidroside treatment groups were much lower than those from the control group (Table 2).
Table 2. Expressions of DEC1, STAT3 and HIF-1α in gastric cancers (x±s).

| Treatment            | Optical density (OD) |
|----------------------|----------------------|
|                      | HIF-1α (x±s)        | STAT3 (x±s) | DEC1 (x±s) |
| 60μmol salidroside   | 0.039±0.013<sup>a</sup> | 0.038±0.015<sup>a</sup> | 0.045±0.012<sup>a</sup> |
| 40μmol salidroside   | 0.072±0.025<sup>b</sup> | 0.096±0.033<sup>b</sup> | 0.093±0.023<sup>b</sup> |
| 20μmol salidroside   | 0.120±0.054<sup>c</sup> | 0.139±0.076<sup>c</sup> | 0.121±0.064<sup>c</sup> |
| Saline               | 0.173±0.057         | 0.198±0.068 | 0.282±0.087 |

<sup>a</sup><sup>P<0.05</sup>, <sup>b</sup><sup>P<0.05</sup>, <sup>c</sup><sup>P<0.05</sup> vs control group.

4. Discussion
It is proved that salidroside, the derivative of curcuma longa can inhibit the growth of some cancers through arresting cell cycle, inducing apoptosis and affecting certain signal pathways [7-9]. Because it has multiple functions and less toxicity [19]. So, we speculated that salidroside should possess multiple functions on gastric carcinomas and it is important to carry out a deep investigation to clarify the cancer inhibitory mechanisms for salidroside. In this research, we found that salidroside suppressed the proliferation of gastric cancer. The results verified that the time and dose-dependent cancer inhibitory functions of salidroside were brought out through eliciting the cancer cells apoptosis and inhibiting the growth of cancer cells. It is further affirmed that salidroside could decrease the expressions of HIF-1α/DEC1/STAT3 signal pathways.

Cancer cell proliferation was suppressed significantly by the treatment with different dosages of salidroside. The inhibitory rates of gastric cancer cells were elevated with the higher concentrations of salidroside and the culturing time extension. The results suggested that the proliferation inhibitory effect on cancer cells of salidroside was dose and time-dependent, and the differences among salidroside treatment groups were significant.

After cultivated with salidroside, the cancer cells showed significantly increased apoptotic percentage with the elevating concentrations of salidroside. Like other cancers, gastric carcinoma also exists cell progression heterogeneity and abnormal apoptosis [20]. Inducing apoptosis in cancer cells can be found after some chemotherapy drugs and herbs treatment [21,22]. These researches indicate that the induction of cancer cell apoptosis is likely to be an effect mechanism for gastric cancers. Our research suggested that cancer proliferation was significantly suppressed by the treatment with salidroside. Results from analysis of flow cytometry indicated that salidroside was able to increase the cancer cell apoptosis. These results imply that the important mechanisms of suppressing the growth of gastric carcinoma from salidroside is to induce the apoptosis of cancer cells.

To verify whether salidroside intervened the three signaling molecules in gastric carcinoma cells, the expressions of the molecules were detected. Signal pathways exert important functions on the development and progression of cancers [23,24]. HIF-1α takes part in hypoxic processes, and exerts important roles in cancer development. Up-regulation of HIF-1α has been confirmed in a number of gastrointestinal cancers, which is indicated in poor prognosis and drug resistance of cancers [11-13]. The JAK/STAT3 signal pathways display effects on promoting cancer angiogenesis, proliferation and invasion. STAT3 signal pathway is activated to suppress apoptosis, promote proliferation and development of many cancers [14,15]. Researches indicated that HIF-1α could cross-link STAT3, interacting on the targeted genes when they are elicited by cytokines [12,13]. It was affirmed that the up-regulating expressions of DEC1, HIF-1α and STAT3 in cancer cells was likely to promote the development of some cancers [25,26]. In view of the studies, we speculated that over-expression of HIF-1α in gastric carcinoma cells is intimately correlated with DEC1 and STAT3. STAT3, HIF-1α and DEC1 molecules act together and constitute HIF-1α/DEC1/STAT3 signal pathways, which are likely to exert vital functions on the ignition and progression of gastric carcinoma. Few studies affirmed the functions of salidroside on gastric carcinoma cells via HIF-1α/DEC1/STAT3 signal pathways.
pathways. Our research confirmed that salidroside was able to decrease the expressions of HIF-1α/DECI/STAT3 signal pathways in gastric carcinoma cells, further inhibiting the growth and development of cancers.

The study confirmed that salidroside could suppress the growth of gastric carcinoma cells, and the important mechanisms lies in inducing the apoptosis and decreasing of the expressions of HIF-1α/DECI/STAT3 signal pathways. The results suggested that salidroside plays the multiple inhibiting effects on cancer growth, which laid a basis for later clinical research. Nevertheless, the profound anti-tumor mechanisms of salidroside still need further investigation.

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