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Silibinin induces apoptosis and inhibits proliferation of SKOV-3 human ovarian cancer cells through suppressing survivin and HLA-G expression

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A B S T R A C T

To investigate the effect of silibinin on the proliferation and apoptosis in human ovarian cancer cell line SKOV-3 and its underlying mechanisms. SKOV-3 cells were treated with various concentrations of silibinin. Cell viability was assessed by MTT assay and the apoptotic distribution was determined by flow cytometry. By administering different concentrations of silibinin, the activation of caspase-3 was measured using Caspase-Glo® 3/7 assay kit and the levels of survivin and HLA-G gene-expression were measured by reverse-transcription real-time PCR. Silibinin inhibited the growth of SKOV-3 cells in a dose- and time-dependent manner. Silibinin effectively induced the apoptosis of SKOV-3 cells by caspase-dependent mechanism, while caused a decrease the survivin and HLA-G expression. These results suggest that silibinin inhibited the proliferation of SKOV-3 cells, and induced the apoptosis by down-regulating survivin and HLA-G expression, with a significant activation of caspase 3.

Introduction

Ovarian cancer is the first most common malignancy of the female reproductive system, besides breast cancer and it is the leading cause of gynecologic cancer deaths worldwide [1-2]. Because early disease is asymptomatic, more than 70% of the patients with ovarian cancer have already reached an advanced stage of disease at the initial diagnosis [2-3].

Natural plant components are excellent sources of complex chemicals with useful properties, including great therapeutic value [4]. Silibinin, a specific polyphenolic flavonolignan, constitutes a major biologically active portion of the plant extract of milk thistle (*Silybum marianum*) that is widely consumed as a dietary supplement [5-6]. Silibinin has both

antioxidant and anti-cancer properties [5, 7]. The anticancer efficacy of silibinin is clearly evident from the published reports against various cancers including prostate, skin, lung, colon, breast, hepatic, ovarian, cervical, kidney and gastric carcinoma, while the underlying mechanisms are very different in different cancer cells [5, 8-9]. The antineoplastic activity of silibinin has led to its evaluation as a potential cancer preventative and therapeutic agent [5, 10]. Silibinin induces cell apoptosis both in a manner-dependent on caspases activation or independent of caspases activation in different human carcinoma cells [9, 11]. Recent evidences suggest that the onset of cellular apoptosis is closely related to the occurrence, progress and metastasis of tumors. Study of the mechanisms of apoptosis in tumor cells is an important field of tumor therapy and molecular cancer biology. Apoptosis is regarded as the preferred mechanism for managing the cancer cells [12-14]. Disturbance in the regulation of apoptotic machinery contributes to the development of tumor and subsequent multi-drug resistance [15]. Anti-apoptotic proteins counteract signaling through the specific apoptosis pathways and provide targets for possible drug discovery and new anticancer interventions [16].

Survivin is a bifunctional protein in that it is an apoptotic suppressor and plays a central role in cell division [16-18]. Several preclinical studies have demonstrated that the down-regulation of survivin expression or function, accomplished by means of various strategies, reduces the tumor growth potential, increased the apoptotic rate and sensitizes the tumor cells to chemotherapeutic drugs and radiations in different human cancer models. Moreover, the first survivin-based antineoplastic inhibitors have recently entered the clinical trials [16, 19-20].

Moreover, one of the mechanisms that ovarian cancer cells evade immune surveillance is by up-regulation of the human leukocyte antigen-G (HLA-G) expression. HLA-G is a non-classical major histocompatibility complex (MHC) class I gene, characterized by a restrictive distribution and accumulated evidence has suggested its biological role in inactivating the immune response [21-22]. Treatment strategies for human tumors have essentially focused on triggering the immune response, and it is of critical importance to characterize the mechanisms that prevent such anti-tumor defense [23].

A previous report has shown that aberrant HLA-G expression in cancer cells plays important role in disease progression and it was associated with tumor metastasis and poor survival in an animal model having ovarian cancer [24].

The underlying mechanism of silibinin in ovarian cancer has not yet been clarified. So, the purpose of this work was to develop an understanding of silibinin's impact on ovarian cancer cells to begin to determine its therapeutic value in preventing or treating this disease. Therefore, we examined the anticancer activities of silibinin and investigated its effects on the survivin and HLA-G expression in SKOV-3 cancer cell line.

Materials and Methods

Materials

Culture medium RPMI 1640, cell culture supplements and fetal bovine serum (FBS) were obtained from Gibco (Life Technologies, USA). Reverse transcriptase, Taq DNA polymerase and Oligo (DT) were ordered from Promega (Mannheim, Germany). Silibinin was purchased from

Squibb (USA), PrimeScript™ RT reagent Kit was obtained from Takara and RealQ PCR 2X Master Mix with green dye was acquired from Ampliqon (Denmark).

Cell culture and silibinin treatment

The SKOV-3 human ovarian carcinoma cell line was obtained from Pasteur Institute (Tehran, Iran) and cultured at 37 °C with 5% CO₂ in RPMI 1640 medium with 10% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin and 0.3 µg/ml glutamine. For silibinin treatment, the cells were exposed to 12.5–300 µg/µl silibinin and then incubated for 24, 48 and 72 h, whereas DMSO solution without silibinin was used as blank reagent.

Cell proliferation assay

After treatment with silibinin, the cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell samples were incubated with 100 µl MTT for 3 h at 37 °C, the supernatant was removed and the formazan crystals, formed in viable cells, were solubilized with 100 µl of DMSO. A 0.1 ml aliquot of each sample was then transferred to 96-well plates and the absorbance of each well was measured at 570 nm with ELISA Reader.

Growth inhibition was measured by dividing the mean absorbance of treated wells per mean absorbance of control wells (drug-free wells), and was expressed as a percentage. The inhibitory concentrations of silibinin against 50% of the cells (IC₅₀) were defined as the drug concentrations, at which the cell growth was inhibited by 50%, as compared with drug-free controls.

Flow cytometric analysis of apoptosis

SKOV-3 cells were plated in 6-well plates under standard culture conditions. After 48

h, the cells were fed with fresh medium and treated with DMSO alone (control experiment) or administered with different doses of silibinin (50, 75 and 100 µg/µl). After 48 h of treatment, the culture medium was aspirated, the cells were quickly washed twice with ice-cold PBS and trypsinized, and finally the cell-pellets were collected for further analyses. For apoptotic determination, the cells (5×10^5) were washed with 1 ml PBS (pH 7.4) and then resuspended in binding buffer, according to the manufacturer's protocols. The cell aliquots were then incubated with Annexin-V-FITC and PI, solutions, and incubated for 15 min at 4 °C in the dark. Then, the apoptotic induction was determined by FACScan flow cytometer with the help of Cell Quest software (FACSCalibur; Becton-Dickinson, San Jose, CA, USA). All the experiments were performed in triplicate.

Caspase-3 activity assay

SKOV-3 cells were treated with 50 µg /µl of silibinin. Then, the cells were collected, cell lysates prepared in CCLR and the protein concentrations were determined using the Bradford method. Subsequently, the caspase-3 activity assay was conducted with Caspase-Glo® 3/7 assay kit, according to the manufacturer's instructions (Promega, Madison, WI, USA). A 5-10 µg of protein in a 50 µl of total volume was mixed with 100 µl of equilibrated Caspase-Glo® 3.7 reagent and incubated for 1 h at the room temperature. Afterwards, the luminescence was measured using a TD-20/20 Luminometer.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cell cultures using the Trizol Reagent, which was about 70% confluent. A 1 mL of Trizol

solution was added to the cell pellet and vortexed. The reaction mix was then incubated at room temperature for 5 minutes, followed by the addition of 200 µL of chloroform and mixed well. Then, the prepared mixture was incubated at room temperature for 5 minutes, centrifuged at 12.000×g for 15 minutes at 4 °C. The aqueous phase was collected into a separate 1.5 mL micro-centrifuge tube and 500 µL of isopropyl alcohol was added to it while mixing. The reaction mixture was incubated again at room temperature for further 10 minutes and centrifuged at 12.000×g for 10 minutes at 4 °C. The RNA pellets were acquired by adding 75% absolute alcohol and stored in RNase-free water at -80 °C for further use. The total RNA concentration and its purity were measured using the UV-Visible Spectrophotometer (Eppendorf, BioPhotometer) at the wavelength of 260-280 nm. The integrity of the isolated RNA was confirmed by running the electrophoresis of individual samples on a 2% agarose gel.

After RNA preparation, the complementary DNA (cDNA) was reverse-transcribed using the 2-steps RT-PCR kit, according to the manufacturer's instructions. The synthesized cDNA was immediately used in a real-time PCR or stored at -80 °C for further use.

Analysis of gene expression by real-time PCR

The expression of survivin, HLA-G and rRNA-5s mRNAs was determined using the real-time PCR. Each cDNA sample was amplified using SYBR Green on the ABI 7500 Fast Real-time PCR System (Applied Biosystem, CA). The reaction conditions consisted of 2 µl of cDNA + 0.5 µl primers in a final volume of 20 µl of super-mix. PCR reaction parameters were as follows: denaturation at 95 °C for 5 minutes,

followed by 50 cycles of denaturation at 95 °C for 10 seconds, annealing at 60C for 30 seconds, and finally the extension at 72 °C for 30 seconds. The whole experiment was performed in triplicate.

For each sample, the ΔCt values were determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. The relative gene expression level was also normalized relative to a positive calibrator, consisting of one of the samples from the calibration curve. The relative gene expression level of the calibrator (ΔCt calibrator) was also determined by subtracting the average of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. The results of the present experiment were expressed as 'N-target' and determined as follows:

$$N\text{-target} = 2^{(\Delta C t_{\text{sample}} - \Delta C t_{\text{calibrator}})}$$

Statistical analysis

The data were analyzed using SPSS software for Windows (version 16.0). Statistical significance of difference in growth inhibition and expression levels of survivin, HLA-G and rRNA-5s between control and treated groups was assessed using Student's *t*-test. A statistically significant difference was considered to be present at $P < 0.05$.

Results and Discussion

Silibinin inhibits cell viability in SKOV-3 cells

The SKOV-3 human ovarian carcinoma cells were exposed to silibinin at the concentrations of 12.5–300 µg/µl for 24, 48 and 72 h and the cytotoxicity was

determined using MTT assays. MTT results have shown that the above stated concentrations of silibinin could significantly induce cytotoxicity in the SKOV-3 cells in a dose-dependent manner. The maximum amount of cytotoxicity was observed when the cells were exposed to 12.5 µg/µl of silibinin for 24 h (Figure 1).

Data analysis of the cytotoxicity assay have shown that the IC₅₀ values of silibinin against the SKOV-3 cells were 150.37, 78.56 and 56.49 µg/µl after the incubation periods of 24, 48, and 72 hours, respectively (P<0.05); indicating a dose- and time-dependent response relationship (Figure 2).

Silibinin-induced apoptosis in SKOV-3 cells

Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger a significant amount of apoptosis. To determine whether silibinin could induce apoptosis in SKOV-3 cells, flow cytometric analysis was performed using Annexin V-FITC conjugated to Propidium Iodide (PI). As shown in Figure 3, the apoptotic cell population increased from 7.93% in control to 30.04%, 36.98% and 34.15% (p all <0.05) after 48 h treatment with 50, 75 and 100 µg/µl of silibinin, respectively.

Effect of silibinin on survivin and HLA-G expression in SKOV-3 cells

Survivin and HLA-G gene expression in SKOV-3 cell line was assessed with the help of real time PCR after the exposure of these cells to 50, 75 and 100 µg/µl silibinin for 48 h, respectively. Continuous measurement of the PCR product was enabled by incorporation of SYBR Green fluorescent dye into the double stranded PCR products. The level of survivin and HLA-G was

normalized to the transcript level of rRNA 5s and ultimately, the $\Delta\Delta Ct$ value was calculated for each sample for the purpose of statistical analysis according to Yuan et al. (2007) [25]. Finally, the $\Delta\Delta Ct$ values were transformed to absolute values using the formula $2^{-\Delta\Delta Ct}$.

The results of real time PCR have shown that the expression of survivin and HLA-G in SKOV-3 cell line were decreased after the incubation of these cells with 50, 75 and 100 µg/µl of silibinin in a dose-dependent manner.

The present study shows that the silibinin could has proved to be a potential antiproliferative agent with the anti-survivin and anti-HLA-G potency in the SKOV-3 ovarian cancer cell line.

Cytotoxicity and drug resistance are the two main obstacles in the way of conventional chemotherapeutic drug-based treatment regimens that are the real challenges in the field of cancer research. As shown by Figure 1, silibinin caused a significant decrease in the growth of SKOV-3 cells. This antineoplastic effect was increased with increasing the concentration of this compound. MTT analysis has shown that the silibinin could demonstrate the significant IC₅₀ potential against SKOV-3 ovarian cancer cell line at 150.37, 78.56 and 56.49 µg/µl after the 24, 48, 72 h, respectively (Figure 2). In other words, it seems that the effect of silibinin on SKOV-3 ovarian cancer cell line is time-and dose-dependent. Other studies have shown that silibinin has a time-dependent inhibitory effect against the growth of various types of cancer cell lines [11, 27-28].

It is well known that chemotherapeutic agents induce apoptosis in most of the cancer cell types via two major ways of

death receptor-mediated pathway and mitochondria-mediated pathway [29-30]. Both pathways converge to a final common pathway, involving the activation of a cascade of proteases called caspases, which can cleave regulatory and structural molecules, and thus induce cell death [31]. Silibinin could induce cellular apoptosis both via the caspases activation or independent of caspases activation [11, 32-33]. We investigated the effect of silibinin on the apoptosis and activation of caspase-3 (an effector caspase). The present results have shown that the caspases-related pathway is involved in the silibinin-triggered apoptosis in SKOV-3 cells.

Silibinin exerts its anticancer effect on different cellular factors and the gene expression as well [34-38]. We have investigated the influence of silibinin on the expression of the survivin and HLA-G, the effective factors that are considered to be one of the major causes of the cancer initiation.

Survivin, a member of the inhibitors of apoptosis proteins (IAP) family [39], is known to inhibit both extrinsic and intrinsic pathways of apoptosis by acting as endogenous inhibitor of different kinds of caspases. Survivin functions as a key regulator of mitosis and programmed cell death [40], and is prominently expressed in transformed cell lines and in almost all kinds of human tumors, related to the lung, gastric, colon, pancreas, prostate and breast tissues. In vitro and in vivo studies have shown that the down-regulation or inactivation of survivin is correlated with the marked inhibition of tumor growth [41]. In our experiment, realtime-PCR indicates that the survivin expression is down regulated at mRNA level in silibinin-treated group. So the down-regulation of survivin might be responsible for the apoptotic induction of SKOV-3 cells by silibinin. Li et al. (2008)

observed the inhibitory effect of silibinin on survivin activity in renal cell carcinoma [42].

We also examined the impact of silibinin on the HLA-G expression. HLA-G expression is significantly seen in the embryonic tissues, adult immune privileged organs, and the cells of the hematopoietic lineage. More interestingly, under pathophysiological conditions, the HLA-G antigens may be expressed on various types of malignant cells, suggesting that HLA-G antigen expression is a strategy used by tumor cells to escape the immune surveillance [43]. Several reports have shown that the non-classical HLA-G class I molecule inhibits NK (natural killer) lytic activity upon its interaction with KIRs (killer inhibitory receptors) that belong to both the C-type lectin and Ig superfamilies. While, the existence of a different, still uncharacterized KIR, able to interact specifically with HLA-G was also postulated [23]. Thus, the expression of HLA-G by the target cells would constitute a powerful mechanism by which the tumors escape from NK immune-surveillance. According to the present analyses, silibinin inhibits the expression levels of HLA-G mRNA. Our data also indicate that the silibinin also decreased the HLA-G expression in SKOV-3 cell.

Conclusion

In conclusion, the findings of present study have demonstrated that silibinin could successfully inhibit the cancer cell proliferation, induce apoptosis by caspase-dependent mechanism and cause the down-regulation of survivin and HLA-G while inducing a considerable amount of apoptosis in SKOV-3 ovarian cancer cell line. Further studies are needed to unveil the underlying mechanisms and for the further confirmation of these results.

Figure.1 Effect of silibinin on SKOV-3 cell viability. SKOV-3 cells were cultured as described in “Materials and Methods” and treated with either DMSO or 12.5–300 $\mu\text{g}/\mu\text{l}$ of silibinin for 24, 48 and 72 h, and cell number was determined at the end of the exposure period

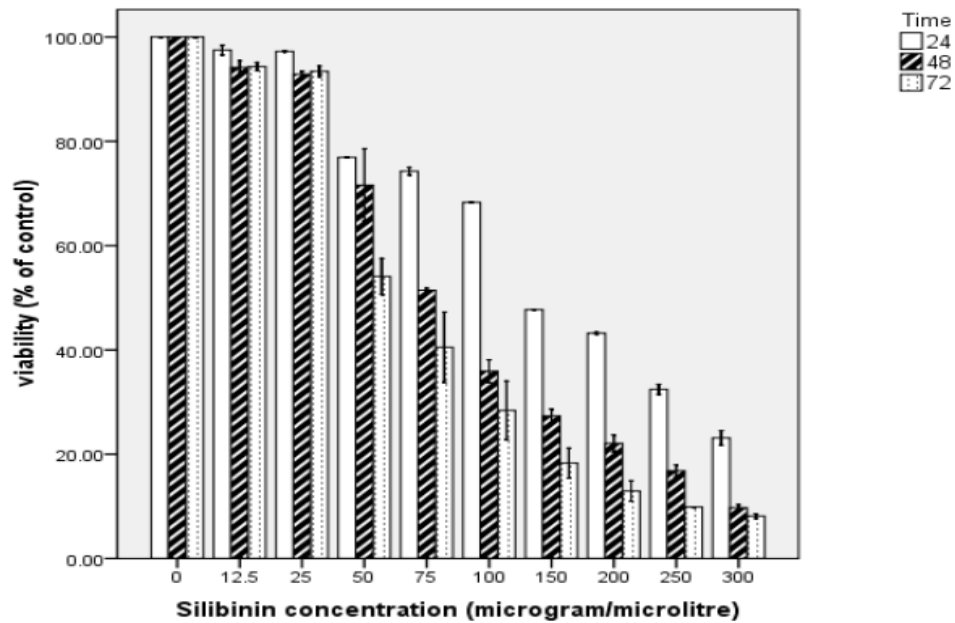


Figure.2 Anticancer activity of silibinin against the breast cancer cell line SKOV-3 after its administration for 24, 48 and 72 hours of incubation, as assessed using the MTT assay. Data are reported as IC₅₀ versus incubation time

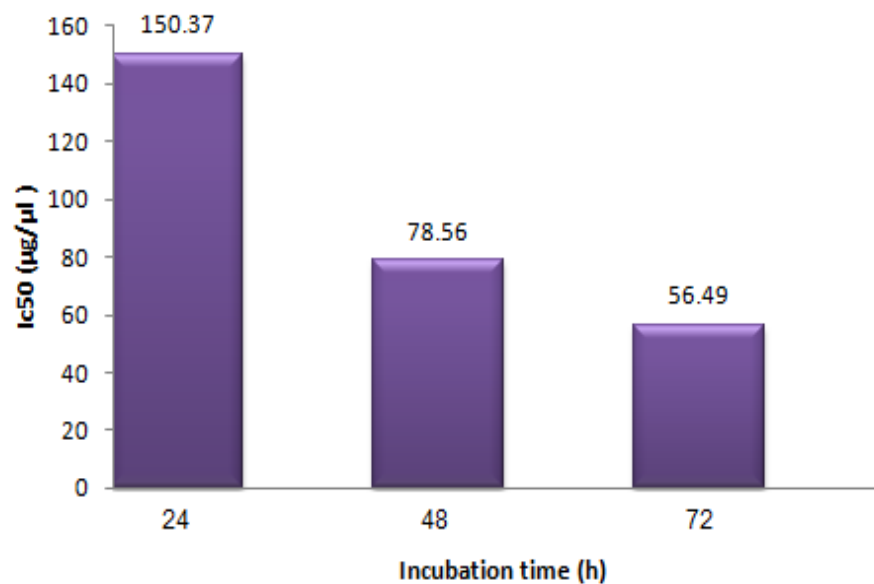


Figure.3 Induction of apoptosis in SKOV-3 cells as detected by flow cytometry. Flow cytometry profile represents Annexin V-FITC staining in x-axis and propidium iodide in y-axis. Dual staining of cells with Annexin V-FITC and propidium iodide enabled categorization of cells into four regions. Region Q1 shows the necrotic cells, Q2 shows the late apoptotic cells, Q3 shows the live cells and Q4 shows the early apoptotic cells. (a) Control cells, (b) the cells exposed to 50 $\mu\text{g}/\mu\text{l}$ silibinin, (c) cells exposed to 75 $\mu\text{g}/\mu\text{l}$ silibinin and (d) cells exposed to 100 $\mu\text{g}/\mu\text{l}$ silibinin for 48 h. Data represent the mean values of three independent experiments

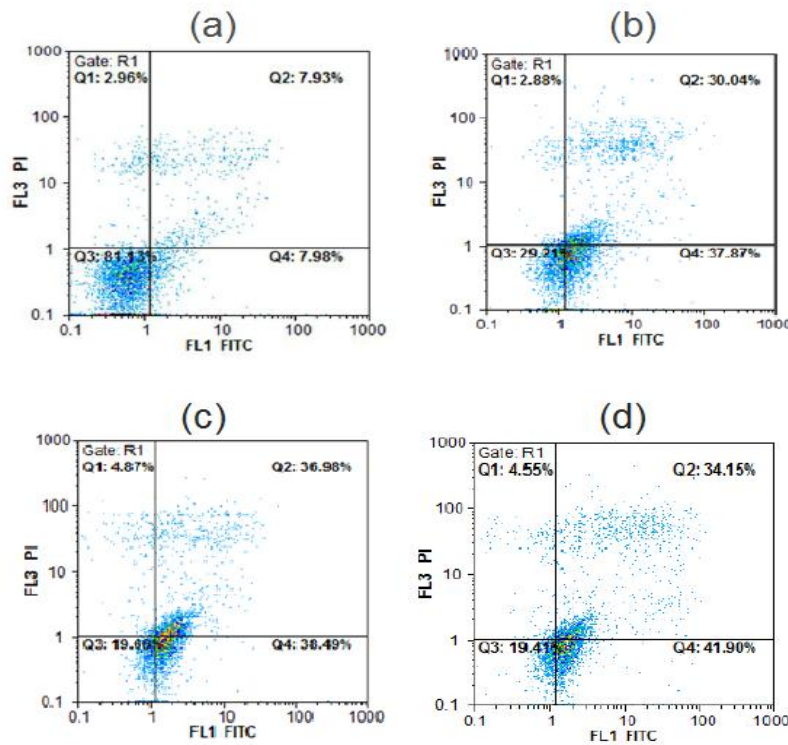
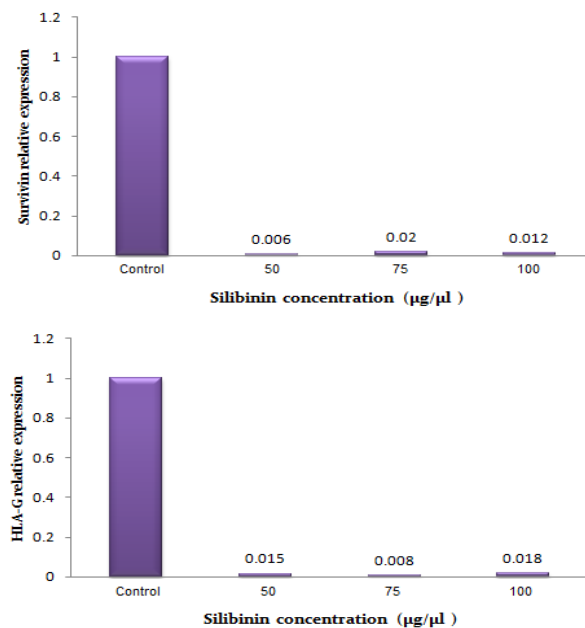


Figure.4 Effects of silibinin on the inhibition of survivin and HLA-G expression in SKOV-3 ovarian cancer cells



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Authors' contributions

Maryam Namazian performed the experiments and statistical analysis. Nasrin Motamed provided guidance, supervision, and suggestions. Elnaz Birjandian wrote the manuscript. All authors discussed the results and approved the final manuscript.

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