

Gypenosides induce cell death and alter gene expression in human oral cancer HSC-3 cells

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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 down-regulated 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.

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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® (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 *in vitro* 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). DiOC₆ 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% CO₂, as described previously (20-22).

Assessment of viability. HSC-3 cells (5x10⁴ cells/well) were maintained in 12-well plates for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ 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™ (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 (5x10⁴ 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% CO₂ 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 (5x10⁴ 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 (5x10⁴ 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 (Trevigen, Inc., Gaithersburg, MD, USA) as described previously (20,21).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$). HSC-3 cells (5x10⁴ 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 DiOC₆ (4 mol/l) and incubated at 37°C for 30 min. Levels of $\Delta\Psi_m$ were assessed using CellQuest™ (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 (5x10⁴ 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 RNeasy 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

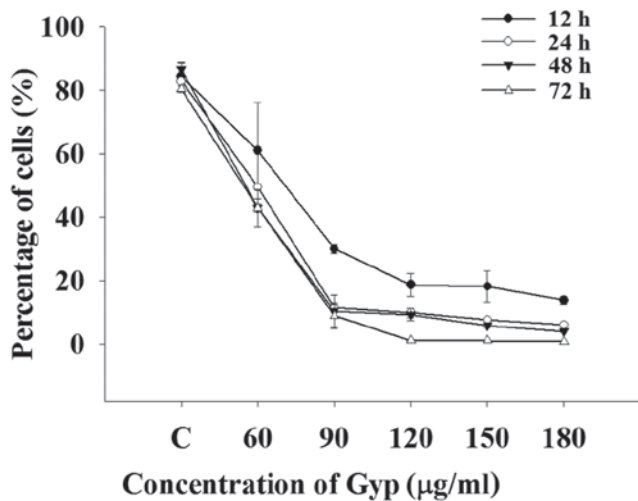


Figure 1. Gyp decreased the percentage of viable cells in human oral cancer HSC-3 cells. Cells were treated with 0, 60, 90, 120, 150 and 180 µg/ml Gyp for 12, 24, 48 and 72 h and cell viability was assessed using flow cytometry. Data are presented as the mean ± standard deviation. Gyp, Gypenosides.

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

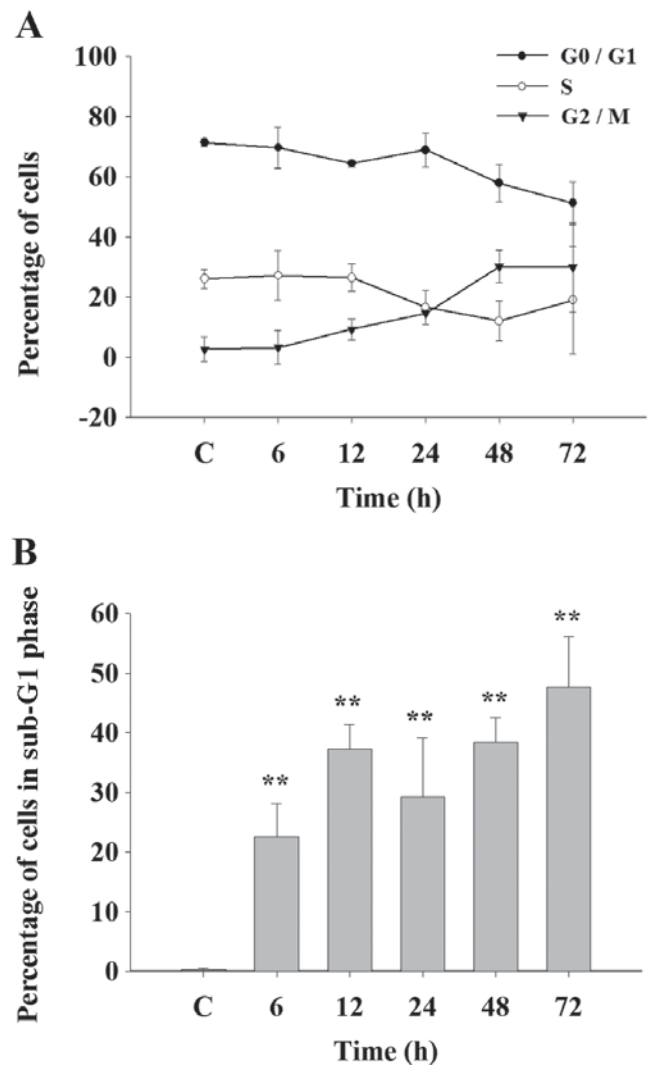


Figure 2. Gyp affects the cell cycle in human oral cancer HSC-3 cells. Cells were exposed to 120 µg/ml Gyp for 0, 6, 12, 24, 48 and 72 h, and subsequently underwent analysis of (A) cell cycle distribution and (B) the proportion of cells in the sub-G1 phase (apoptotic cells) via flow cytometry. Data are presented as the mean ± standard deviation. ** $P < 0.01$ vs. C. Gyp, Gypenosides; C, control cells.

(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.

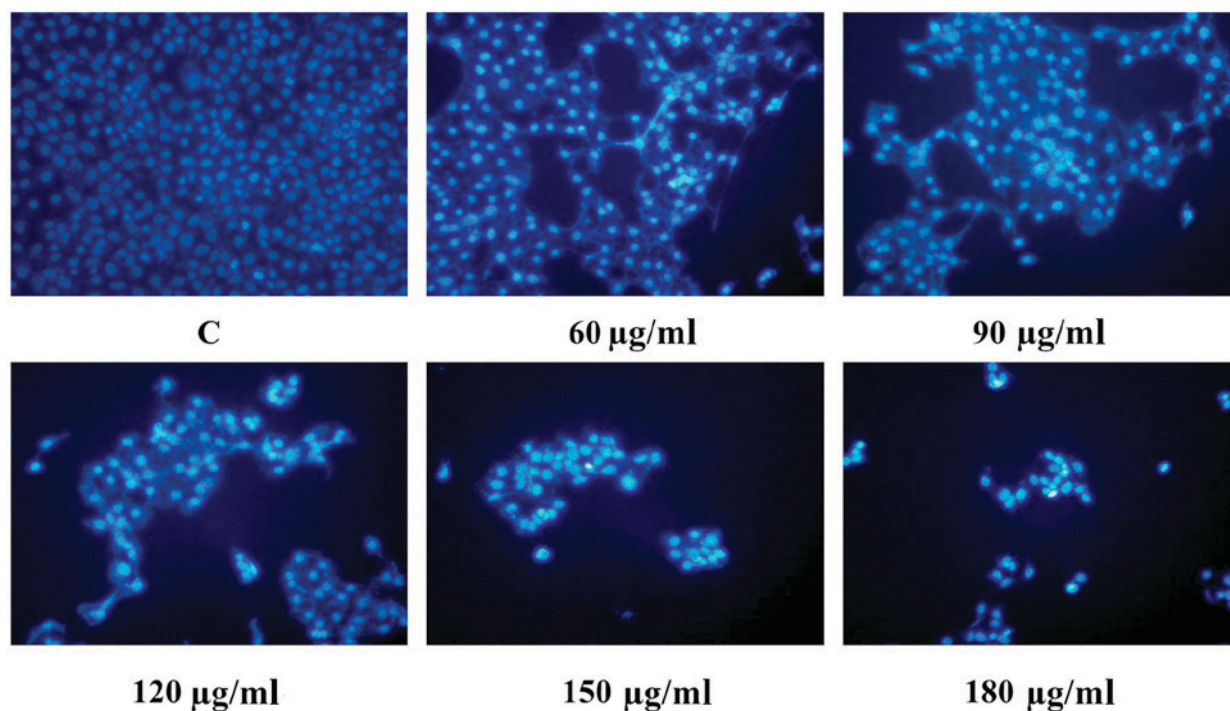


Figure 3. Gyp induces chromatin condensation in human oral cancer HSC-3 cells. Cells were exposed to 0, 60, 90, 120, 150 and 180 $\mu\text{g/ml}$ Gyp for 24 h, harvested and stained with 6-diamidino-2-phenylindole. Cells were examined and photographed using a fluorescence microscope (magnification, x200). Gyp, Gypenosides; C, control cells.

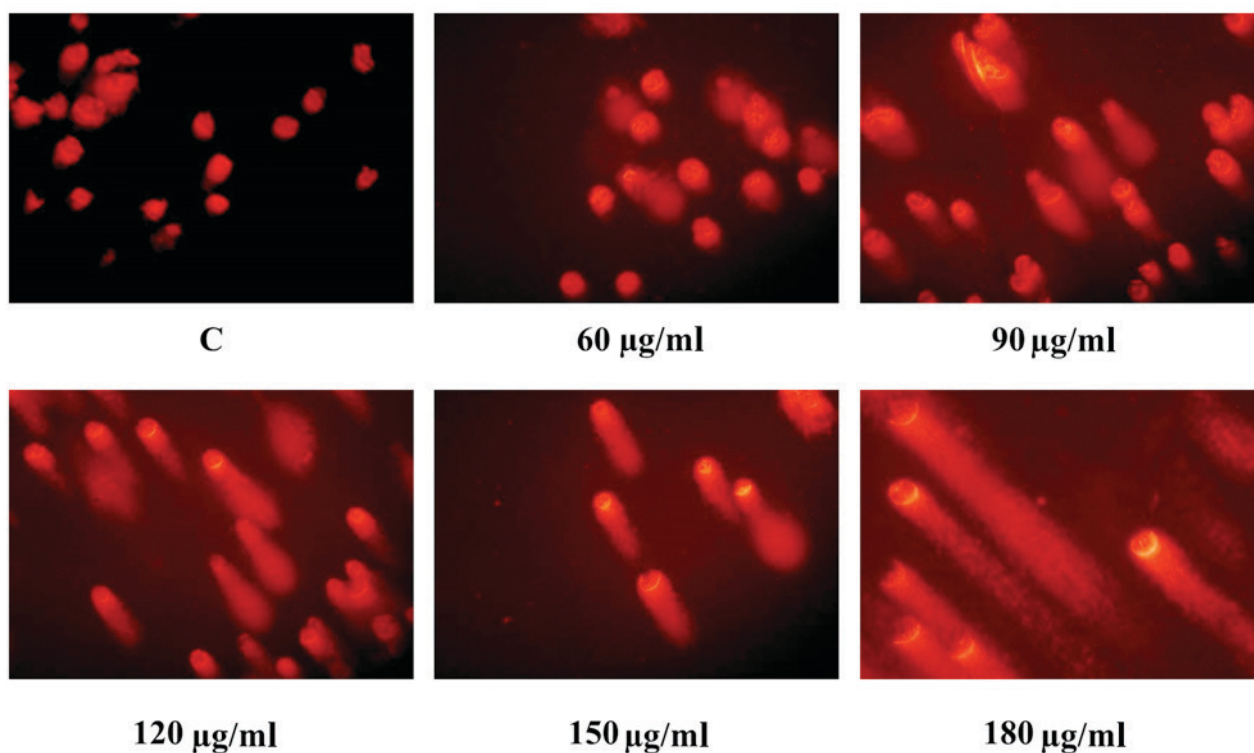


Figure 4. Gyp induces DNA damage in human oral cancer HSC-3 cells. Cells were exposed to 0, 60, 90, 120, 150 and 180 $\mu\text{g/ml}$ Gyp for 24 h and harvested. DNA damage was examined by Comet assay. Cells were examined and photographed using a fluorescence microscope (magnification, x200). Gyp, Gypenosides; C, control cells.

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 $\mu\text{g/ml}$) for 24 h and DNA damage was assessing 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	

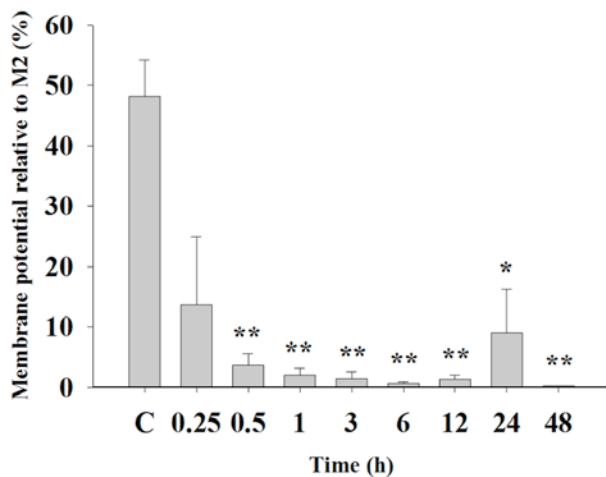


Figure 5. Gyp induced changes in the mitochondrial membrane potential in human oral cancer HSC-3 cells. Cells were treated with 120 µg/ml Gyp for 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h, collected and stained with DiOC₆. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. C. Gyp, Gypenosides;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

the production of the comet tail (Fig. 4). Higher concentrations of Gyp led to greater DNA damage, as indicated by the presence of longer comet tails (Fig. 4).

Gyp decreases the $\Delta\Psi_m$ in HSC-3 cells. HSC-3 cells were treated with 120 µg/ml Gyp for 0, 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h. The levels of $\Delta\Psi_m$ production were analyzed and quantified using flow cytometry. Levels of $\Delta\Psi_m$ were significantly decreased in HSC-3 cells treated with Gyp (P<0.05; Fig. 5) and this decrease occurred in a time-dependent manner.

Gyp affects gene expression in HSC-3 cells in vitro. HSC-3 cells were treated with or without 120 µg/ml Gyp and subsequently underwent cDNA microarray analysis of gene expression. A total of 953 genes upregulated ≥2-fold, 20 genes were upregulated ≥10-fold and 118 genes were

upregulated between 4 and 10-fold (Table I). By contrast, 2,039 genes were downregulated ≥2-fold, 23 genes were downregulated >10-fold and 276 genes were downregulated between 4 and 10-fold (Table I). Genes in HSC-3 cells that were highly influenced by Gyp treatment *in vitro* are listed in Table II. Among those affected genes, 10 were upregulated >13-fold as follows: GTP binding protein overexpressed in skeletal muscle (*GEM*), serpin peptidase inhibitor clade E member 1 (*SERPINE1*), ras homolog family member B (*RHOB*), kelch repeat and BTB domain containing 8 (*KBTBD8*), interleukin (*IL*)11, activating transcription factor (*ATF*)3, cytochrome P450 family 1, subfamily A, member 1 (*CYP1A1*), ADP-ribosylation factor-like 14 (*ARL14*), transfer RNA selenocysteine 2 (*TRNAU2*), and syntaxin 11 (*STX11*). However, the following 10 genes were downregulated >14-fold: Six transmembrane epithelial antigen of prostate family member 4 (*STEAP4*), γ-aminobutyric acid A receptor ε (*GABRE*), serpin peptidase inhibitor clade B, member 13 (*SERPINE13*), transcriptional-regulating factor 1 (*TRERF1*), apolipoprotein L1 (*APOL1*), follistatin (*FST*), *LOC100506718*, microRNA (*MIR*)205, *neuregulin* (*NRG*)1 and G protein-coupled receptor (*GPR*)110.

Gyp treated HSC-3 cells demonstrates the top alteration in gene expression scored by the number of pathway networks. GeneGo analysis is presented in Figs. 6-8. Experimental data were mapped on the processes. Upregulation was marked as red and downregulation was marked as blue circles of different intensities, which indicated the different levels of inhibition in HSC-3 cells following incubation with Gyp (120 µg/ml) *in vitro*. Fig. 6 represents the top scored network from Gyp (120 µg/ml) vs. control using the Analyze Networks (AN) algorithm, Fig. 7 represents second scored AN network from Gyp (120 µg/ml) vs. control and Fig. 8 represents the third scored AN network from Gyp (120 µg/ml) vs. control.

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

Table II. Representative genes in HSC3 cells treated with gypenosides.

Probe set ID	Gene symbol	Fold-change	Gene description
8151816	GEM	75.42	GTP binding protein overexpressed in skeletal muscle
8135069	SERPINE1	20.25	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
8040473	RHOB	18.04	Ras homolog family member B
8080911	KBTBD8	15.22	Kelch repeat and BTB domain containing 8
8039484	IL11	14.96	Interleukin 11
7909610	ATF3	14.49	Activating transcription factor 3
7990391	CYP1A1	14.44	Cytochrome P450, family 1, subfamily A, polypeptide 1
8083743	ARL14	13.88	ADP-ribosylation factor-like 14
8073680	TRNAU2	13.23	Transfer RNA selenocysteine 2 (anticodon UCA)
8122457	STX11	13.08	Syntaxin 11
8140840	STEAP4	-14.19	Six transmembrane epithelial antigen of prostate family member 4
8175683	GABRE	-14.58	γ -aminobutyric acid A receptor ϵ ; microRNA 452; microRNA 224
8021603	SERPINE13	-14.69	Serpin peptidase inhibitor, clade B (ovalbumin), member 13
8126428	TRERF1	-14.71	Transcriptional regulating factor 1
8072735	APOL1	-14.85	Apolipoprotein L, 1
8105302	FST	-15.22	Follistatin
7976073	LOC100506718	-15.61	Uncharacterized LOC100506718; fibronectin leucine rich transmembrane protein 2
7909422	MIR205	-16.38	MicroRNA 205
8145766	NRG1	-19.69	Neuregulin 1
8126820	GPR110	-22.05	G protein-coupled receptor 110

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 $\Delta\Psi_m$. 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, *CYP1A1* was increased 14.44-fold, *ARL14* was increased 13.88-fold, *TRNAU2* 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, *SERPINE13* was decreased 14.69-fold, *TRERF1* was decreased 14.71-fold, *APOL1* was decreased 14.85-fold, *FST* was decreased 15.22-fold, *LOC100506718* 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

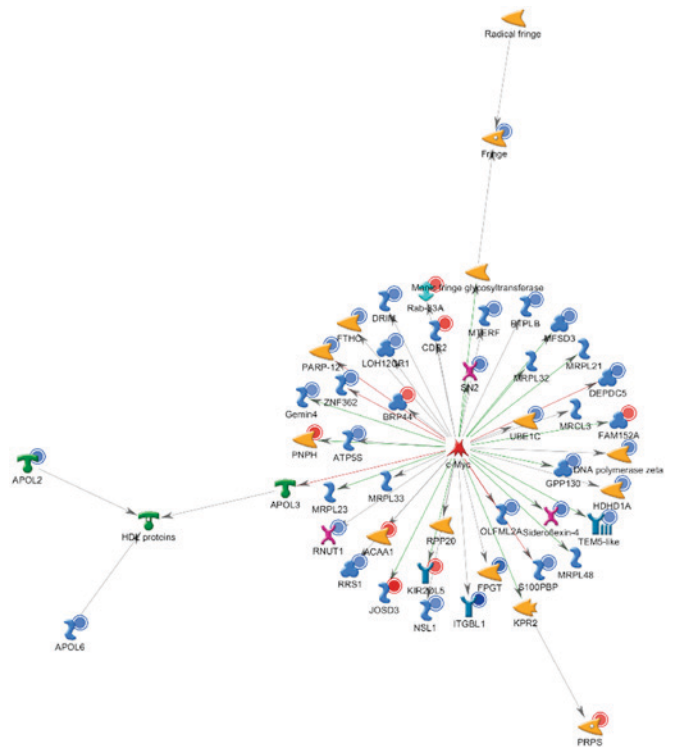
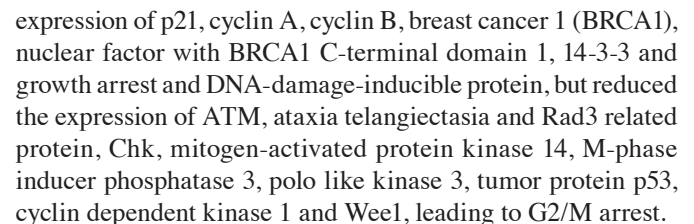


Figure 6. The top scored (by the number of pathways) network from Gyp (120 $\mu\text{g/ml}$) vs. control using the Analyze Networks algorithm on GeneGo software. Thick cyan lines indicate the fragments of canonical pathways. Red circles indicate upregulated gene expression. Blue circles indicate downregulated gene expression.



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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Figure 7. The second scored (by the number of pathways) network from Gyp (120 $\mu\text{g/ml}$) vs. control using the Analyze Networks algorithm on GeneGo software. Thick cyan lines indicate the fragments of canonical pathways. Red circles indicate upregulated gene expression. Blue circles indicate downregulated gene expression.

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