Abstract. Gypenosides (Gyp), the primary components of Gynostemma pentaphyllum Makino, have long been used as a Chinese herbal medicine. In the present study, the effects of Gyp on cell viability, the cell cycle, cell apoptosis, DNA damage and chromatin condensation were investigated in vitro using human oral cancer HSC-3 cells. The results of the present study indicated that Gyp induces cell death, G2/M phase arrest and apoptosis in HSC-3 cells in a dose-dependent manner. It was also demonstrated that Gyp decreased the depolarization of mitochondrial membrane potential in a time-dependent manner. A cDNA microarray assay was performed and the results indicated that a number of genes were upregulated following Gyp treatment. The greatest increase was a 75.42-fold increase in the expression of GTP binding protein in skeletal muscle. Levels of the following proteins were also increased by Gyp: Serpine peptidase inhibitor, clade E, member 1 by 20.25-fold; ras homolog family member B by 18.04-fold, kelch repeat and BTB domain containing 8 by 15.22-fold; interleukin 11 by 14.96-fold; activating transcription factor 3 by 14.49-fold; cytochrome P450, family 1 by 14.44-fold; ADP-ribosylation factor-like 14 by 13.88-fold; transfer RNA selenocysteine 2 by 13.23-fold; and syntaxin 11 by 13.08-fold. However, the following genes were downregulated by GYP: Six-transmembrane epithelial antigen of prostate family member 4, 14.19-fold; γ-aminobutyric acid A receptor by 14.58-fold; transcriptional-regulating factor 1 by 14.69-fold; serpin peptidase inhibitor, clade B, member 13 by 14.71-fold; apolipoprotein L 1 by 14.85-fold; follistatin by 15.22-fold; uncharacterized LOC100506718; fibronectin leucine rich transmembrane protein 2 by 15.61-fold; microRNA 205 by 16.38-fold; neuregulin 1 by 19.69-fold; and G protein-coupled receptor 110 by 22.05-fold. These changes in gene expression illustrate the effects of Gyp at the genetic level and identify potential targets for oral cancer therapy.

Introduction

Oral cancer is a major cause of cancer-associated mortality in humans worldwide and the fifth most prevalent cause of cancer-associated mortality in Taiwanese males (1). Chewing the betel nut, a popular practice in Taiwan, has been recognized as a major factor contributing to oral cancer development (2). The current treatments available for oral cancer include surgery, radiotherapy, chemotherapy and a combination of radiotherapy and chemotherapy (3); however, these therapies induce numerous side effects. Investigators have therefore shifted their attention to developing chemotherapeutic agents derived from natural sources. At present, plant-derived anti-cancer drugs clinically used to treat patients with cancer
include Taxol<sup>®</sup> (paclitaxel) and Taxol derivatives, which are synthesized from Taxus brevifolia (4,5).

The primary components of Gypenosides (Gyp) are extracted from Gynostemma pentaphyllum (Thunb.) Makino (Cucurbitaceae). This plant has been used as a traditional Chinese medicine for many years and has been found to exhibit biological activities including antioxidant effects, prevention of cardiovascular disease and antitumor activity (6,7). Numerous studies have reported that Gyp treatment exhibits positive effects in the treatment of cardiovascular disease (8), hypolipoproteinemia (9,10), hepatitis (11) and cancer (12). Furthermore, it has been demonstrated that Gyp induces cell death and apoptosis in human hepatoma Hep3B (7) and Huh7 (13) cells, prostate cancer PC-3 cells (14), tongue cancer SCC4 cells (15) and murine leukemia WEHI-3 cells (16). It has been reported that Gyp induces cardiotoxic and central inhibitory effects in rats and functions by inhibiting the microsomal Na+ and K+-ATPase activities of the heart and brain (17). Furthermore, Gyp induces cell apoptosis via mitochondria-dependent pathways and the activation of caspase-3 in human colon cancer cells (18).

Recently, it was reported that Gyp induces cell cycle arrest and apoptosis in human liver cancer A549 cells, most likely via the p53-independent pathway(s) (19). A number of studies have identified the potential pathway by which Gyp induces cytotoxic effects on cancer cells; however, the molecular mechanisms underlying its anti-cancer activity remain unclear. Furthermore, to the best of our knowledge, there have been no studies investigating the effects of Gyp on human oral cancer cells. Thus, the aim of the present study was to investigate the effects of Gyp on human oral cancer HSC-3 cells <em>in vitro</em> and the mechanisms underlying the association between the induction of cell cycle arrest and apoptosis with gene expression.

**Materials and methods**

**Chemicals, reagents and cell culture.** Gyp was extracted from Gynostemma pentaphyllum Makino that was provided by Professor Jung-Chou Chen (China Medical University, Taichung, Taiwan) as described previously (18). Dimethyl sulfoxide (DMSO), Tris-HCl, propidium iodide (PI), trypan blue, Triton X-100, ribonuclease-A, penicillin-streptomycin and trypsin-EDTA were all purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). DiOC<sub>6</sub> and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc.

**Cell culture.** The human oral squamous cell carcinoma HSC-3 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin) in 75T tissue culture flasks, dispensed into new flasks every 2 to 3 days and all cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, as described previously (20-22).

**Assessment of viability.** HSC-3 cells (5x10<sup>4</sup> cells/well) were maintained in 12-well plates for 24 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and subsequently incubated with 0, 60, 90, 120, 150 or 180 µg/ml Gyp for 12, 24, 48 or 72 h. Control cells were treated with DMSO alone. Following incubation, cells were harvested and stained with propidium iodide (PI, 5 µg/ml). Cell viability was assessed using CellQuest<sup>®</sup> (version 5.2.1; BD Biosciences, San Jose, CA, USA) and flow cytometry (BD Biosciences) following a previously described protocol (20,21).

**Cell cycle and sub-G1 examined by flow cytometry.** HSC-3 cells (5x10<sup>4</sup> cells/well) in the 12-well plate were incubated with 0 or 120 µg/ml Gyp for 6, 12, 24, 48 and 72 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and subsequently collected for cell cycle distribution assays. The percentage of cells in the sub-G1 (apoptosis), G0/G1, S- and G2/M phases were measured using ModFit LT software (version 3.0; BD Biosciences) and flow cytometry (BD Biosciences), as described previously (20,21,23).

**DAPI staining.** HSC-3 cells (5x10<sup>4</sup> cells/well) in a 12-well plate were treated with 0, 60, 90, 120, 150 or 180 µg/ml Gyp at 37°C for 24 h, stained with DAPI (37°C, 15 min) and assessed using fluorescence microscopy as described previously (20,21).

**Comet assay.** HSC-3 cells (5x10<sup>4</sup> cells/well) were treated with 0, 60, 90, 120, 150 or 180 µg/ml Gyp at 37°C for 24 h and subsequently harvested to examine the DNA damage with the Comet assay kit (Trengiven, Inc., Gaithersburg, MD, USA) as described previously (20,21).

**Detection of mitochondrial membrane potential (ΔΨ<sub>m</sub>).** HSC-3 cells (5x10<sup>4</sup> cells/well) were treated with 120 µg/ml Gyp at 37°C for 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h. Cells were collected from each treatment, washed twice with PBS, re-suspended in 500 µl DiOC<sub>6</sub> (4 mol/l) and incubated at 37°C for 30 min. Levels of ΔΨ<sub>m</sub> were assessed using CellQuest<sup>®</sup> (version 5.2.1; BD Biosciences) by flow cytometry (BD Biosciences) as described previously (20,21).

**cDNA microarray assay used for gene expression in HSC-3 cells following exposure to Gyp.** HSC-3 cells (5x10<sup>4</sup> cells/well) were maintained in a 12-well culture plate in DMEM medium for 24 h and subsequently incubated with 0 or 120 µg/ml Gyp for 24 h at 37°C. Following incubation, cells were collected from the control and Gyp treated-groups and total RNA was extracted using the Qiagen RNAeasy Mini kit (P/N 74104; Qiagen Inc., Valencia, CA, USA) and quantity and purity were assessed at 260 and 280 nm using a spectrophotometer (Nanodrop 1000; Thermo Fisher Scientific, Inc.) (24). Total RNA was used to perform cDNA reverse transcription, synthesis, amplification, fragmentation and terminal labeling with the GeneChip WT Sense Target Labeling and Control reagents (Qiagen, Inc.). Labeling and microarray hybridization were performed on the chip (Affymetrix GeneChip Human Gene 1.0 ST array; Affymetrix, Inc., Santa Clara, CA, USA) as previously described (24). The resulting localized concentrations of fluorescent molecules on the chip were further detected and quantified using an
Affymetrix GeneChip® Scanner 3000 (Affymetrix; Thermo Fisher Scientific, Inc.). The data were further analyzed using Expression Console software version 1.1.2 (Affymetrix; Thermo Fisher Scientific, Inc.) with default RMA parameters (24). Upregulated and downregulated gene expression in HSC-3 cells following exposure to Gyp were examined and changes of ≥2-fold were recorded, with + signifying upregulation and - signifying downregulation.

GeneGo analysis. The list containing the 2,992 unique Gyp, complete with Affymetrix transcript identifiers, was uploaded onto GeneGo MetaCore™ software (version 5.0; GeneGo, Inc., St. Joseph, MI, USA). GeneGo recognizes the Affymetrix identifiers and maps the Gyp to the MetaCore™ data analysis suite, generating maps to describe common pathways or molecular connections between Gyp on the list. Graphical representations of the molecular relationships between genes were generated using the GeneGo pathway analysis (24).

Statistical analysis. All results are expressed as the mean ± standard deviation. Statistical analysis was performed using an unpaired Student’s t-test and SigmaPlot version 10.0 (Systat Software, Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Gyp affects the viability of HSC-3 cells. Following incubation with various concentrations of Gyp (0, 60, 90, 120, 150 and 180 µg/ml) for 12, 24, 48 and 72 h, HSC-3 cells were collected for PI staining and to measure cell viability using flow cytometry. The results indicated that cell viability decreased in a time- and dose-dependent manner compared with control (untreated) cells (Fig. 1).

Gyp affects cell cycle arrest and apoptosis in HSC-3 cells. The results indicated that 48-72 h Gyp treatment (120 µg/ml) induced a decrease in the percentage of cells in the G0/G1 (enhanced G0/G1 peak) and S-phases and an increase in the percentage of cells in the G2/M phase (Fig. 2A). Cells treated with 120 µg/ml Gyp for 6-72 h contained a significantly higher percentage of apoptotic cells in the sub-G1 phase compared with the control group (P<0.01; Fig. 2B). Cells in the sub-G1 phase are apoptotic (25); therefore treatment with 120 µg/ml Gyp induced apoptosis in HSC-3 cells.

Gyp induces chromatin condensation in HSC-3 cells. HSC-3 cells were treated with various concentrations of Gyp (0, 60, 90, 120, 150 and 180 µg/ml) for 24 h and stained with DAPI. The results demonstrated that Gyp markedly induced chromatin condensation (cell apoptosis) in HSC-3 cells in a dose dependent manner based on the images obtained via fluorescent microscopy (Fig. 3). Gyp induced nuclear condensation and the incorporation of labeled nucleotide into the DNA, indicating apoptosis, whereas control cells were negative for DAPI staining.
Gyp induces DNA damage in HSC-3 cells. HSC-3 cells were treated with various concentrations of Gyp (0, 60, 90, 120, 150 or 180 µg/ml) for 24 h and DNA damage was assessed using the Comet assay. In cells with damaged DNA, a Comet assay will show longer comet tails (26). The results indicated that Gyp induced marked DNA damage in HSC-3 cells based on
Table I. Number of genes by the fold-change in HSC3 cells treated with gypenosides.

| Fold-change | Number of genes | Total |
|-------------|-----------------|-------|
| ≥20         | 2               | 953   |
| ≥10 and <20 | 18              |       |
| ≥5 and <10  | 62              |       |
| ≥4 and <5   | 56              |       |
| ≥3 and <4   | 139             |       |
| ≥2 and <3   | 676             |       |
| >-3 and ≤-2 | 1,358           | 2,039 |
| >-4 and ≤-3 | 382             |       |
| >-5 and ≤-4 | 143             |       |
| >-10 and ≤-5| 133             |       |
| >-20 and ≤-10| 22             |       |
| ≤-20        | 1               |       |

Figure 5. Gyp induced changes in the mitochondrial membrane potential in human oral cancer HSC-3 cells. Cells were treated with 120 µg/ml Gyp for 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h, collected and stained with DiOC₆. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. C. Gyp, Gypenosides; C, control cells.

Discussion

It has been reported that Gyp has anti-inflammatory (27), antithrombotic (28), antioxidative (27) and anticancer (29-32) properties. Furthermore, Gyp induces apoptosis in human hepatoma cells via the upregulation of Bax and Bcl-2 homologous antagonist/killer, and downregulation of Bcl-2 to induce mitochondrial cytochrome c release and activation of the caspase cascade (31). It has been reported that Gyp induces apoptosis via the mitochondria-dependent pathway in human colon cancer COLO 205 cells (15). Furthermore, Gyp inhibits cell migration in human colon cancer SW620 and esophageal cancer Eca-109 cells (33). A previous study by the present authors demonstrated that Gyp induced G0/G1 arrest via the checkpoint kinase (Chk)2 pathway and induced apoptosis in human tongue cancer SCC-4 cells via endoplasmic reticulum stress and the mitochondria-dependent pathway (15). However, to the best of our knowledge, there have been no studies identifying the effects of Gyp on human oral cancer HSC-3 cells and its effects on gene expression. In the present study, the effects of Gyp on human oral cancer HSC-3 cells were studied and it was demonstrated that Gyp decreased the percentage of viable HSC-3 cells, increased G0/G1 phase arrest and
 decreased the number of HSC-3 cells in the G2/M phase. It was also determined that Gyp induced chromatin condensation and DNA damage in HSC-3 cells and decreased the ΔΨm. These results suggest that Gyp induces cytotoxic effects in human oral cancer HSC-3 cells, which is in accordance with a previous study by the present authors demonstrating that Gyp induced cytotoxic effects in human oral cancer SCC-4 cells in vitro (15). Although a number of studies have demonstrated that Gyp induces cytotoxic effects including cell cycle arrest and apoptosis in human cancer cell lines, to the best of our knowledge, no studies exist determining how Gyp affects gene expression in human oral cancer cells. In the present study, gene expression in human oral HSC-3 cancer cells was examined following exposure to Gyp. The results demonstrated that 953 genes were markedly upregulated and 2039 genes were markedly downregulated.

The highest increase in gene expression observed was 75.42-fold in GEM, while SERPINE1 was increased 20.25-fold, RHOB was increased 18.04-fold, KBTBD8 was increased 15.22-fold, IL11 was increased 14.96-fold, ATF3 was increased 14.49-fold, GEM was increased 13.08-fold, STEAP4 was increased 13.23-fold and STX11 was increased 13.08-fold. However, STEAP4 expression was decreased 14.19-fold, GABRE was decreased 14.58-fold, SERPINB13 was decreased 14.69-fold, TRERF1 was decreased 15.61-fold, MIR205 was decreased 16.38-fold, NRG1 was decreased 19.69-fold and GPR110 was decreased 22.05-fold.

The results of the GeneGo analysis indicated that Gyp affects gene expression in human oral cancer HSC-3 cells in vitro. Gyp affects the expression of genes, such as increasing the expression of insulin-like growth factor-1 receptor, which is highly expressed in cancer (34), increasing plasminogen activator urokinase signaling, which mediates Treg suppressor function via signal transducer and activator of transcription 5 and extracellular signal-related kinase signaling pathways (35), and increasing integrin outside-in signaling (36). It has previously been reported that during outside-in signaling, the binding of intracellular adhesion molecule-1 to lymphocyte function-associated antigen 1 is able to trigger the transmission of signals from the extracellular space into the cytoplasm and alter gene expression and cellular metabolism (37), thus increasing ErbB-family signaling. It has been reported that following ligand binding to the ErbB receptor extracellular domain, signal transduction occurs, promoting hetero- or homo-dimerization amongst family members (38) and stimulating epidermal growth factor (EGF) signaling pathways. Furthermore, it has been determined that activation of EGF receptor by EGF stimulates various signal transduction pathways to induce cell mitogenesis and survival, and also increases Ezrin levels (39,40). Ezrin, Radixin and Moesin have been reported to serve as scaffolds on the actin microfilaments to integral membrane proteins in mammalian cells for signaling molecules to regulate cell migration, proliferation, adhesion, and polarity (41,42). In the present study, Gyp also affected the expression of genes associated with the regulation of the G2/M checkpoint by ATM/ATR. Gyp increased the
expression of p21, cyclin A, cyclin B, breast cancer 1 (BRCA1), nuclear factor with BRCA1 C-terminal domain 1, 14-3-3 and growth arrest and DNA-damage-inducible protein, but reduced the expression of ATM, ataxia telangiectasia and Rad3 related protein, Chk, mitogen-activated protein kinase 14, M-phase inducer phosphatase 3, polo like kinase 3, tumor protein p53, cyclin dependent kinase 1 and Wee1, leading to G2/M arrest.

In conclusion, the present study demonstrated that the expression of genes in HSC-3 cells associated with DNA damage and repair, cell cycle checkpoints, cell proliferation and cell metastasis were affected by Gyp treatment. Identifying which genes were upregulated and which were downregulated provides information about the possible signaling pathways and complex interactions underlying the cytotoxic mechanisms of Gyp at the genetic level.

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