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Original Article

Effect of sea cucumber glycosides on ovarian cancer cell line SKOV3: an *in vitro* and *in silico* study

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Abstract: Background: Ovarian cancer (OC) is a life-threatening pathological condition in women through the world. The treatment of ovarian cancer is limited to surgical resection, radiotherapy, and chemotherapy. Sea cucumbers are natural and a health food with a well-known potential for the development of anticancer pharmaceuticals. Sea cucumber glycosides are the anticancer substance in sea cucumbers. The present study was designed to evaluate the effect of sea cucumber glucosides on ovarian cancer in the cell line SKOV3. Methods: Comprehensive bioinformatic analysis were performed to predict the pharmacological effect of sea cucumber on OC. Dose screening, microarray analysis and cytological experiments were performed to investigate the detailed effects of sea cucumber glucosides on OC. Results: Sea cucumber glycosides were found to have apoptotic, anti-proliferated, anti-invading and migration effects on SKOV3 ovarian cells. By using microarray analysis, we speculate that the anti-cancer effect of sea cucumber glycosides on ovarian cancer might be related to the p38-MAPK pathway. Conclusion: In the present study, we showed that sea cucumber glycosides have anti-cancer effects by inducing the apoptosis rate, reducing cell proliferation and cell invasion, and we also identified the possible cellular pathway for the sea cucumber glycoside action by *in silico* study. Our study provides a new potential in the treatment of ovarian cancer by natural products.

Keywords: Sea cucumber glycosides, ovarian cancer, apoptosis, microarray analysis

Introduction

Ovarian cancer (OC) is one of the top cancer-related causes of death in women worldwide [1]. Although there have been advances in research and treatment strategy towards this malignant disease, the mortality rate of OC is still high [2]. Chemotherapeutic or biologic treatments are some of the limited therapeutic approaches, however drug resistance has been become a daunting challenge in OC treatment [3].

Natural products are important potential anti-cancer targets. The sea cucumber is a species of aquatic marine life in the class *Holothuroidea*, and it has long been used as health-beneficial food and in pharmaceuticals in East Asia. In ancient Chinese medical manuscripts, sea cucumber was described as an agent with anti-cancer, anti-hypertension, immunoregulation, antioxidant and wound healing effects [4]. Sea cucumber contains many biologically and pharmacologically active components, such as glycosides and mucopolysaccharides. It's glycosides are major secondary metabolites as well as the material base of its chemical defense, the propertie of which are known for being anti-tumor, immune to fungi, virus and cytotoxin, good for immunoregulation and hemolysis [5]. Moreover, their special chemical structures and various biological activities not only have aroused wide attention from researchers, but also have provided a new type of raw material for the development of marine biological drugs.

Previous studies have shown that sea cucumber glycosides can inhibit the growth of many human tumor cell lines *in vitro*, such as U-87-

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MG, HCT-8, leukemia P-388, KB, Schabel, Mel-28, A-549, MICF-1, HT-29, IA9, CAKI-1, SK-MEL, PC-3, lymphoidal leukemia L 1210, MCF-7, MKN-28, HCT-116, U87MG, HepG2, HeLa, THP-1, KB-VIN, HCT-8, C33A, and some others [6].

Therefor, in the present study, we aim to investigate the therapeutic potential of sea cucumber glycosides in regards to OC. By using the OC cell line SKOV3, we revealed the anti-cancer effect of sea cucumber glycosides, and demonstrated the underlying mechanisms of this anti-cancer effect via microarray analysis.

Materials and methods

Extraction of sea cucumber glycosides

Specimens of the sea cucumber *thelenota ananas* were collected off the Yongxing Islands in the South China Sea in May 2015. The sea cucumber was identified by Mr. Yizhen Yan (Research Center for Marine Drugs, School of Medicine, Shanghai Jiao Tong University) and was frozen shortly after collection. The sea cucumber *thelenota ananas* (wet weight 15 g) was cut into pieces and the active compound was extracted with methanol at room temperature. The obtained methanol extract was suspended in water at 15°C. The precipitate was vacuum filtered and washed with MeOH to give the glycoside mixture. The main component was reviewed in [7].

Screening of sea cucumber glycoside dose

The effect of sea cucumber glycosides on the proliferation of ovarian cancer SKOV3 cells (kindly provided from Shanghai Institute of Oncology) was detected by MTT assay, and the doses of subsequent studies were screened. SKOV3 cells were plated in McCOY's 5A medium and cultured at 37°C under 5% CO_2 (the same below). In the logarithmic growth period, SKOV3 cells were digested and passaged with 0.25% trypsin, and then digested with 0.25% trypsin. The cells were collected and resuscitated with the culture medium to obtain a single cell suspension with a density of 2×10^5/ml. The cells were seeded in 96-well plates at 100 μl/well. After overnight culture, the cells were randomly divided into different dose groups of sea cucumber glycoside (300, 100, 50, 30, 10, 3, 1 μg/ml), and three replicate wells were set in each group. After 24 hours of culture, the culture medium was discarded, MTT reagent was added according to the instructions of the kit, and the absorbance (A), of each well was detected by enzyme-linked immunosorbent assay (ELISA) at 490 nm. The cell proliferation rate and 50% cell growth inhibition concentration (IC_{50}) were calculated. Cell proliferation rate = (experimental group OD value - blank group OD value)/(control group OD value - blank group OD value) × 100%. The above test was repeated 3 times.

Extraction of total RNA from SKOV3 cells and microarray processing

SKOV3 cells in the logarithmic growth phase were resuscitated with McCOY's 5A medium and inoculated in 6-well plate at 6×10^5/well. After the cells adhered to the well chamber walls, the cells were randomly divided into the control group and the sea cucumber glycoside group (The dose setting refers to the IC_{50} value under "3.2"), and 3 multiple wells were set in each group. The control group was given PBS, and the administration group was given sea cucumber glycosides and cultured for 24 hours. After collecting the cells, Trizol RNA extraction kit was used and the total RNA was extracted from each group according to the manufacturer's instructions. RNA integrity was calculated by ultra-micro spectrophotometer.

Genome-wide mRNA expression profile was obtained by microarray analysis with the Agilent SurePrint G3 Human Gene Expression v3 (8*60K) in accordance with the manufacturer's instructions.

Analysis of microarray data

Based on Agilent's chip, including 6 samples (3 experimental groups and 3 control groups) principal component analysis was performed with function "prcomp" in R 3.6.1. Differentially expressed genes (DEGs) between the experimental and control groups were analyzed using the GEO2R online tool (http://www.ncbi.nlm.nih.gov/geo/geo2r). The screening conditions for the DEGs were absolute value of Log_2 (Fold Change) > 1 and *P* value obtained from Welch's t test < 0.05. The app of ClueGO [8] in CytoScape 3.7.1 was employed in Gene Ontology (GO)-based gene set enrichment anal-

ysis. $P < 0.05$ was considered statistically significant.

Network analysis between pharmacology of sea cucumber glycosides and ovarian cancer

Genes related to OC were selected in the GeneCards database (https://www.genecards.org/). The ingredients and their targeted genes were downloaded from Traditional Chinese Medicine Systems Pharmacology (TCMSP) Database (https://tcmspw.com/tcmsp.php). The network analysis of protein-protein interaction was performed in STRING database Version 11.0b (https://string-db.org/). GO-based gene set enrichment analysis was performed in Metascape (http://metascape.org) [9].

Cytology experiment

Apoptosis: The effect of different doses of sea cucumber glycosides on apoptosis of human ovarian cancer cell line SKOV3 was detected by Hoechst (Invitrogen H1399)/PI staining (DOJINDO P378). The cells were cultured to the logarithmic growth phase and were in good condition. The cells were digested, re-suspended in the cell pellet with medium, and adjusted to the density of 2×10^5/ml. A total of 100 μl of cell suspension was added to each well of a 96-well plate and incubated at 37°C. After the cells were attached, different doses of drugs (30, 35, 40 μg/ml) are administered. After 24 h, 100 μl of staining solution was added (Hoechst 10 μg/ml, PI 20 μM) to each well, and stained for 20 min at 37°C in the dark. The apoptosis program in Acumen power supply was used for assessing the effect.

Cell viability assay: The effect of different doses of sea cucumber glycosides on the proliferation of human ovarian cancer cell line SKOV3 was detected by MTT assay. The blank group (without cells), control group (containing cells, no drug), and experimental group (drug treatment group) were set. The cells were cultured to a logarithmic growth phase, and the cells were in good condition; then the cells were digested, and the cell pellet was resuspended in the medium to adjust the cell density to 2×10^5/ml; 100 μl of the cell suspension was added to each well of a 96-well plate and cultured at 37°C. After the cells were attached, different doses of the drug (30, 35, 40 μg/ml) were administered. After 24 h, the medium was discarded, MTT (working concentration 0.5 mg/ml) was added, and after incubation at 37°C for 4 hours, 150 μL of DMSO was added to each well, and shaken on a shaker at low speed for 10 min. The OD value of each well was measured at 490 nm on an enzyme-linked immunosorbent assay to calculate the cell proliferation rate. Cell proliferation rate = (experiment group OD value - blank group OD value)/(control group OD value - blank group OD value) × 100%.

Cell invasion: Transwell chambers were used to detect cell invasiveness. Matrigel at a concentration of 100 mg/l diluted with McCOY's 5A was added to the Transwell chamber and humidified at 37°C. The cells in the control group, the low-dose group, the middle-dose group and the high-dose group were taken and suspended in a serum-free medium. A total of 10^5 cells (200 μl) were added to the upper chamber of the Transwell chamber, and 600 ul of the volume fraction of 0.1 fetal bovine serum (BI 04-001-1ACS) of McCOY's 5A was added to the lower chamber. Next, cells were placed at 37°C and continued to culture for 24 h in a CO_2 incubator. The chamber was removed, and the cells were wiped off with a cotton swab. Methanol was used to fix the cells, with Hoechst staining, and 5 fields were selected under a light microscope to observe the number of invading cells.

Cell migration: Five straight lines were drawn on the back of the 6-well plate with a marker pen, and the inoculation density was 5×10^5 cells/well plate. After completely adhering to the wall, the drug treatment was carried out: control group, low dose group (30 μg/ml), middle dose group (35 μg/ml), high dose group (40 μg/ml). After 24 h, scratches were made with a 10 μl tip perpendicular to the horizontal line behind the 6-well plate. After washing the detached cells with PBS, the medium was added and cultured in a 37°C, 5% CO_2 incubator. The fields of view were taken at 0 h, 12 h, and 24 h, respectively, and photographed three times. Image J software measured the scratch width and calculated the cell migration rate. Cell mobility = (0 h width -12 h or 24 h width)/0 h width × 100%.

Statistical analysis

All experiments were carried out in triplicate. Results were expressed as mean ± standard deviation (SD). The Sidak's multiple comparisons test was used to compare parameters between the different study groups using Graphpad prism 7.0 under one-way analysis of va-

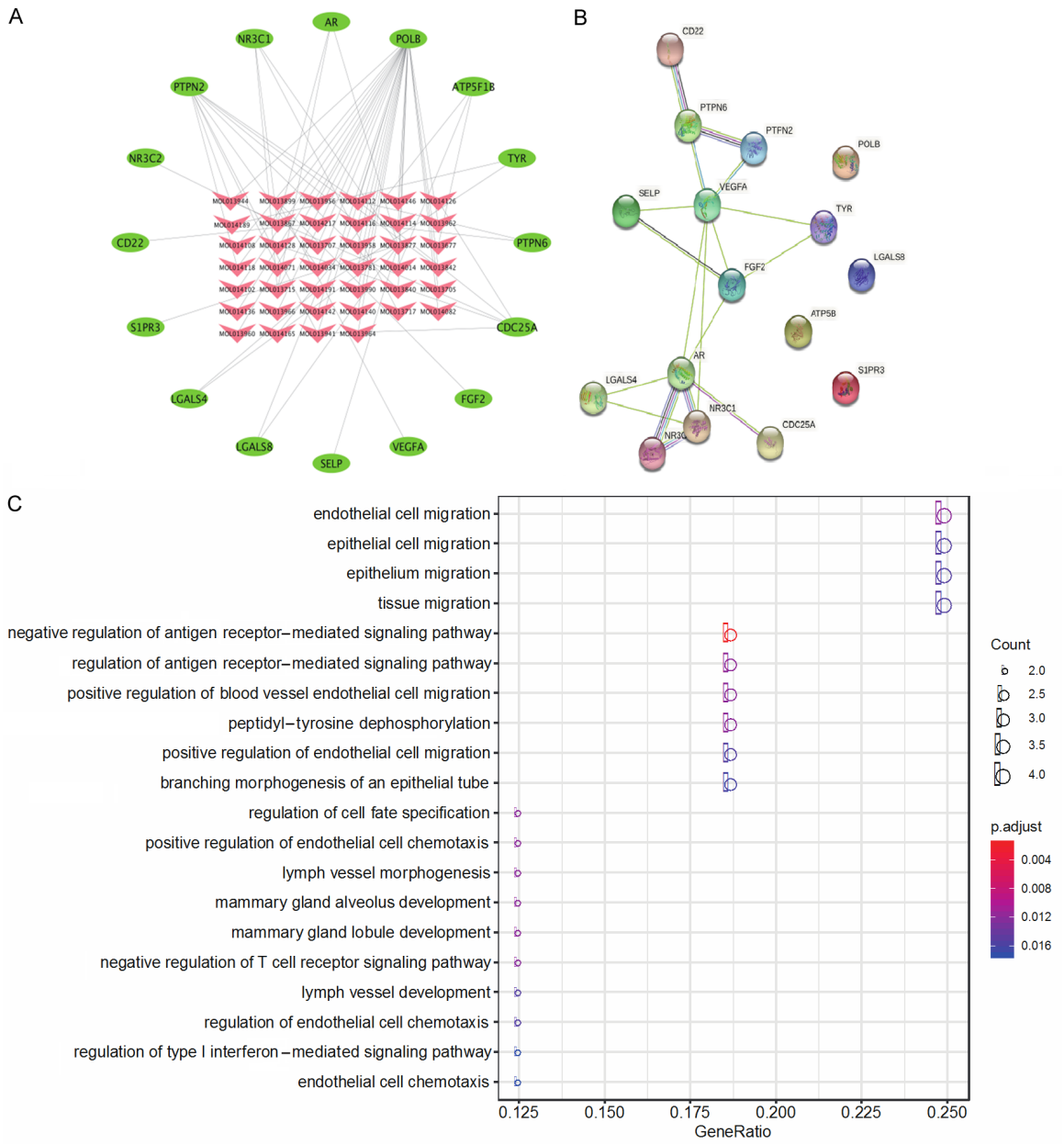

Figure 1. Pharmacological network analysis between sea cucumber glycosides and ovarian cancer. A. Network between sea cucumber ingredients and those targeted genes. B. Protein-protein interaction network between sea cucumber ingredient-targeted genes and OC-related genes. C. The bubble plot of GO-based gene set enrichment analysis of sea cucumber ingredient-targeted genes intersected with OC-related genes.

riance (ANOVA). A *P* value of less than 0.05 was considered statistically significant.

Results

Compound-target-disease network analysis of sea cucumber glycosides and ovarian cancer

We screened the genes related to OC in the GeneCards database, and found 1,541 genes, which the relevance score was more than 10 (Supplementary File 1). Then, we searched the drug-targeted genes of ingredients in the sea cucumber from the TCMSP Database. By setting the Oral Bioavailability (OB) > 30% and Drug-Likeness > 0.18, we selected 102 ingredients targeting 21 proteins (**Figure 1A**). By intersecting the genes related to OC and those sea cucumber ingredient-targeted ones, we found

Table 1. Effect of sea cucumber glycosides on the proliferation of SKOV3 cells

Concentration (μg/ml)	Cell proliferation rate		
1	66.11%	74.43%	84.51%
3	138.42%	100.95%	68.74%
10	96.13%	89.77%	85.39%
30	79.69%	98.10%	106.43%
50	3.87%	2.56%	8.25%
100	0.00%	0.00%	0.00%
300	0.00%	0.00%	0.00%

14 core genes and generated the interacting network of those genes based on STRING database (**Figure 1B**). By performing a Gene Ontology (GO)-based gene set enrichment analysis, we found that those genes were enriched in biological process related to cell migration and vessel morphogenesis (**Figure 1C**), suggesting that the pharmacological effect of sea cucumber glycosides on OC may be involved in cell chemotaxis and tube morphological development.

Dose screening of sea cucumber glycosides on cytotoxicity

We then tested the best dose of sea cucumber glycosides by screening. Compared with the 1 μg/ml sea cucumber glycoside group, the cell survival rate of the 3 μg/ml sea cucumber glycoside group was significantly increased, and the cell survival rates of the 10, 30, 50, 100, 300 μg/ml sea cucumber glycosides group were significantly decreased, and the differences were statistically significant ($P < 0.05$), as shown in **Table 1** and **Figure 2A**. In addition, the IC_{50} value of sea cucumber glycoside was 47.66 μg/ml, so 47.66 μg/ml of sea cucumber glycoside was finally selected for subsequent experiments.

Bioinformatics analysis of the pharmacological effect of sea cucumber glycosides

After 47.66 μg/ml sea cucumber glycoside administration for 24 h, we extracted the total RNA of those cells, and by GeneChips, we generated the gene expression profile of SKOV3 cells treated by sea cucumber glycosides. We firstly performed PCA for the six datasets, and found that the SKOV3 cells treated with sea cucumber glycosides were separated from the control group (**Figure 2B**). Then, we calculated the differentially expressed genes between the two groups. There were 245 genes with different expression in SKOV3 cells, of which 131 were up-regulated (fold change > 2, Welch t test *P* value < 0.05) and 114 were down-regulated (fold change < 0.5, Welch t test *P* value < 0.05), as shown in **Figure 2C**.

We then performed the GO-based gene set enrichment analysis to investigate the possible function of sea cucumber glycosides on SKOV3 cells.

On the category Biological Processes of GO, we found that the upregulated genes were mainly enriched in the GO terms related to fibroblast growth factor receptor signaling pathway, p38-MAPK cascade, negative regulation of osteoblast differentiation and wound healing, while the downregulated genes were involved in the GO terms related to positive regulation of smooth muscle contraction, energy homeostasis and cell fate commitment (**Figure 3A**, **3B**).

Cytological experiment results

In this study, sea cucumber glycosides were used to intervene in ovarian cancer SKOV3 cells to detect the apoptosis rate of ovarian cancer cells. The apoptosis rate of ovarian cancer cells in the low concentration group (30 μg/ml), middle concentration group (35 μg/ml) and high concentration group (40 μg/ml) was higher than that in control group. The apoptotic rate of ovarian cancer in the high concentration group was statistically higher than that in the drug control group, the low concentration group and the middle concentration group ($P < 0.05$; **Figure 4A**, **4B**).

The effect of sea cucumber glycosides on the proliferation of ovarian cancer SKOV3 cells is shown in **Figure 4C**. The proliferation rate of ovarian cancer cells in the low concentration group, medium concentration group and high concentration group was lower than that of the control group. The proliferation rate of ovarian cancer cells in the high concentration group was statistically lower than that in the control group, low concentration group and middle concentration group ($P < 0.05$).

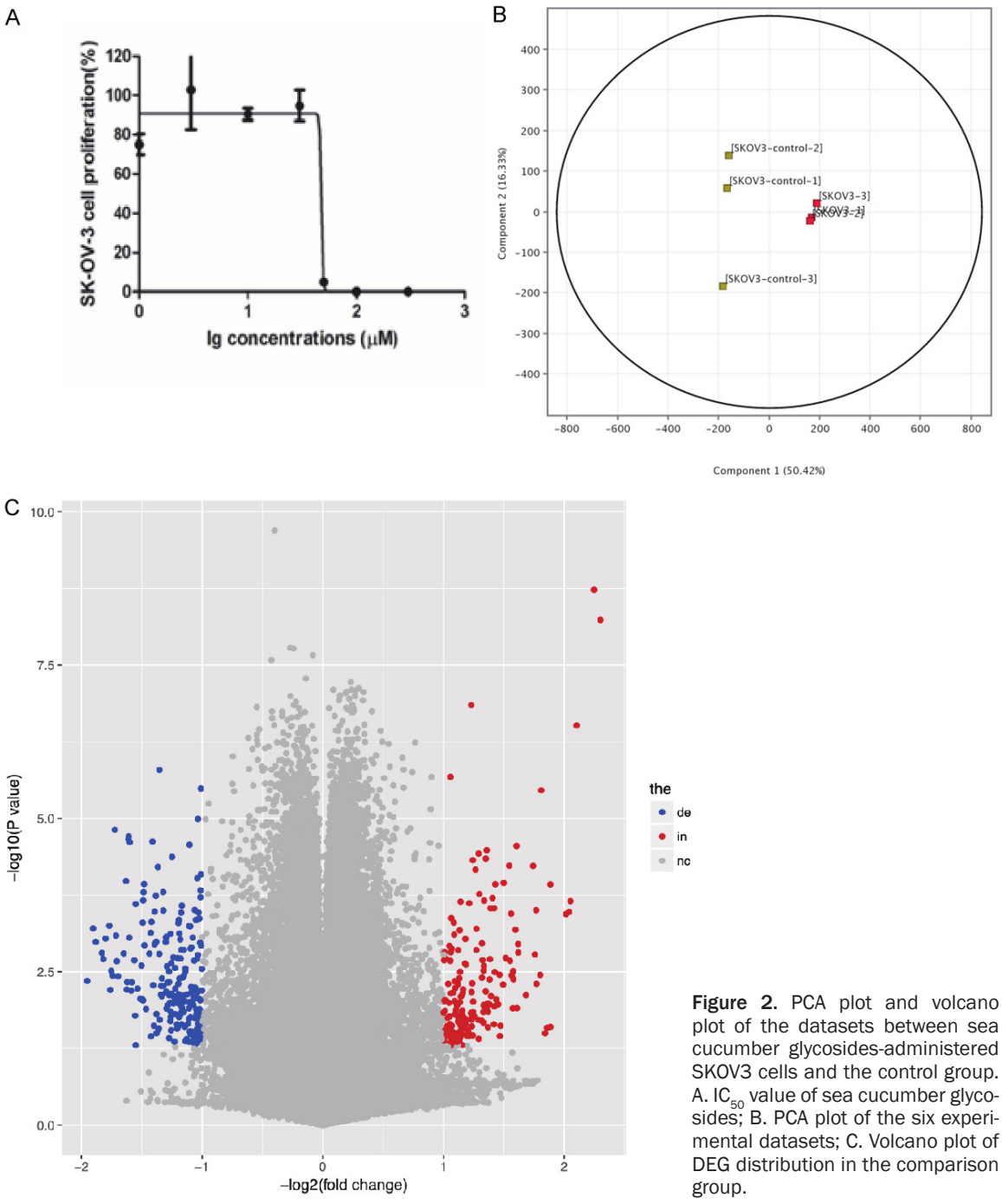

Figure 2. PCA plot and volcano plot of the datasets between sea cucumber glycosides-administered SKOV3 cells and the control group. A. IC_{50} value of sea cucumber glycosides; B. PCA plot of the six experimental datasets; C. Volcano plot of DEG distribution in the comparison group.

The effect of sea cucumber glycosides on the invasive ability of ovarian cancer SKOV3 cells is shown in **Figure 4D**, **4E.** The results showed that the invasive ability of SKV03 cells in the high dose group was significantly lower than that in the control group, low dose group and middle dose group, and the difference was statistically significant ($P < 0.05$).

The effect of sea cucumber glycoside on the migration of ovarian cancer SKOV3 cells is shown in **Figure 4F**, **4G**. The results showed that the invasive ability of SKV03 cells in the high dose group and middle dose were significantly higher than that in the control group and low dose group, and the difference was statistically significant ($P < 0.05$).

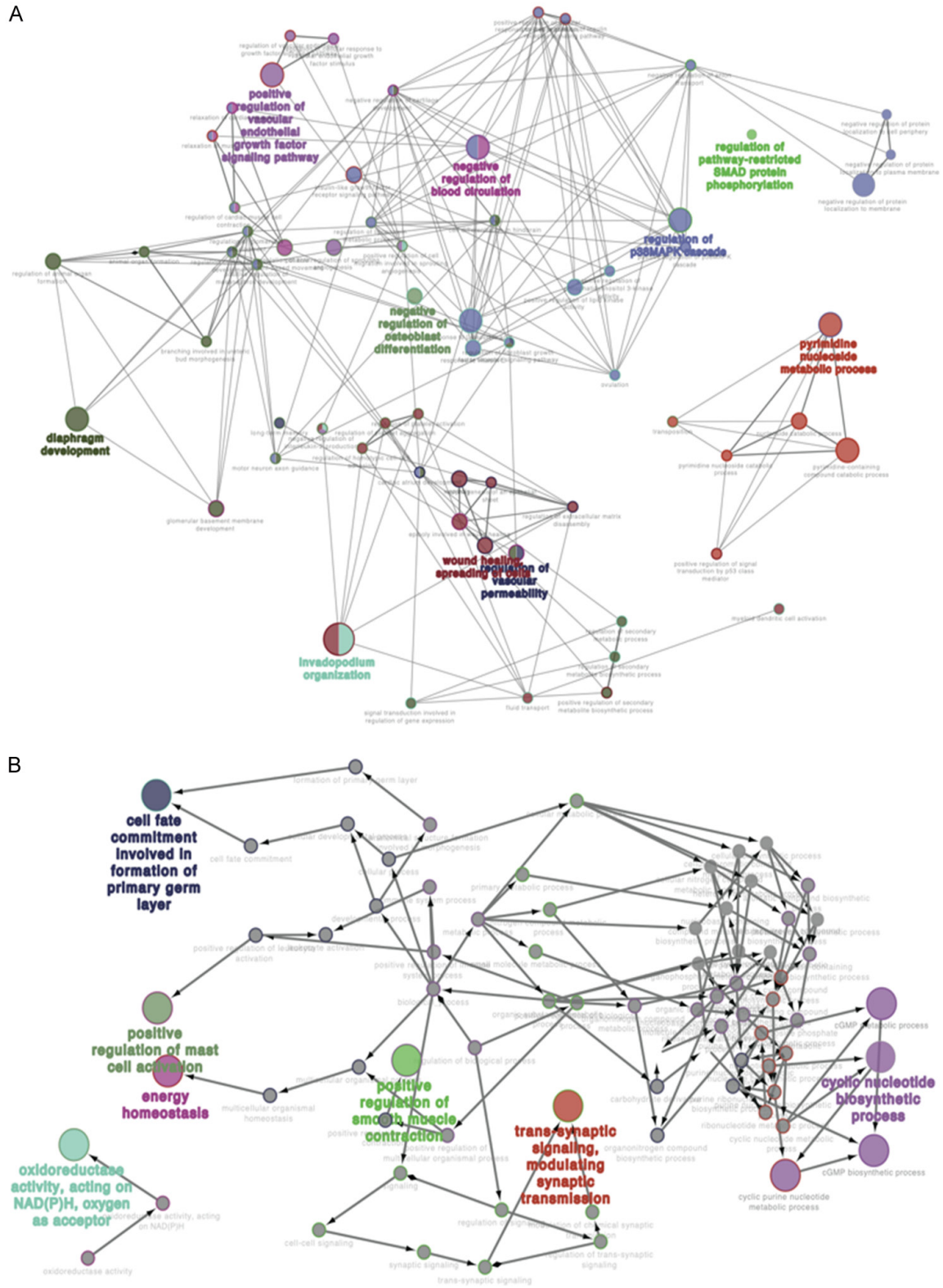

Figure 3. The GO-based gene set enrichment analysis of DEGs. A. The GO-based gene set enrichment analysis of upregulated DEGs. B. The GO-based gene set enrichment analysis of downregulated DEGs. The colored GO terms are enriched. The line in the directed acyclic graph means the parent-children relationship.

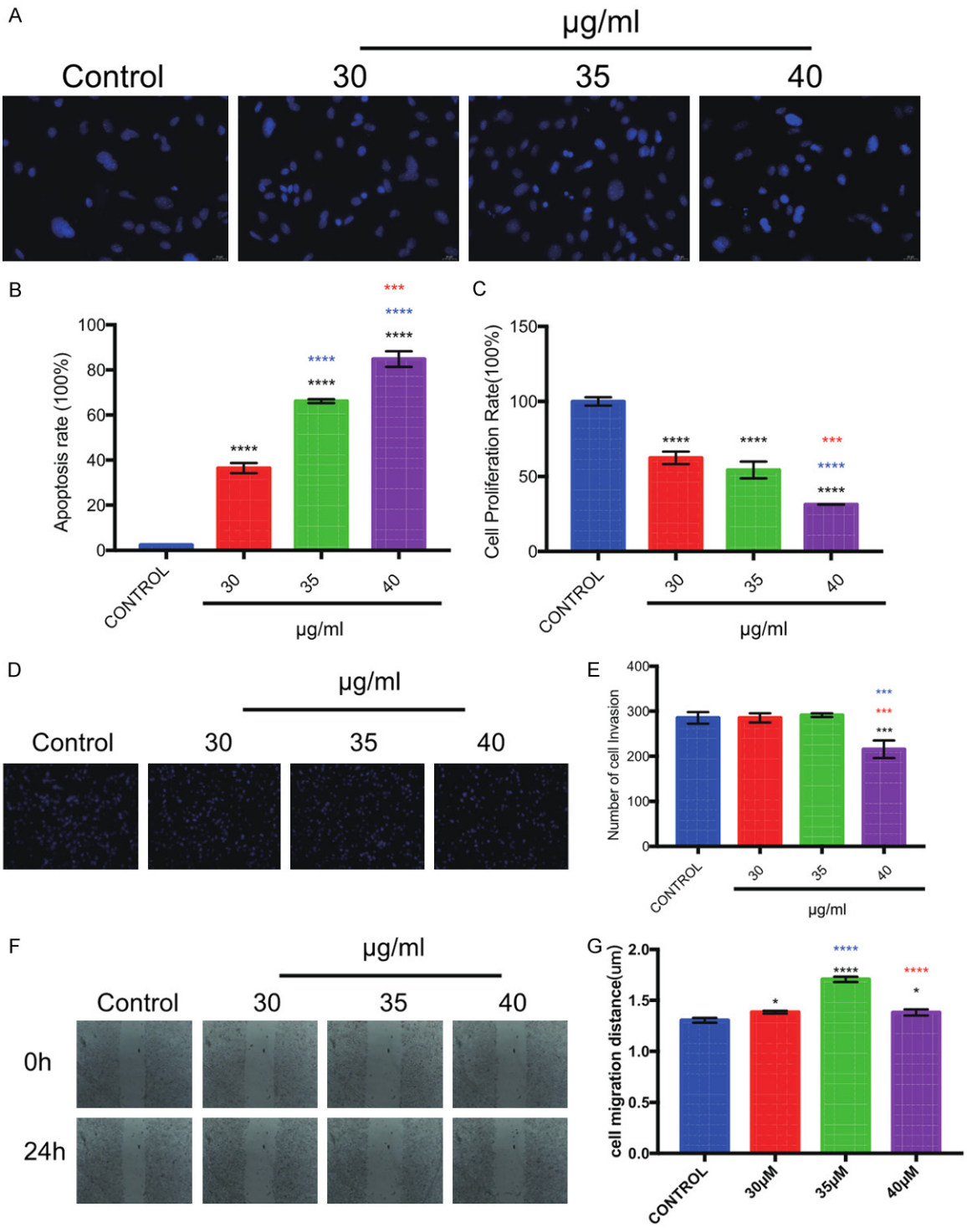

Figure 4. The anti-cancer effects of sea cucumber glycosides. A. Hoechst staining of apoptosis cells. B. Comparison of apoptotic rates. Black*: compared to the control group. Blue*: compared to the low dose group (30 μg/ml). Red*: compared to the middle dose group (35 μg/ml). ****: P < 0.0001. C. Comparison of cell proliferation rate. Black*: compared to the control group. Blue*: compared to the low dose group (30 μg/ml). Red*: compared to the middle dose group (35 μg/ml). ****: P < 0.0001; ***: P < 0.001. D. Hoechst staining of invasive cells. E. Comparison of cell invasion. Black*: compared to the control group. Blue*: compared to the low dose group (30 μg/ml). Red*: compared to the middle dose group (35 μg/ml). ***: P < 0.001. F. Would healing of cell migration. G. Comparison of cell migration. Black*: compared to the control group. Blue*: compared to the low dose group (30 μg/ml). Red*: compared to the middle dose group (35 μg/ml). ***: P < 0.001.

Discussion

Sea cucumber glycosides have multiple effects on various pathological conditions [5]. For the anti-cancer effect, previous studies have shown that sea cucumber glycosides can inhibit the growth of many human tumor cell lines *in vitro* [6]. For the first time, we revealed the inhibitory effect of sea cucumber glycosides on ovarian cancer cell line SKVO3.

Sea cucumber glycosides contain a series of compounds and each has a distinctive biological effect. These compounds exhibit various biological and pharmacoligcal effects including cytotoxic, anti-fungal, anti-biotic, anti-viral, hemolytic and anti-neoplastic properties [4]. Holothurin can inhibit the growth of epidermal carcinoma tumor cells [9]. Holothurin A and 24-dehydroechinoside A, two isoforms of holothurin, exhibited significant inhibition of metastasis *in vitro* and *in vivo* [10]. Another series of compounds in sea cucumber glycosides are philinopsides A, B, E and F, and pentactasides I, II and III, which were shown to have significant cytotoxicity *in vitro* against such tumor cell lines as U87MG, A-549, P-388, MCF-7, HCT-116, and MKN-28 [11, 12]. Colochiroside A revealed strong anti-cancer activity against mouse H22 and S180 sarcoma cells [13]. Stichoposide C induces apoptosis of human leukemia and colorectal cancer cells and HL-60 leukemia xenograft models [14]. Frondoside A induced apoptosis of human pancreatic cancer cells by activating the caspase cascade and inhibiting MDA-MB-231 cell migration and invasion in a concentration-dependent manner [15]. Those glycosides mentioned above have the properties to induce apoptosis, as well as inhibit proliferation, cell migration and cell invasion. Since the sea cucumber extract used in the present study is a mixture of glycosides, the detailed active components against SKOV3 need a further investigation.

To investigate the detailed mechanism of the sea cucumber glycosides against ovarian cancer the gene set enrichment analysis results revealed that the up-regulated biological processes were mainly related to the p38-MAPK pathway and negative regulation of cell migration, which is in consistent with our results of *in vitro* experiments (**Figure 3A**). We showed that sea cucumber glycosides can induce apoptosis in SKOV3 cells in a dose-dependent manner (**Figure 4B**). The p38-MAPK cascade is a key pathway involved in cellular apoptosis [16]. In ovarian cancer, the apoptotic pathways, including the activators, such as Bcl-2 family, and inhibitors, like the key regulators nuclear factor kappa B and MAPK were changed [17, 18]. The protein level of Bcl-2 was higher in the multicellular spheroids, other than adherent cells in SKOV3 cell line [19]. By using multicellular spheroids from SKOV3 cell lines we showed that suspended and adherent cells were more susceptible to death when treated with cisplatin compared to multicellular spheroids. The p38-MAPK pathway is involved in the inhibitory pathways of apoptosis. Upregulation of the p38-MAPK pathway caused the initiation of apoptosis [20], and the induction of p38 phosphorylation by compounds, such as delphinidin and resveratrol, which can inhibit the proliferation, and reduce the cell migration and invasion in SKOV3 [21, 22]. Although there was evidence that activation of p38-MAPK pathway can lead to cellular apoptosis in ovarian cancer cells, more studies should be performed upon patient-derived cells to verify this mechanism, and the relationship between activation of the p38-MAPK pathway and the induction of apoptosis in SKOV3 cells following the administration of sea cucumber glycosides should also be further investigated.

In conclusion, although the medical modality of ovarian cancer treatment is limited, we found a new approach with the glycosides from a marine animal, the sea cucumber. In the present study, we showed that sea cucumber glycosides have anti-cancer effects by inducing the apoptosis rate, as well as reducing cell proliferation and cell invasion. By performing gene set enrichment analysis, we speculate the apoptotic induction from sea cucumber glycosides might be related to the upregulation of the p38-MAPK pathway. Our study provides a new potential in the treatment of ovarian cancer by natural products.

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Disclosure of conflict of interest

None.

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