

Glucose restriction combined with chemotherapy decreases telomere length and cancer antigen-125 secretion in ovarian carcinoma

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Abstract. Although chemotherapy is the standard treatment for ovarian cancer (OC), recent studies have focused on its coupling with hypoglycemic drugs to decrease glucose availability. Similarly to cancer antigen 125 (Ca-125), telomerase, the key protein for telomere lengthening, is overexpressed in 90% of OC cases. The aim of the present study was to investigate the effect of the combination of glucose restriction and chemotherapy on telomere length and Ca-125 secretion in OC cells. SKOV-3, OVCAR-3 and Igrov-1 cells were treated with 20 μ M cisplatin and 100 nM paclitaxel for 48 h in three different glucose concentrations: i) 4.5 g/l, ii) 1 g/l and iii) 0.5 g/l. The same treatment was repeated once per week for 6 consecutive weeks. The surviving cells were considered platinum-taxane escape (PTES) cells. The expression levels of telomerase and Ca-125 in treated and PTES cells were quantified by qPCR, and Ca-125 secretion by ELISA. Telomere

length was evaluated by qPCR according to the Cawthon method. The modulation of Ca-125 by telomerase was assessed using inhibitors, small interfering RNA and transfection with human telomerase reverse transcriptase (hTERT) vectors. The implication of phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/Akt/mTOR) in Ca-125 modulation was investigated using specific inhibitors. An increase in hTERT and Ca-125 expression levels (range, 1.5-3 fold) was observed in short-term treated cells. However, an opposite effect was detected in PTES cells, where the rate of decrease in the expression levels of hTERT and Ca-125 reached 60% after treatment in 0.5 g/l glucose. Moreover, telomere length was decreased by 30% in cells treated with 0.5 g/l glucose. Inhibition of hTERT expression significantly decreased Ca-125 secretion, suggesting a potential modulation of Ca-125 by hTERT. The inhibition of the PI3K/Akt/mTOR pathway also decreased Ca-125 secretion; however, the effect of this treatment was not enhanced when coupled with telomerase inhibitors. In conclusion, the combination of chemotherapy and glucose restriction was observed to decrease Ca-125 secretion and telomerase expression leading to shortening in telomere length. Thus, decreasing glucose availability for OC cells during treatment may lead to a better clinical outcome and potentially improve the prognosis of patients with OC.

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Abbreviations: BMP2, bone morphogenetic protein 2; Ca-125, cancer antigen 125; CDDP, cisplatin; ERCC1, excision repair cross-complementation group 1; hTERT, human telomerase reverse transcriptase; IL-6, interleukin-6; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; NK, natural killer; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTES, platinum-taxane escape; PTX, paclitaxel; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA

Key words: chemotherapy, glucose restriction, platinum-taxane escape cells, cancer antigen-125, telomerase

Introduction

Ovarian cancer (OC) is one of the most commonly diagnosed types of gynecological cancer worldwide and had the highest mortality rates among all types of cancer of the female reproductive system in 2017 (1). Despite the poorly understood etiology of this cancer type, certain risk factors have been established, which include genetic predisposition and age. By contrast, contraceptive administration and increased parity have been found to be major protective factors (2). The most common subtypes of OC include serous, mucinous, endometrioid and clear cell carcinoma (3). Since OC is asymptomatic during its early stages, it is most commonly diagnosed at late

stages, leading to a poor prognosis and short overall survival time (4). In total, >80% of OC cases exhibit a positive response to standard chemotherapy along with platinum-based and taxane molecules (5); nevertheless, chemoresistance remains a major limitation in cancer treatment (6).

Although cancer antigen 125 (Ca-125) is the major biomarker used for diagnosis and disease monitoring in OC, this glycoprotein is also highly expressed in other types of malignancies, including breast cancer, mesothelioma, non-Hodgkin's lymphoma and gastric cancer (7-11), and is less expressed in some benign cases, including pregnancy and ovulatory cycles (12,13). Despite the functions of Ca-125 being poorly understood, certain studies have suggested the implication of this glycoprotein in the cell-mediated immune response. Ca-125 binds to natural killer (NK) cells via galectin-1 and other antibodies, leading to a reduction in CD16 expression and suppression of the NK cell cytotoxic responses (14,15). Moreover, Ca-125 is able to bind to mesothelin, a 40-kDa protein expressed by mesothelial cells and certain types of cancer cells, including epithelial OC cells. Taking into account that peritoneal cells express the membrane-bound form of mesothelin, this interaction between both proteins may increase the risk of metastasis of Ca-125-expressing tumor cells into the peritoneal cavity (16,17). Furthermore, overexpression of the mucin 16 gene coding for Ca-125 has been observed to increase the risk of resistance to certain chemotherapeutic agents, including cisplatin (18). Ca-125, which is modulated by various factors, regulates the activity of mechanistic target of rapamycin (mTOR) kinase, which in turn controls the expression of its downstream target c-MYC. In addition, knockdown of the MUC16 gene in pancreatic cancer cells has been shown to lead to a decrease in glucose uptake and lactate secretion, and to an alteration of cellular proliferation and metabolism (19).

In >80% of different types of cancer case, telomerase is expressed by cancer cells to ensure their immortalization (20). This enzyme promotes telomere length stabilization and elongation through *de novo* synthesis of repeats lost after DNA replication or oxidative stress (21). Recently, inhibitors of telomerase activity, including BIBR1532, have been investigated as potential adjuvants to platinum-based chemotherapy in OC cells *in vitro* (22). The catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), regulates telomerase activity through the variation of its expression (23). In fact, hTERT mRNA expression is regulated by a number of proteins; however, the transcription factor c-MYC serves an essential role in the activation of this expression by forming a complex with the MYC-associated factor X protein and binding to the E boxes of the promoter region (24). The expression of c-MYC is linked to various signaling pathways, including the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt)/mTOR, Wnt/ β catenin and mitogen-activated protein kinase signaling pathways (25,26).

Increased glucose metabolism is a distinct characteristic of highly proliferative cells, including cancer cells, stem cells and immune cells (27). Even in the presence of oxygen, cancer cells tend to metabolize glucose into lactate instead of undergoing oxidative phosphorylation. This process is termed the Warburg effect (28). Since one of the eight hallmark characteristics of cancer cells is their ability to reprogram glucose

metabolism (29), the effect of glucose restriction on cancer cell proliferation, apoptosis and response to treatment has been recently studied. In fact, glucose levels in cancer patients may be an important prognostic indicator. In OC, increased expression of glucose transporter 1 (GLUT1), a transmembrane protein responsible for glucose uptake, is related to shorter survival time in patients with OC (30). In addition, decreasing glucose availability for colon cancer cells has been reported to contribute to an increase in cell death (31). Moreover, the combination of glucose restriction and autophagy inhibition has been shown to result in decreased tumor growth and cancer cell proliferation (32). Furthermore, a decrease in telomerase activity and a higher response rate to the telomerase inhibitor BIBR-1532 was observed in breast cancer cells cultured in medium with low glucose concentration (33).

Considering the fact that most relapse cases in patients with OC occur due to chemoresistance, mechanisms aiding in reversing resistance or preventing its occurrence should be investigated. Several studies have proven the involvement of hTERT in cancer cell immortalization. Moreover, Ca-125 affects the response of cancer cells to chemotherapy and glucose restriction decreases cancer cell proliferation and viability (34). Based on these facts, the effect of chemotherapy combined with glucose restriction on the expression and activity of Ca-125 and telomerase was assessed in the present study. Additionally, the modulation of Ca-125 expression by hTERT and the possible involvement of the PI3K/Akt/mTOR signaling pathway were investigated.

Materials and methods

Cell culture and drugs. The present study was performed on 3 OC cell lines, namely the Igrov-1 (Institut Gustave Roussy), SKOV-3 and Ovar-3 (both American Type Culture Collection) cell lines. SKOV-3 and Igrov-1 cells were cultured in 4.5 g/l DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (PS; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol, whereas Ovar-3 cells were cultured in F12 medium supplemented with 20% FBS and 1% PS (Sigma-Aldrich; Merck KGaA). All cells were incubated in a humidified incubator at 37°C with 5% CO₂. CDDP (Sigma-Aldrich; Merck KGaA) was freshly dissolved in 0.9% NaCl solution, whereas PTX (Sigma-Aldrich; Merck KGaA) was prepared in DMSO and stored at -20°C.

Short-term combinatorial treatment. The three OC cell types were seeded in 6-well plates (2x10⁵ cells/well) and treated with 20 μ M cisplatin (CDDP) and 100 nM paclitaxel (PTX) for 48 h at 37°C at 80% confluence. This treatment was performed in three different cell culture media: i) DMEM with 4.5 g/l glucose, ii) DMEM with 1 g/l glucose and iii) DMEM with 0.5 g/l glucose. The negative control corresponded to non-treated cells. The supernatant was subsequently collected and the treated cells were subjected to RNA extraction using Nucleospin RNA Extraction kit (Macherey-Nagel, GmbH).

Generation of platinum-taxane escape (PTES) cells (35). To generate PTES cells, 1x10⁵ SKOV-3, Ovar-3 and Igrov-1 cells were seeded in 6-well plates and treated at 37°C with 20 μ M CDDP for 1 h, followed by 100 nM PTX for 3 h once per week

Table I. List of primer sequences.

Gene	Primer sequence (5'-3')
hTERT	F: CGGAAGAGTGTCTGGAGCAA R: CTCCCACGACGTAGTCCATG
Ca-125	F: CTGCATGTACTCCCATCTCTTCAA GAGAGAGATGGGAGTAGATGCAG R: CTGCATCTACTCCCATCTCTCTC TTGAAGAGATGGGAGTAGATGCAG
IL-6	F: TCAATATTAGAGTCTCAACCCCCA R: TTCTCTTTTCGTTCCCGGTGG
IL-8	F: CCACCGGAGCACTCCATAAG R: GATGGTTCCTTCCGGTGGTT
BMP2	F: TTTCAATGGACGTGTCCCCG R: AGCAGCAACGCTAGAAGAA
ERCC1	F: AGGCACAAGTAACAGGCTCAC R: AAGGTCGTAATTCCTTTGCAC
GAPDH	F: TGAGCCAGATAGGCTGGAA R: TAACGCAGGCGATGTTGTC
GLUT-3	F: CCCAGATCTTTGGTCTGGAA R: AAGGGCTGCACTTTGTAGGA
GLUT-1	F: GATGATGCGGGAGAAGAAGG R: AAGACAGCGTTGATGCCAGAC
HIF1 α	F: TATGAGCCAGAAGAAGCTTTAGGC R: CACCTCTTTTGCAAGCATCCTG

hTERT, human telomerase reverse transcriptase; Ca-125, cancer antigen 125; IL-6, interleukin-6; IL-8, interleukin-8; BMP2, bone morphogenetic protein 2; ERCC1, excision repair cross-complementation group 1; GLUT-3, glucose transporter 3; GLUT-1, glucose transporter 1; HIF1 α , hypoxia inducible factor subunit α ; F, forward; R, reverse.

different glucose concentrations. The comparison between the control and treated cells in the same glucose conditions revealed an increase in the mRNA expression of hTERT in the treated cells (range, 1.5-2.5 fold; Fig. 1A). Moreover, this short-term treatment also led to an increase in Ca-125 mRNA levels (range, 1.5-3 fold; Fig. 1B). As presented in Fig. 1C, an approximate 2-fold increase was observed in the secretion of Ca-125 in the Ovar-3 cells; however, Ca-125 secretion by the SKOV-3 and Igrov-1 cells was undetectable by ELISA. Nevertheless, no significant difference was observed among the cells treated in different glucose conditions, indicating that the expression of both proteins was not affected by the decrease in glucose levels during the treatment.

Generation of PTES cells. For the development of OC cell lines with double resistance to cisplatin and paclitaxel, the SKOV-3, Ovar-3 and Igrov-1 cell lines were treated with CDDP and PTX once per week for 6 consecutive weeks. The doses of the chemotherapeutic agents (20 μ M CDDP and 100 nM PTX) were chosen according to the peak plasma levels reached when 100 mg/m² of CDDP and 175 mg/m² of PTX were administered intravenously in patients with ovarian cancer (35). The cells were treated with CDDP for 1 h and PTX for 3 h.

The treatment durations correspond to the half-life of these chemotherapeutic agents in the human body (35). The treatment was performed in culture media of the three cell lines with the following glucose concentrations, which correspond to the physiological alterations observed in blood glucose levels: i) 4.5 g/l glucose, high; ii) 1 g/l glucose, low; and (iii) 0.5 g/l glucose, fasting.

After 6 weeks of treatment, the remaining living cells were considered as PTES cells. Six PTES cell types were generated: PTES SKOV-3 cultured in three different glucose concentrations and PTES Igrov-1 cultured in these same conditions. Despite their resistance to clinically relevant concentrations of cisplatin, PTES Ovar-3 cells could not be generated. In order to confirm the decrease in the sensitivity of PTES cells to additional chemotherapeutic challenges, these cells were subjected to a single exposure of higher doses of CDDP, PTX and a combination of both treatments in the same glucose conditions as the initial treatment. According to the obtained results, SKOV-3 and Igrov-1 PTES cells required higher concentrations of the chemotherapeutic treatment to reach the same levels of proliferation observed in the parental cells treated with lower doses (Fig. 2). The chemoresistance profile of the PTES cells was evaluated by quantifying the mRNA expression levels of the IL-6, IL-8 and BMP2 proteins, implicated in environment-mediated drug resistance, and the ERCC1 protein, which is directly involved in DNA repair (39). The obtained results revealed a significant increase in the expression of these mRNAs in all PTES cells, indicating that these cells may have developed resistance to the chemotherapeutic treatment (Fig. 3). Additional investigations concerning the effect of the combination of chemotherapy with induced hypo-, normo- and hyperglycemia on the expression of GLUT1, GLUT3 and HIF1 α were performed by measuring the mRNA expression levels of these genes in control and PTES cells. The results revealed a significant increase in the expression of both glucose transporters in PTES cells treated with the three glucose concentrations, with no significant difference between them (Fig. 4A and B). However, HIF1 α expression level was significantly increased only in SKOV-3 4.5 PTES cells (Fig. 4C).

Decrease of telomere length and hTERT and Ca-125 expression in PTES cells. Next, the effect of this combined long-term (6 weeks) treatment on the expression of hTERT, the catalytic subunit of telomerase, was assessed in all PTES cell lines. The results demonstrated a 20% decrease in the hTERT mRNA expression levels of PTES SKOV-3 cells treated with 4.5 g/l of glucose; however, this decrease reached 40% with 0.5 g/l of glucose. A similar effect was observed in the PTES Igrov-1 cells, where the reduction ranged from 15% with 4.5 g/l glucose to 60% with fasting glucose concentrations (Fig. 5A). Telomere length was subsequently evaluated to assess the effect of this reduction on telomerase expression. A non-significant decrease was observed in PTES SKOV-3 and Igrov-1 cultured with 4.5 and 1 g/l glucose. However, the decrease in telomere length was greater in the both PTES cell lines treated with fasting glucose concentrations (Fig. 5B). Additionally, the Ca-125 mRNA expression levels were quantified in chemosensitive and chemoresistant cells. It was observed that Ca-125 expression decreased in a similar manner as the hTERT

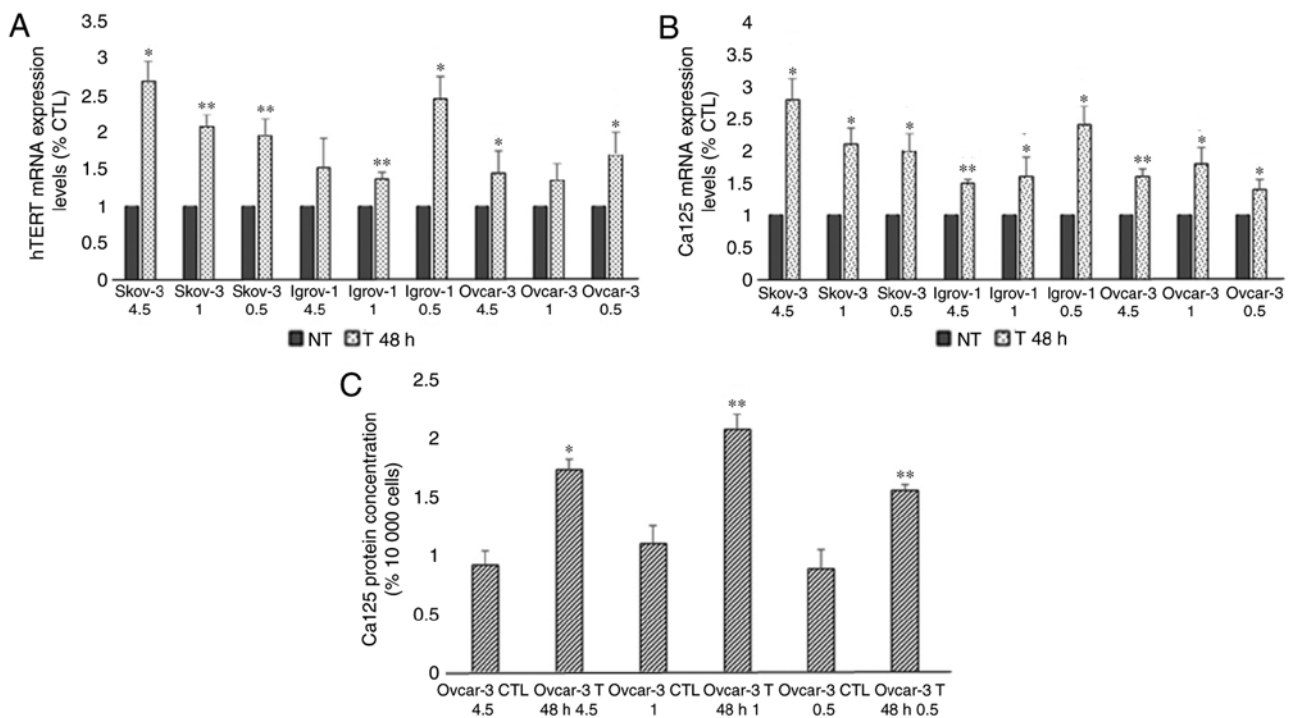


Figure 1. Effect of 48-h combinatory treatment of CDDP, PTX and glucose restriction on hTERT and Ca-125 expression and secretion. SKOV-3, Ovar-3 and Igrov-1 cells were seeded in 6 well-plates. These cells were treated with three different glucose concentrations (4.5, 1 and 0.5 g/l) with 20 μ M CDDP and 100 nM PTX for 48 h. Supernatant was collected following the treatment in order to quantify secreted Ca-125 using ELISA. The cells were then harvested, and RNA was isolated for hTERT and Ca-125 mRNA quantification by reverse transcription-quantitative polymerase chain reaction. (A and B) hTERT and Ca-125 mRNA expression levels were found to be increased in all treated cells; these results did not appear to be significantly affected by the different glucose levels. (C) A similar effect was observed with regard to Ca-125 secretion in the treated Ovar-3 cells. The values displayed in this figure refer to values adjusted to 10,000 cells, since cell number usually affects the levels of secreted proteins. Cell count was performed following cell harvesting and values were normalized to 10,000 cells. Each value represents the mean from three different experiments. The control group was set to 1, and the remaining values were compared with the control. The results are presented as the mean \pm SD. * P <0.05 vs. control group, and ** P <0.01 vs. control group. CTL, control; CDDP, cisplatin; PTX, paclitaxel; hTERT, human telomerase reverse transcriptase; Ca-125, cancer antigen 125; NT, non-treated cells.

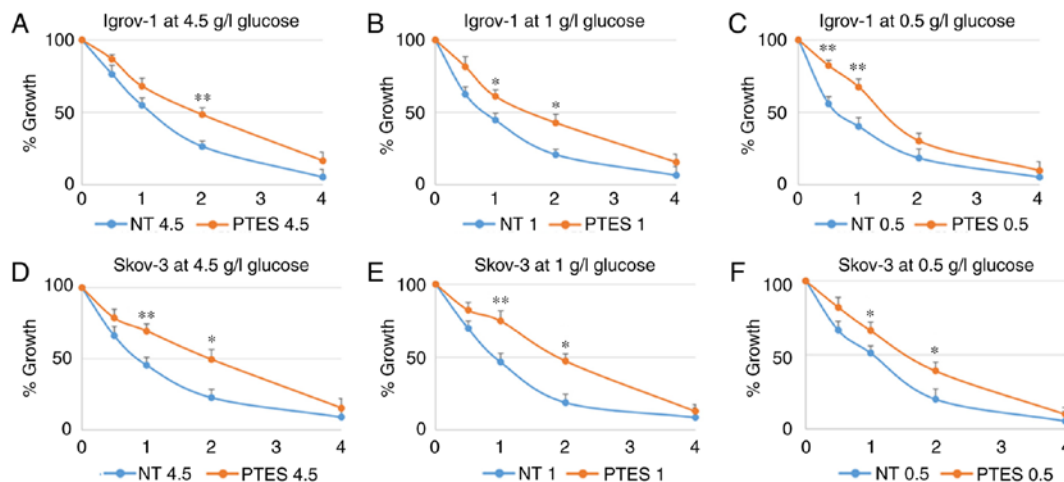


Figure 2. Characterization of SKOV-3 and Igrov-1 cells with induced resistance to CDDP and PTX. Igrov-1 (A-C) and SKOV-3 (D-F) control and PTES cells were treated with a single exposure of increasing doses of CDDP + PTX. The numbers presented on the x-axis (1, 2, 3 and 4) correspond to CDP1, CDP2, CDP3 and CDP4, respectively. CDP 1 indicates the treatment of PTES cells with one single physiological dose of the chemotherapeutic agents (20 μ M CDDP for 1 h + 100 nM PTX for 3 h). CDP 2 indicates the treatment of PTES cells with twice the physiological dose, CDP 3 with triple the dose, and CDP 4 with four times the physiological dose. The control group value was set to 1 and the remaining values were compared with the control. The results are presented as the mean \pm SD. * P <0.05 compared with the control group and ** P <0.01 compared with the control group. CDDP, cisplatin; PTX, paclitaxel; PTES, platinum-taxane escape; NT, non-treated cells.

expression; however, the reduction ranged between 60 and 80% in the PTES SKOV-3 cells and between 65 and 85% in the PTES Igrov-1 cells (Fig. 5C).

Effect of hTERT inhibition on Ca-125 expression and secretion. Based on the aforementioned results, a parallel change in the expression of hTERT and Ca-125 was observed after both

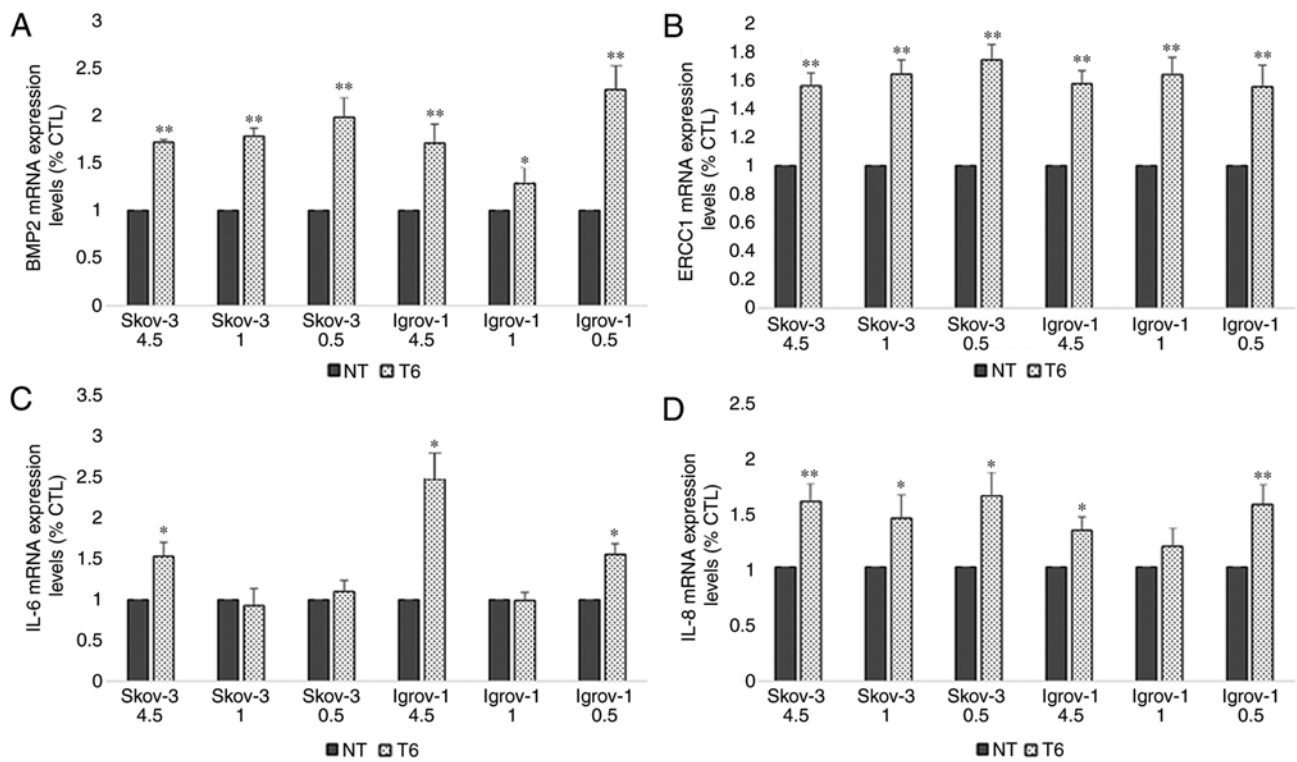


Figure 3. Effect of 6-week treatment on markers of chemoresistance in PTES cells. Cells from the three ovarian cancer cell lines were seeded in 6-well plates. These cells were cultured with 4.5, 1 and 0.5 g/l glucose concentrations 48 h prior to the treatment. Next, they were treated with 20 μ M CDDP for 1 h, followed by 100 nM PTX for 3 h. This procedure was repeated once per week for six consecutive weeks. Subsequently, the cells were harvested and subjected to RNA extraction, followed by reverse transcription-quantitative polymerase chain reaction for (A) BMP2, (B) ERCC1, (C) IL-6 and (D) IL-8. A significant increase was observed in the expression of the majority of these proteins under the different conditions. The observed values represent the mean from three different experiments. Using the $2^{-\Delta\Delta C_q}$ method, the control group was set to 1, and the remaining results were compared with the control. The results are presented as the mean \pm SD. * $P < 0.05$ vs. control group, and ** $P < 0.01$ vs. control group. PTES, platinum-taxane escape; CDDP, cisplatin; PTX, paclitaxel; BMP2, bone morphogenetic protein 2; IL-6, interleukin-6; IL-8, interleukin-8; ERCC1, excision repair cross-complementation group 1.

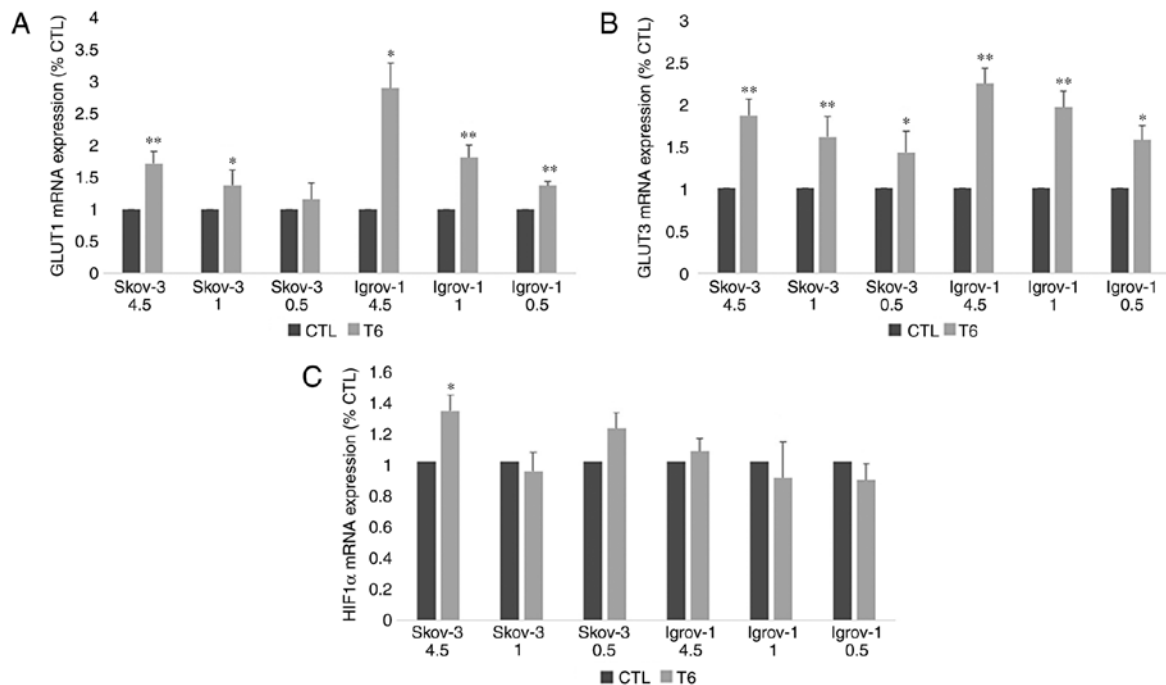


Figure 4. Effect of 6-week treatment in hyper-, normo- and hypoglycemic conditions on glucose transporters and HIF1 α expression in PTES cells. Control and PTES cells were harvested and subjected to RNA extraction, followed by reverse transcription-quantitative polymerase chain reaction for GLUT1 (A), GLUT3 (B) and HIF1 α (C). A significant increase was observed in the expression of the glucose transporters under the different conditions. The observed values represent the mean from three different experiments. Using the $2^{-\Delta\Delta C_q}$ method, the control group was set to 1, and the remaining results were compared with the control. The results are presented as the mean \pm SD. * $P < 0.05$ vs. control group, and ** $P < 0.01$ vs. control group. CTL, control; HIF1 α , hypoxia inducible factor 1 subunit α ; PTES, platinum-taxane escape; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3.

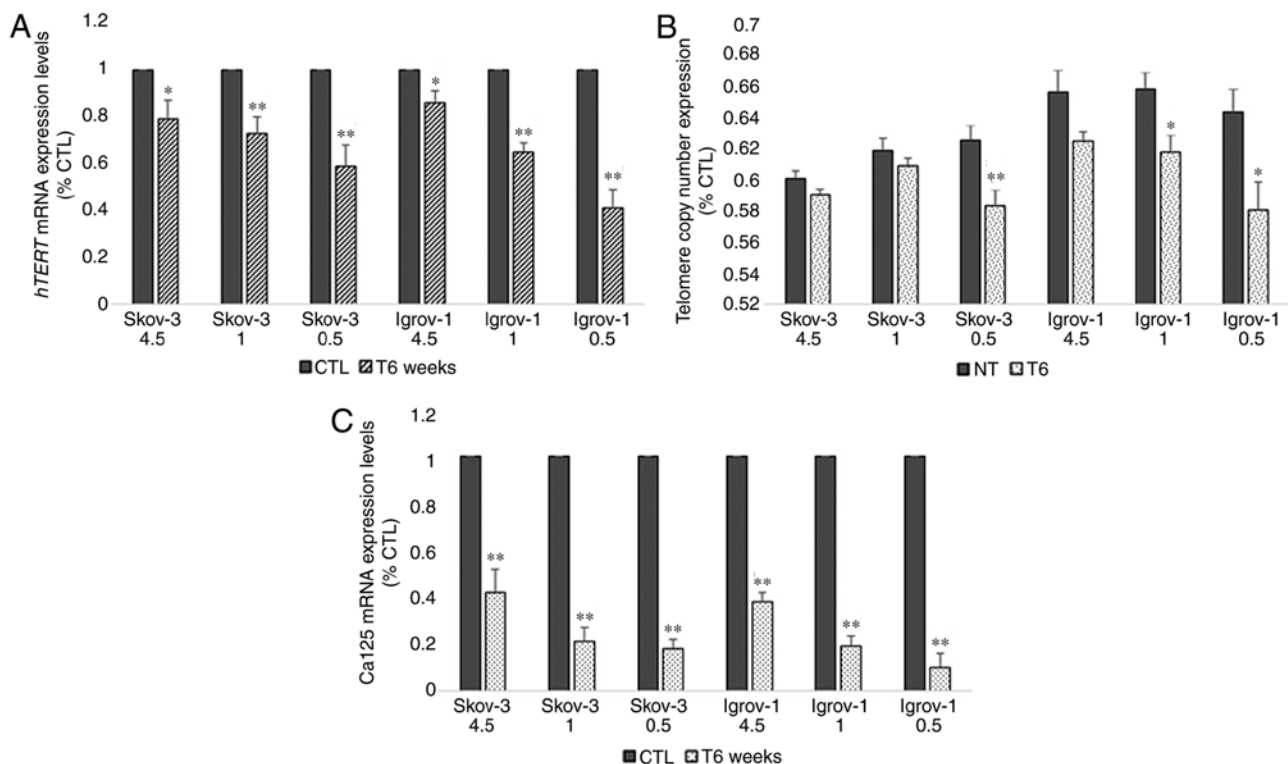


Figure 5. hTERT and Ca-125 mRNA expression and telomere length in PTES cells. Cells from the 6 PTES cell lines and their corresponding controls were harvested, subjected to RNA extraction to quantify hTERT and Ca-125 mRNA expression by reverse transcription-quantitative polymerase chain reaction and subjected to DNA extraction for telomere length quantification by the telomere length assay following Cawthon method. (A and C) A significant decrease was observed in the mRNA expression of both hTERT and Ca-125; the lowest levels were observed in cells treated with 0.5 g/l glucose. (B) The decrease in telomere length was observed in all PTES cells; however, a significant decrease was demonstrated only in cells treated with fasting glucose concentrations. The values observed represent the mean of three different experiments. The results are presented as the mean \pm SD. * $P < 0.05$ vs. control group, and ** $P < 0.01$ vs. control group. CTL, control; hTERT, human telomerase reverse transcriptase; PTES, platinum-taxane escape; Ca-125, cancer antigen 125.

short-term and long-term treatments. Taking into consideration that telomerase regulates the expression of various genes, the role of hTERT in the modulation of Ca-125 expression and secretion was investigated. Thus, chemosensitive cells from the three cell lines were treated with three telomerase inhibitors: BIBR1532 at 5 and 10 μ M, costunolide at 5 and 10 μ M and MST-312 at 1 and 2 μ M. The concentrations of these inhibitors were selected according to a dose-response curve, which demonstrated that they did not exert any cytotoxic effects. The results indicated a significant decrease in the mRNA and protein expression of Ca-125 in the three cell lines after treatment with all three inhibitors compared with the control. However, the highest effect was observed with BIBR1532 at 5 μ M for SKOV-3 and Ovar-3 and at 10 μ M for Igrov-1 (Fig. 6A and B). Transfection of all three cell lines with hTERT siRNA was performed to confirm the direct involvement of hTERT in Ca-125 modulation and to exclude the possibility of extratelomeric action of the hTERT inhibitors. In order to make sure that the transfection was successful, hTERT mRNA expression was assessed in control and transfected cells. The results (Fig. 7A) demonstrated a significant decrease (~90%) in hTERT expression level in transfected cells compared with control cells. As expected, the mRNA expression pattern obtained by gene silencing was similar to that obtained after treatment with the inhibitors (Fig. 6C and D). A significant decrease was observed in the mRNA expression of Ca-125 in the three cell lines, and in the protein expression of Ca-125 in Ovar-3 cell line following hTERT silencing.

Effect of hTERT overexpression on Ca-125 expression and secretion. After investigating the effect of hTERT inhibition, the SKOV-3, Ovar-3 and Igrov-1 cells were transfected with pBabe-neo-hTERT to evaluate the effect of telomerase overexpression on Ca-125 expression and secretion. In order to make sure that the transfection was successful, hTERT mRNA expression was assessed in control and transfected cells. The results (Fig. 7B) demonstrated a significant 25-30 fold increase in hTERT expression level in transfected cells compared with control cells. The results indicated a significant increase affecting both the mRNA and protein levels of Ca-125 after 48 h of transfection. Notably, an increase of ~30% in the SKOV-3 and Ovar-3 cells, and an increase of 50% in Igrov-1 cells was observed with regards to Ca-125 mRNA expression, compared with the control (Fig. 6E and F).

Effect of PI3K/Akt/mTOR signaling pathway inhibition on Ca-125 expression and secretion. As aforementioned, previous studies have investigated the potential link between Ca-125 and the protein mTOR. Since this protein is implicated in the PI3K/Akt/mTOR signaling pathway and a mutual modulation links hTERT to this pathway (40), the regulation of Ca-125 expression and secretion by PI3K/Akt/mTOR was examined. The SKOV-3, Ovar-3 and Igrov-1 cells were treated with various inhibitors specific to proteins in this pathway for 48 h. The decrease in Ca-125 mRNA expression reached 60% after treatment with PI3K and Akt inhibitors (PI828, GSK 2126458

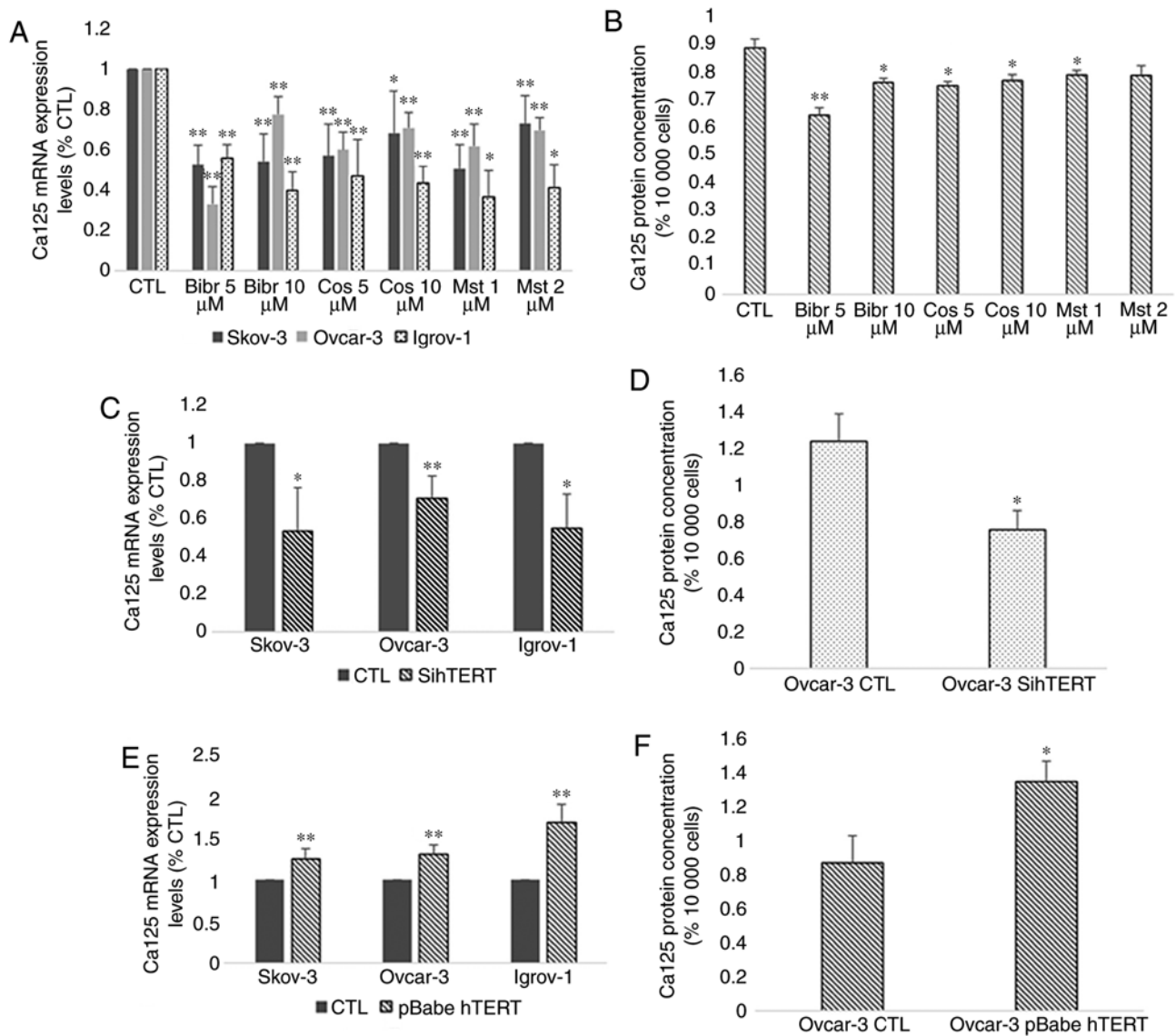


Figure 6. Effect of hTERT inhibition and overexpression on Ca-125 mRNA and protein expression. Cells from the three cell lines were cultured in 6-well plates. At 80% confluence, these cells were treated with telomerase inhibitors BIBR1532 (5 and 10 μ M), costunolide (5 and 10 μ M) and MST-312 (1 and 2 μ M). Next, the supernatant was collected, and the cells were harvested for RNA extraction. Ca-125 mRNA expression was assayed by reverse transcription-quantitative polymerase chain reaction and protein secretion levels by ELISA. (A and B) A significant decrease was observed after treatment with all inhibitors compared with the control; however, the greatest effect was caused by BIBR1532. In addition, the cells were transfected with siRNA for hTERT and pBabe-neo-hTERT to evaluate the effect of both gene silencing and gene overexpression on Ca-125. (C and D) The effect of siTERT was similar to the effect of hTERT inhibitors. (E and F) However, transfection with pBabe-neo-hTERT caused a significant increase in both mRNA and protein levels of Ca-125 compared with the control. The values displayed in this figure refer to values adjusted to 10,000 cells, since cell number usually affects the levels of secreted proteins. Cell count was performed following cell harvesting and values were normalized to 10,000 cells. Each experiment was performed at least three times, and the values included in these graphs represent the mean from these experiments. The results are presented as the mean \pm SD. * P <0.05 vs. control group, and ** P <0.01 vs. control group. CTL, control; hTERT, human telomerase reverse transcriptase; siTERT, siRNA for hTERT; siRNA, small interfering RNA; Ca-125, cancer antigen 125.

and Wortmanin for PI3K, and GSK 69029 for Akt) in the Igrov-1 cells and 40% with the mTOR inhibitor rapamycin (Fig. 8A). mRNA expression and protein secretion in the treated Ovar-3 cells exhibited similar results (Fig. 8A and B, respectively). Thus, the PI3K/Akt/mTOR signaling pathway was indicated as a potential regulator of Ca-125 in OC cells.

Effect of the combination of telomerase and PI3K/Akt/mTOR inhibitors on Ca-125. hTERT and the PI3K/Akt/mTOR pathway are both regulators of Ca-125 in OC cells. Taking into account that telomerase is an activator of this signaling pathway, the possible regulation of Ca-125 by hTERT via

PI3K/Akt/mTOR was investigated. The PTES cells were treated with telomerase inhibitors (BIBR-1532, Costunolide and MST-312) combined with PI3K, Akt and mTOR inhibitors for 48 h. However, this combination did not potentiate the effects observed on Ca-125 mRNA and protein levels in all three cell lines (Fig. 9).

Discussion

Resistance to chemotherapy is a major limitation in the treatment of various types of cancer, including OC. Several mechanisms are responsible for the onset and development of

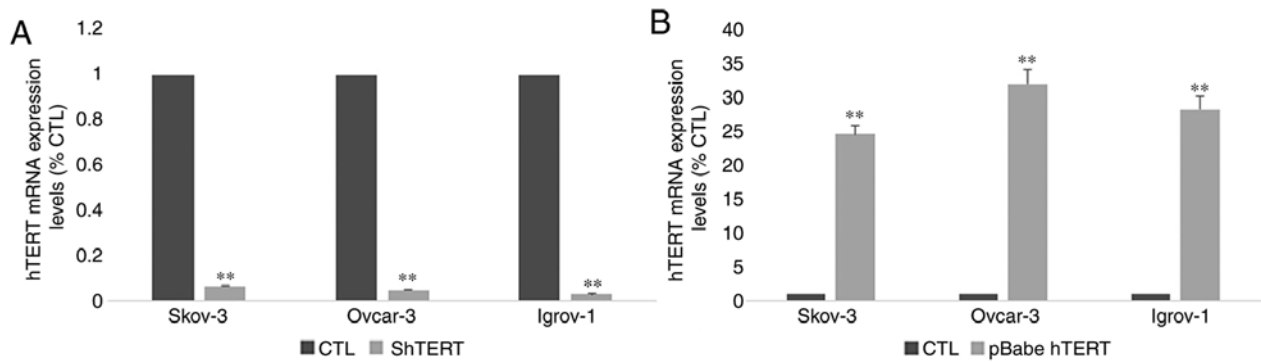


Figure 7. Effect of sihTERT and pBabe-neo-hTERT transfection on hTERT mRNA expression levels. After harvesting transfected cells, mRNA expression of hTERT was assessed using quantitative polymerase chain reaction, in order to ensure that the procedure was completed successfully. (A) The results demonstrated a significant decrease of >90% in hTERT mRNA expression levels after silencing the expression of this gene using sihTERT. (B) On the contrary, hTERT expression levels exhibited a ~25-fold increase after transfection of the three types of ovarian cancer cells with pBabe-neo-hTERT. Each experiment was performed at least 3 times, and the values included in these graphs represent the mean from these experiments. The results are presented as the mean \pm SD. **P<0.01 vs. control group. hTERT, human telomerase reverse transcriptase; siRNA, small interfering RNA.

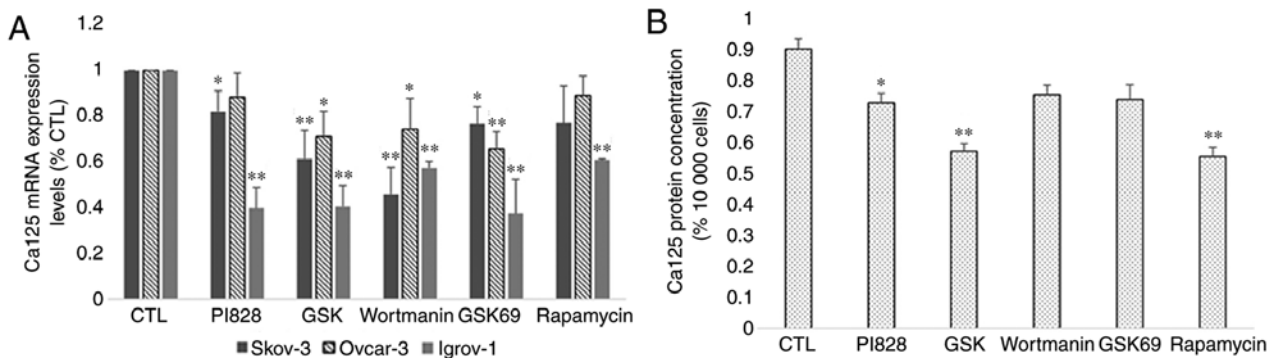


Figure 8. Effect of PI3K/Akt/mTOR inhibitors on Ca-125 mRNA and protein expression. SKOV-3, Ovar-3 and Igrov-1 cells were seeded in 6-well plates. At 80% confluence, these cells were treated with inhibitors of PI3K (PI828, wortmanin and GSK; 10 μ M), Akt (GSK690693; 100 nM) and mTOR (rapamycin; 200 nM) for 48 h. Next, the supernatant was collected for quantification of secreted Ca-125 by ELISA, and the cells were harvested for RNA extraction, followed by reverse transcription-quantitative polymerase chain reaction for quantifying Ca-125 mRNA expression. (A) A significant decrease was observed in the majority of cells treated with the inhibitors; however, Igrov-1 exhibited the lowest values. (B) This decrease was also observed in protein secretion in the treated Ovar-3 cells. Ca-125 level in the supernatants of SKOV-3 and Igrov-1 cell lines were undetectable using ELISA. The values displayed in this figure refer to values adjusted to 10,000 cells, since cell number usually affects the levels of secreted proteins. Cell count was performed following cell harvesting and values were normalized to 10,000 cells. Each experiment was performed at least 3 times, and the values listed in these graphs represent the mean from these experiments. The results are presented as the mean \pm SD. *P<0.05 vs. control group, and **P<0.01 vs. control group. Ca-125, cancer antigen 125.

chemoresistance, including the activation of DNA repair mechanisms (41), the development of sequential genetic alterations inducing transient environment-mediated drug resistance (42) and the presence of cancer-initiating cells embedded within the tumor (43). To overcome chemoresistance, a previous study focused on the development of new strategies and treatment combinations, including the establishment of second-line chemotherapy or coupling standard treatment regimens with immunotherapy (44). Another recently explored strategy is based on the targeting of cancer cell metabolism to improve its response to therapeutics. A previous study demonstrated the effect of targeting certain glycolytic enzymes to overcome resistance to trastuzumab in breast cancer cells (45). Furthermore, a previous study on breast cancer highlighted the potential use of lactate dehydrogenase A as a target to reverse resistance to PTX (46). Thus, glucose availability and metabolism in cancer serve important roles in the sensitivity of cells to chemotherapeutic drugs. Based on these facts, the effect of chemotherapy combined with glucose restriction

on the development of chemoresistance was evaluated in the present study. Moreover, the outcome of this combinatorial treatment on the immortality of cancer cells was investigated by studying telomerase expression and activity, and the ability of these cells to secrete the serum marker Ca-125. The PTES cells were initially treated with the chemotherapeutic agents CDDP and PTX for 48 h in 3 different glucose concentrations. The three concentrations were chosen so as to mimic the physiology of a patient following high, low and fasting glucose diets. Following this short-term treatment, expression of both hTERT and Ca-125 increased significantly. A similar effect on hTERT expression was observed in a previous study after treatment of hepatocellular carcinoma cells with low doses of cisplatin for 24 h. It was reported that increased expression of the transcription factor c-MYC resulted in hTERT upregulation (47). In addition, higher Ca-125 levels after short-term treatment may be explained by the implication of this protein in the modulation of the response of OC cells to genotoxic drugs, including cisplatin (48). However, no significant

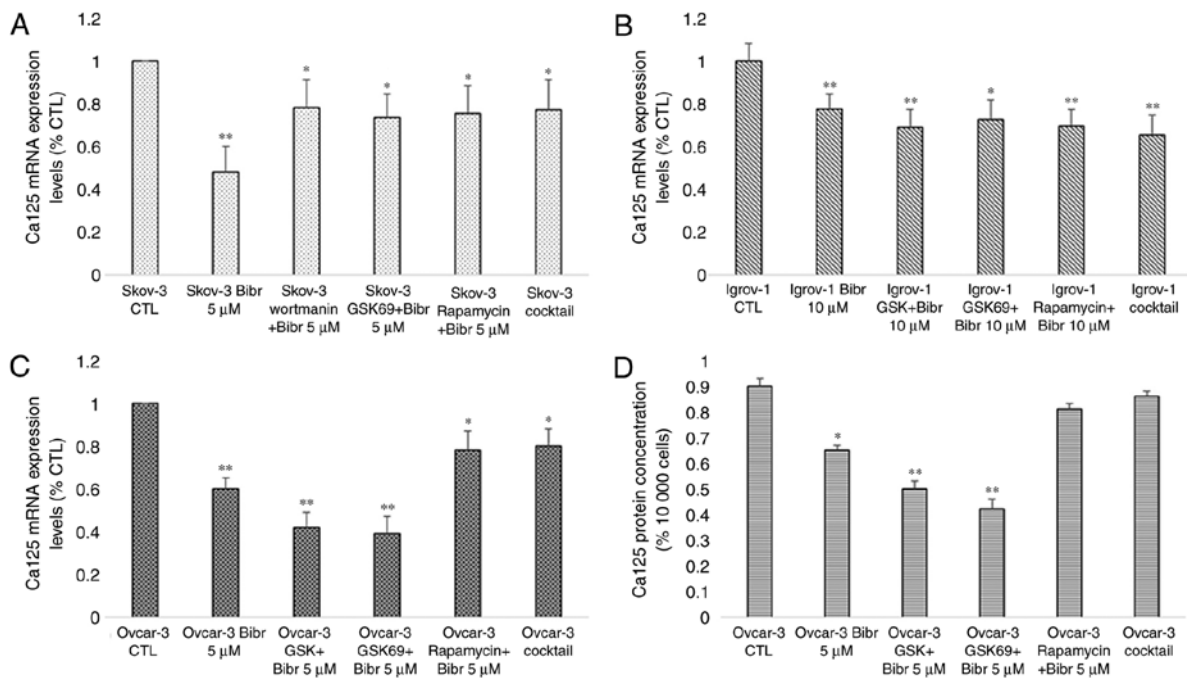


Figure 9. Effect of hTERT and PI3K/Akt/mTOR simultaneous inhibition on Ca-125 expression and secretion. Cells were seeded in 6-well plates and treated at 80% confluence with a combination of hTERT and PI3K/Akt/mTOR pathway inhibitors. Inhibitors of telomerase and PI3K were chosen according to the highest effect on Ca-125 observed in the previous experiments. RNA extraction followed by reverse transcription-quantitative polymerase chain reaction was performed for mRNA expression and ELISA for Ca-125 protein secretion. (A-C) Combination of the inhibitors did not exhibit an additive nor a synergic effect on Ca-125 mRNA expression. (D) A similar result was observed with regard to the protein secretion levels in the Ovcar-3 cells. The term 'Cocktail' refers to the combination of all inhibitors together in one treatment. The values displayed in this figure refer to values adjusted to 10,000 cells, since cell number usually affects the levels of secreted proteins. Cell count was performed following cell harvesting and values were normalized to 10,000 cells. Each experiment was performed at least 3 times, and the values listed in these graphs represent the mean from these experiments. The results are presented as the mean \pm SD. * P <0.05 vs. control group, and ** P <0.01 vs. control group. hTERT, human telomerase reverse transcriptase; Ca-125, cancer antigen 125.

difference was detected among the cells treated with various glucose concentrations. This may be explained by the short period of exposure to glucose restriction and cell adaptation that requires additional time for metabolic flux changes.

After the long-term treatment of cells with specific concentrations of CDDP and PTX, the expression levels of IL-6, IL-8, BMP2 and ERCC1, which are implicated in the initiation and development of resistance to chemotherapy, were evaluated. The increased expression of all these proteins observed in the PTES cells compared with the control explains the ability of these cells to survive 6 weeks of treatment. These proteins are associated with various mechanisms implicated in chemoresistance. For instance, ERCC1 is involved in the nucleotide excision repair mechanism. Interleukin expression corresponds to the environment-mediated drug resistance developed by the PTES cells (49). However, no significant difference was observed in the expression of these markers among the PTES cells treated with various glucose concentrations. Thus, decreasing glucose availability during treatment may not directly affect the ability of cancer cells to develop chemoresistance.

Following several cellular divisions, the telomeric ends of the chromosomes reach a critical length, leading to the activation of apoptosis. However, telomerase serves a key role in the elongation of these telomeres in cancer cells. To assess the effect of the combinatorial treatment on the immortalization of the PTES cells, the mRNA expression of the catalytic subunit hTERT was examined in the present study. Previous studies revealed that telomerase expression was increased

in osteosarcoma cisplatin-resistant cells, thus leading to the suppression of cisplatin-induced apoptosis (50). However, hTERT expression was significantly decreased in the PTES cells in the present study. This decrease was greater in the PTES cells treated with fasting glucose concentrations. To further investigate the effect of this reduction, the length of the telomeres in control and resistant cells was assessed. A decrease in telomere length was observed; however, the shortening of telomeres was statistically significant only in the PTES cells treated with fasting glucose concentrations. The non-significant results for 4.5 and 1 g/l glucose concentrations may be explained by the fact that slight decreases affecting telomerase expression and availability do not necessarily lead to a shortening in telomeres, since telomerase has the ability to elongate the chromosomal telomeric ends, even if present in low quantities (51). Based on these findings, it may be suggested that the combination of chemotherapy and glucose restriction could lead to a decrease in both hTERT expression and telomere length in PTES cells, which in turn may result in a decrease in cancer cell immortalization.

Ca-125 is a key regulator of the metastasis of OC cells into the peritoneal cavity due to its strong affinity to mesothelin. Moreover, the sensitivity of OC cells to cisplatin has been narrowly linked to the expression of the surface marker Ca-125. Based on these facts, the changes in the expression of Ca-125 were investigated in both PTES and control groups. A decrease was observed in Ca-125 expression, which was lowest after treatment with fasting glucose concentrations. These

findings are contradictory to those of previous studies stating that increased expression of the Ca-125 gene leads to a decrease in the sensitivity of cells to genotoxic drugs, including cisplatin. Other studies have demonstrated that increased levels of Ca-125 at the end of the chemotherapeutic treatment are considered as a marker of poor prognosis and are associated with shorter progression-free and overall survival rates (52). The results of the present study revealed that the combination of chemotherapy with fasting glucose levels during the treatment resulted in the lowest Ca-125 expression in all three types of PTES cells. Thus, this combination may contribute to improved survival rates in patients with OC, coupled with decreased levels of cancer cell immortalization due to the shortening of telomeres.

Based on the aforementioned results, telomerase and Ca-125 expression varied similarly after both short- and long-term treatments. To explain the low levels of Ca-125 observed after 6 weeks of chemotherapy, the possible regulation of Ca-125 gene expression by hTERT was investigated. In addition to its canonical function, one of the non-canonical functions of hTERT is its ability to regulate the expression of several genes in cancer (53). The decrease observed after the inhibition of hTERT by inhibitors (BIBR-1532, Costunolide and MST-312) and the silencing of the gene by hTERT siRNA demonstrated that hTERT regulates Ca-125 expression in OC. Moreover, the overexpression of Ca-125 after transfection of the PTES cells with pBabe-neo-hTERT verified the association between both proteins. A previous study reported that Ca-125 serum levels were higher in patients with telomerase-positive OC compared with patients with telomerase-negative OC (54). Moreover, a clinical study linking hTERT expression in ovarian biopsies and serum Ca-125 to the grade and stage of OC revealed a linear relationship between these two biomarkers (55). Therefore, it may be postulated that the decrease in hTERT expression in PTES cells may account for the decline in Ca-125 expression and secretion.

Telomerase regulates the expression of several genes either directly or via its ability to control various signaling pathways, including the PI3K/Akt/mTOR pathway. Previous studies have highlighted the modulation of Ca-125 expression by mTOR via the transcription factor c-MYC; therefore, the possible implication of the PI3K/Akt/mTOR pathway in the modulation of Ca-125 by hTERT was investigated. The results observed after the treatment with inhibitors of the pathway alone indicate a possible implication of this pathway in the regulation of Ca-125 expression. However, when combined with hTERT inhibitors, the treatment did not significantly potentiate the effect of the signaling pathway inhibitors when used as a mono-treatment. Thus, other signaling pathways may be involved in the regulation of Ca-125 by hTERT. A possible pathway may be the nuclear factor- κ B pathway. In fact, mesothelin, a protein modulated by the activation of this pathway, interacts with Ca-125 to promote peritoneal metastasis (56).

To the best of our knowledge, the present study was the first to demonstrate the effect of chemotherapy combined with glucose restriction on the immortalization and metastasis of OC cells. When neoadjuvant or adjuvant chemotherapy was administered under fasting glucose conditions, telomerase expression was found to significantly decrease, thus contributing to the shortening of chromosomal telomeric ends, which in turn decreases the immortalization of cancer cells. Since telomerase serves an essential role in cisplatin resistance,

this decrease may improve the response to chemotherapy. Moreover, this combined treatment was demonstrated to decrease Ca-125 expression and secretion, which could contribute to improved prognosis, since it is able to reduce the risk of peritoneal metastasis. Furthermore, since the combination of chemotherapy with hypoglycemic drugs is currently used in the treatment of various cancer types, including OC, the present study indicates a potential advantage in using this treatment regimen in both neoadjuvant and adjuvant forms.

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Availability of data and materials

All data generated or analyzed during this study are included in the published article.

Authors' contributions

SA performed cell culture, cell treatments, RT-qPCR, ELISA and transfections, participated in the design of the study and wrote the manuscript. DA participated in the design of the study and manuscript. RT supervised and participated in the experimental work (cell culture and treatments), troubleshooting and data analysis. MDA made substantial contributions to analysis and interpretation of data. MM participated in the design of the manuscript. ENA and GH were responsible for the clinical data interpretation. GC made substantial contributions to interpretation of clinical data. GH designed the study, provided guidance and edited the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Saint Joseph University (Beirut, Lebanon).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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