Gypenosides Inhibited Invasion and Migration of Human Tongue Cancer SCC4 Cells through Down-regulation of NFκB and Matrix Metalloproteinase-9

KUNG-WEN LU1, MING-LI TSAI1, JUNG-CHOU CHEN1, SHU-CHUN HSU2, TE-CHUN HSIA3, MENG-WEI LIN8, AN-CHENG HUANG4, YUNG-HSIEN CHANG5, SIU-WAN Ip6, HSU-FENG LU7 and JING-GUNG CHUNG8,9

1Chinese Medical Research Institute, 2Graduate Institute of Chinese Pharmaceutical Sciences, Departments of 6Nutrition, and 8Biological Science and Technology, China Medical University, Taichung, Taiwan; 3Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan; 4Department of Pharmacy, Han-Ming Hospital, Changhua, Taiwan; 5Department of Clinical Pathology, Chueh Hsin Rehabilitation Medical Center, Taipei, Taiwan; 6Department of Biotechnology, Asia University, Taichung Hsien, Taiwan, R.O.C.

Abstract. Gypenosides (Gyp), components of Gynostemma pentaphyllum Makino, were found to induce suppression of human tongue squamous cell carcinoma SCC4 cell growth and induce apoptosis in response to overexpression of reactive oxygen species, calcium (Ca$^{2+}$) and to decrease mitochondrial membrane potential in vitro. In this study, the effect of Gyp on cell migration and invasion of human tongue SCC4 cells was examined. SCC4 cells treated in vitro with Gyp migrated and invaded less than cells treated with phosphate-buffered saline (PBS) as a control. Gyp inhibited migration and invasion by down-regulating the production of RAS, NFκB, COX2, ERK1/2 and MMP-9 relative to PBS only. These results show that Gyp inhibits invasion and migration of human tongue SCC4 cells by down-regulating proteins associated with these processes, resulting in reduced metastasis.

Gypenosides (Gyp) are the major components in extracts from Gynostemma pentaphyllum Makino, a popular folk medicine in the Chinese population. Gyp had been used for treating hepatitis (1), hyperlipoproteinemia (2, 3), cardiovascular disease (4) and cancer (5). Gyp has also anti-inflammatory (6), antithrombotic (7), antioxidative (8) and anticancer activities (9-12), and was found to inhibit N-acetyltransferase activity and gene expression (13), and heart and brain microsomal Na(+) and K(+)-ATPase activities in rats (14). Recently, we have found that Gyp induced cell cycle arrest and apoptosis in human tongue squamous cell carcinoma SCC4 cells (15). There is no available information to address the effects of Gyp on the invasion and migration of human tongue cancer cells. Therefore, the human tongue SCC4 cell line was selected for examining the mechanisms underlying invasion and migration, with particular focus on the RAS, NFκB, COX2, MMP-2 and MMP-9.

Materials and Methods

Chemicals and reagents. Gyp was extracted from Gynostemma pentaphyllum Makino as described elsewhere (16). Dimethyl sulfoxide (DMSO), potassium phosphates, propidium iodide (PI), ribonuclease-A, Tris-HCl, trypan blue and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TE buffer was purchased from Merek Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM/F12), glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human oral squamous carcinoma cells (SCC4). The human oral squamous cell carcinoma SCC4 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured at 37 °C under a humidified 5% CO$_2$ and 95% air atmosphere in DMEM/F12 containing 10% fetal calf serum (FCS) in 75 cm$^2$ tissue culture flasks with 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine as described previously (17).
Effects of Gyp on the migration of SCC4 cells in vitro. Approximately $5 \times 10^4$ SCC4 cells/ml were plated in 6-well plates for 24 h, then the cells in individual wells were wounded by scratching with a pipette tip and the cells were incubated with DMEM/F12 medium containing no FBS and treated with or without Gyp (60, 90, 120, 150 or 180 µg/ml) for 48 h. The cells were photographed under phase-contrast microscopy (x100 magnification).

Effects of Gyp on the invasion of SCC4 cells in vitro. The in vitro invasion assay was carried out following the method of Huang et al. (11) and Hsu et al. (17). Twenty-four-well Transwell inserts with 8 µm porosity polycarbonate filters (Millipore, Billerica, MA, USA) were re-coated with 30 µg Englebreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel Basement Membrane Matrix) at room temperature for 1 h to form a genuine reconstituted basement membrane. The SCC4 cells ($10^4$ cells/0.4 ml DMEM/F12) were placed in the upper compartment and incubated with vehicle or Gyp (60 or 120 µg/ml). The plates were then incubated at 37°C for 48 h in a humidified atmosphere with 95% air and 5% CO₂. The cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and stained with 2% crystal violet. The cells on the upper surface of the filter were removed by wiping with a cotton swab, and the cells that penetrated through the matrigel to the lower surface of the filter were counted under a light microscope at x200 magnification (16). Each treatment was assayed in duplicate, and three independent experiments were carried out (17).

Effects of Gyp on the expressions of SOS, RAS, NFκB, iNOS, COX2, MMP-2, MMP-9 and ERK1/2 from SCC4 cells. Approximately $5 \times 10^6$ SCC4 cells/ml were treated with 150 µg/ml Gyp for 0, 6, 12, 24, 48 and 72 h, before isolating the cells to detect the proteins associated with invasion and migration (SOS, RAS, NFκB, iNOS, COX2, MMP-2, MMP-9 and ERK1/2). Isolated cells with or without Gyp treatment were lysed and the protein levels quantified. All samples were separated using sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (17-19).
Results

Effects of Gyp on the migration of SCC4 cells in vitro. The effects of Gyp on cell migration was investigated using the wound-healing assay (Figure 1). Higher Gyp concentrations and longer incubation time led to greater inhibition of cell migration in SCC4 cells. These effects were dose- and time-dependent.

Effects of Gyp on the invasion of SCC4 cells in vitro. The results shown in Figure 2A indicate that SCC4 cells invaded from the upper to the lower chamber in the absence of Gyp (control group). Penetration of the EHS-coated filter by SCC4 cells was inhibited in the presence of Gyp. The effect was higher at 120 μg/ml than at 60 μg/ml. The quantification of cells in the lower chamber showed that Gyp significantly inhibited SCC4 cell invasion (Figure 2B) and this effect was dose-dependent.

Effects of Gyp on the expressions of SOS, RAS, NFκB, iNOS, COX2, MMP-2, MMP-9 and ERK1/2 by SCC4 cells. To understand the molecular mechanism of Gyp-inhibited invasion and migration of the SCC4 cells, the expressions of invasion- and migration-associated proteins during treatment with Gyp were examined by Western blotting. The results indicated that the levels of SOS, NFκB and MMP-9 were lower than the corresponding control (Figure 4). These effects may have led to inhibition of invasion and migration.

Discussion

Many patients retain residual disease after surgery and this can eventually lead to metastasis. Therefore, the prevention of cancer metastasis is as important target for improving a patient’s prognosis. In our previous studies, we had found that Gyp inhibited growth and induced apoptosis of hepatocellular carcinoma (HCC) cells in a dose- and time-dependent manner (15). However, there is no available information to address the effects of Gyp on the invasion and migration of cancer cells. In the present study, we investigated whether Gyp inhibited the invasion and migration of human tongue SCC4 cells. We also confirmed that Gyp inhibited the proliferation of SCC4 cells by using the MTT assay (data not shown). Our results from Western blotting showed that Gyp inhibited the invasion and migration of human tongue SCC4 cells. It is well-known that NFκB, iNOS and COX2 (Figure 3) are associated with the proliferation of tumor cells (20-22) and their inhibition can lead to proliferation of tumor cells. Our results also showed that Gyp inhibited the levels of MMP-9 which are associated with the cell invasion and migration and those observations may explain the reason why Gyp inhibited the migration and invasion (Figures 1 and 2). It has been reported that cancer cell-matrix interaction is a critical step in the promotion of cell migration (20, 21) and proteolytic degradation of the extracellular matrix (ECM) is a critical event during tumor invasion and metastasis. MMP-2 and MMP-9, and gelatinases, are involved in metastasis (23). The results from Western blotting indicate that Gyp affected RAS and NFκB which may led to the inhibition of MMP-9. The reason for examining the effects of Gyp on MMP-9 is that both enzymes are recognized as important targets for the development of anticancer drugs and both enzymes are also associated with aggressive, advanced, invasive or metastatic tumor phenotype (24). Our data also showed that Gyp inhibited the levels of RAS and ERK1/2 in SCC4 cells. It has also been reported that RAS and ERK are involved in cell invasion and migration (25).

In this study Gyp inhibition of invasion and metastasis-associated proteases in a human tongue SCC4 cell line is reported and the possible signal pathway is shown in Figure 4. This study also provides additional information on the anti-metastatic potential of Gyp beyond its antitumor activity.
Figure 3. continued
Lu et al: Gypenoside inhibited Cell Invasion and Migration of SCC4 Cells

Figure 3. continued

E

<table>
<thead>
<tr>
<th>Gypenosides (150 µg/mL)</th>
<th>iNOS</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 6 12 24 48 72 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

iNOS

![iNOS Bar Graph](chart)

F

<table>
<thead>
<tr>
<th>Gypenosides (150 µg/mL)</th>
<th>COX2</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 6 12 24 48 72 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

COX2

![COX2 Bar Graph](chart)

G

<table>
<thead>
<tr>
<th>Gypenosides (150 µg/mL)</th>
<th>MMP-2</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 6 12 24 48 72 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MMP-2

![MMP-2 Bar Graph](chart)

H

<table>
<thead>
<tr>
<th>Gypenosides (150 µg/mL)</th>
<th>MMP-9 active form</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 6 12 24 48 72 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MMP-9

![MMP-9 Bar Graph](chart)
References


