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ORIGINAL ARTICLE

Topoisomerase I Inhibitor Suppress Tumor Growth in Chemoresistant Ovarian Cancer-Initiating Cells

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Background: To investigate the role of a topoisomerase I inhibitor (topotecan) in chemoresistant ovarian cancer-initiating cells. **Materials and Methods:** We isolated ovarian cancer-initiating cells (CP70 side-population cells) from the CP70 cell line using FACS Aria-based sorting and cultured them in suspension to form spheroids (CP70 side-population sphere [SPS]). Gene expression was assessed by microarray, to identify potentially effective chemotherapeutic drugs. An MTS assay was used to evaluate cell growth. **Results:** CP70 SPS cells showed significant resistance to the chemotherapeutic drugs cisplatin and paclitaxel. Microarray analysis demonstrated a high expression of topoisomerase-related genes in CP70 SPS cells. Topotecan inhibited ovarian cancer-initiating cells (CP70 SPS) *in vitro* more than it did their parental CP70 cells. This result was confirmed in tissues from human patients. **Conclusions:** Chemoresistant ovarian cancer-initiating cells exhibited high expression levels of topoisomerase, which could be an alternative target of adjuvant therapy for patients with chemoresistant ovarian cancer.

Key words: Chemoresistance, ovarian cancer-initiating cells, topoisomerase I inhibitor, topotecan

INTRODUCTION

Ovarian cancer is the main cause of cancer-associated mortality in gynecological malignancies. The combination of cytoreductive surgery and chemotherapy using paclitaxel and carboplatin is the principal treatment of epithelial ovarian cancer (EOC).[1] Although, the overall and complete response rates in advanced disease are 80% and 40-60%, respectively, most patients eventually relapse after first-line treatment with paclitaxel and carboplatin, with a median progression-free survival of 18 months.[2]

It is currently considered that repopulation of cancer stem cells (CSCs) causes the recurrence of cancer.[3] CSCs are a subset of tumor cells that can self-renew and cause tumor initiation and relapse.[4-6] These cells are resistant to many treatments, including chemotherapy and radiotherapy.[7] Accordingly, these therapies fail because they just kill differentiated tumors, without eliminating CSCs, which survive and generate new tumors subsequently.

Patients with platinum refractory and resistant tumors are strong candidates for novel investigational approaches and studies of drug resistance. Single-agent therapy is a standard treatment in these patients. Weekly topotecan is a well-tolerated and effective regimen for platinum-resistant ovarian cancer, with considerably less hematological toxicity compared with historical data for the 5 days regimen. The response rate to topotecan therapy for platinum-resistant ovarian cancer is 20-25%.[8,9] The reason for the responsiveness of platinum-resistant ovarian cancer to topotecan remains unknown. This study was performed to elucidate the role of a topoisomerase I inhibitor (topotecan) in the treatment of chemoresistant ovarian cancer-initiating cells.

MATERIALS AND METHODS

Cell lines

We obtained the human ovarian cancer cell line CP70 from Dr Tim Huang's laboratory (Ohio State University, Columbus, OH, USA). Cells were maintained at 37°C

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(5% CO_2/air atmosphere) in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum.

Measurement of cytotoxicity

The cells were plated at 5×10^3 cells per well in 96-well plates 1 day before drug treatment, and then cotreated with topotecan for 24 h. After washing, we incubated the cells with fresh medium for 72 h, followed by staining with the Cell Titer 96 Aqueous One Solution Reagent (Promega), which contains a novel tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt; MTS) and an electron-coupling reagent (phenazine ethosulfate).

To measure the amount of soluble formazan produced by the cellular reduction of MTS, absorbance was recorded at 490 nM using a 96-well plate reader. We assayed each reaction at least three times. The results were expressed as the ratio of cytotoxicity to the concentration of topotecan, as a percentage of 1- MTS reduction in treated samples compared with untreated samples for the drug alone.

Side-population and sphere-formation analysis

CP70 side-population spheres (SPS) cells were isolated from the CP70 cell line according to our previous study.[10] Briefly, a side population (SP) derived from ovarian cancer cells was obtained using flow cytometry and Hoechst 33342 staining. Cells were cultured in suspension condition (100,000 cells/mL) and allowed to recover at 37°C for 1 h before treatment with the Hoechst dye. Hoechst 33342 (Sigma) was added at a final concentration of 2.5 μg/mL and the cells were incubated at 37°C for 1 h. The ABCG2 inhibitor, GF120918, was added at a final concentration of 50 μg/mL, to confirm the gating area on flow cytometry. Side-population cells exhibited low staining with Hoechst 33342. After sorting, they were plated immediately in ultralow attachment plates (Corning) at a density of 20,000 viable cells/mL in a serum-free mammary epithelial growth medium (MEGM, BioWhittaker) supplemented with B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF) (BD Biosciences), and 4 μg/mL heparin (Sigma) until the formation of spheres (SPSs).

Clinical specimen

Following informed consent, tumor samples were obtained from patients undergoing surgical treatment at Tri-Service General Hospital in accordance with the appropriate Institutional Review Boards. Within 1 h after surgical removal, tumors were washed and enzymatically dissociated into single cells. Red blood cells were removed by differential centrifugation. Tumor cells were cultured in either NBE media consisting of neurobasal media (Invitrogen), N2 and B27 supplements (0.53 each; Invitrogen), human recombinant bFGF and EGF (50 ng/ml each; R and D systems), or serum media consisting of Dulbecco's modified eagle medium media (Invitrogen) with 10% fetal bovine serum (Cellgro). For ovasphere culture, uncoated plastic dishes were used. For adherent culture of ovasphere, the plates were precoated with a poly-L-lysine/laminin mixture (Invitrogen).

Statistical analysis

The mean and the standard error of the mean are reported. Data were compared using two-tailed Student's *t*-tests. Significance was set at $P < 0.05$.

RESULTS

CP70 side-population spheres cells were resistant to chemotherapeutic drugs

A SP of the CP70 ovarian cancer cell line was isolated using Hoechst 33342 dye exclusion by flow cytometry.[10] The SP was cultured in serum-free medium in a low attachment culture dish until the formation of spheres, which were termed CP70 SPSs [Figure 1]. In our data, CP70 SPS cells showed significant resistance to the chemotherapeutic drugs cisplatin and paclitaxel ($P < 0.05$), which are used commonly in clinical practice to treat patients with ovarian cancer [Figure 2]. This suggests that CP70 SPS populations are more resistant to chemotherapeutic drugs compared with the parental CP70 cells.

Microarray analysis of CP70 side-population spheres compared with CP70 cells

To identify potential chemotherapeutic drugs for chemoresistant ovarian cancer-initiating cells, a microarray

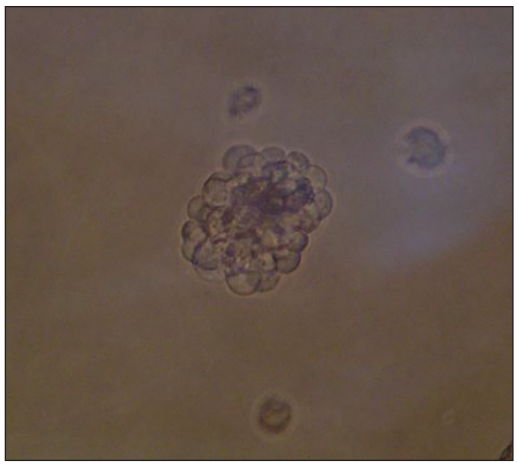

Figure 1. CP70 cells were cultured in suspension condition to form a spheroid tumor, termed CP70 side-population spheres

analysis was performed to evaluate the expression profile of CP70 SPS cells. Topoisomerase-related genes were highly expressed, as demonstrated in a heat map [Figure 3]. Furthermore, we used RT–PCR to evaluate the expression level of messenger ribonucleic acids (RNAs), which revealed that topoisomerase-related genes (*TOP1MT*, *TOP1P2,* and *TOPORS*) were overexpressed in CP70 SPS compared with CP70 cells [Figure 4]. These data indicate that topoisomerase-related genes are highly expressed in tumor-initiating cells.

Topotecan inhibited the growth of chemotherapy-resistant ovarian cancer-initiating cells

Next, we examined the effects of topotecan treatment on CP70 and CP70 SPS cells. Cells were cultured in the presence of increasing concentrations of topotecan for 3 days. After incubation, cell viability was measured with the MTS assay.

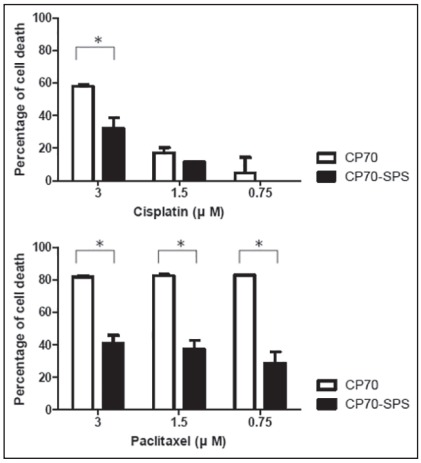

Figure 2. CP70 side-population spheres cells were resistant to chemotherapeutic drugs of cisplatin and paclitaxel (*$P < 0.05$)

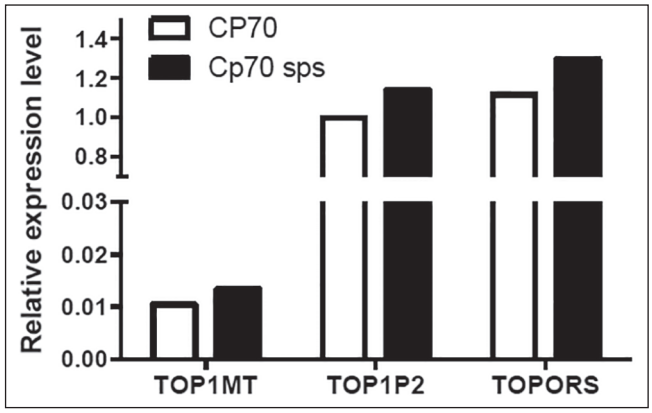

Figure 4. Relative messenger ribonucleic acid expression level (normalized to GAPDH) of topoisomerase-related genes in CP70 side-population spheres and CP70 cells. *TOP1MT*, topoisomerase (deoxyribonucleic acid [DNA]) I, mitochondrial; *TOP1P2*, topoisomerase (DNA) I pseudogene 2; *TOPORS*, topoisomerase I binding, arginine/serine-rich

Topotecan exerted a more pronounced growth inhibition (by 13.5 and 12%, respectively) on CP70 SPS than it did on CP70 (by 3.29 and 0.02%, respectively) cells at a concentration of 0.02 and 0.01 μM ($P < 0.05$) [Figure 5]. In addition, we incubated CP70 SPS cells in adherent conditions for differentiation. Topotecan had an inhibitory effect on cell growth in the cells with stem status compared with those in the differentiated condition ($P < 0.05$) [Figure 6]. These results demonstrated that topotecan had a selective inhibitory effect on SPS, suggesting that topotecan has a more pronounced effect on cancer-initiating cells.

Next, we investigated the effect of topotecan on tumor cells derived from human patients with ovarian cancer. Similar to what was observed in the *in vitro* study, topotecan at different concentrations had a greater inhibitory effect on SPS cells than differentiated cells isolated from the tissue of patients with ovarian cancer [Figure 7]. These results demonstrated that topotecan inhibited SPS cells derived from tumors from patients with ovarian cancer.

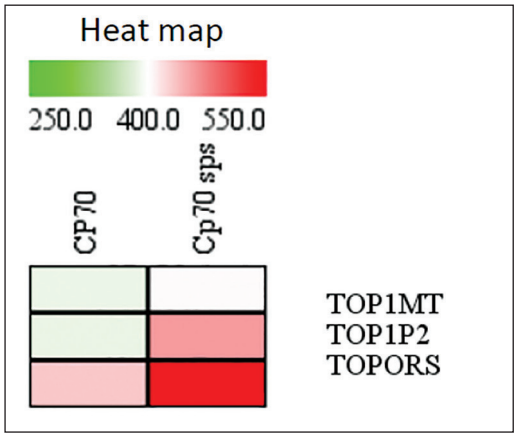

Figure 3. Heat maps illustrating the high expression of topoisomerase-related genes in chemoresistant ovarian cancer-initiating cells. Each colored element corresponds to one gene. Higher expression is shown in deep red; lower expression is shown in light red

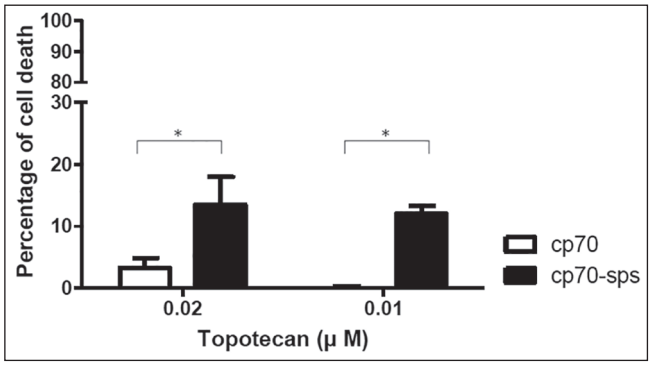

Figure 5. Effect of topotecan on cancer cell death in CP70 and CP70 side-population spheres cells (*$P < 0.05$)

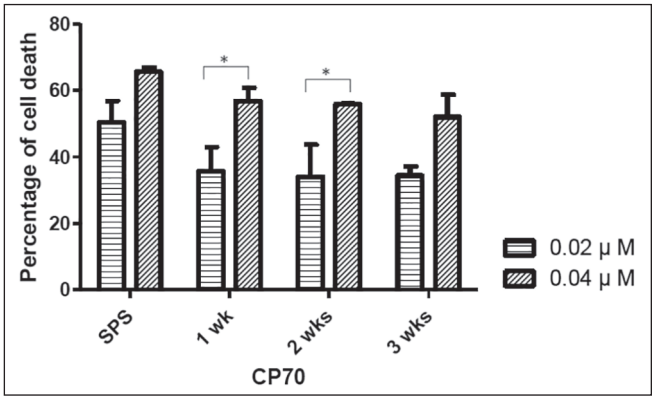

Figure 6. Tumor-suppressive effect of topotecan in CP70 side-population spheres cells differentiated to CP70 cells (*$P < 0.05$)

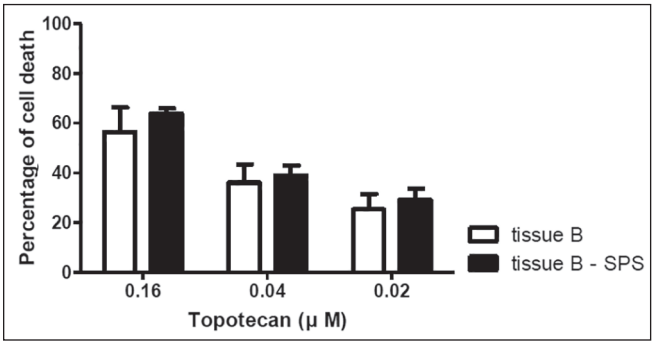

Figure 7. Inhibitory effects of topotecan on side-population spheres cells derived from tumors of human patients with ovarian cancer

DISCUSSION

The origin and pathogenesis of EOC have long been studied but remain poorly understood. The relapse of ovarian cancer in most patients is likely the result of the sparing of ovarian CSCs. The analysis of tumor samples from patients who had undergone first-line chemotherapy showed an enrichment of stem cell markers, such as CD133, CD44, and ALDH1, compared with samples obtained during surgical cytoreduction.[11] Another study showed that ovarian CSCs located close to the stroma form a possible stem cell niche.[12] To date, no definitive markers of ovarian CSCs have been identified; hence, testing for susceptibility to various chemotherapeutic agents has been inconclusive. The high expression levels of CSC markers were enhanced in a population of cells that responded to cisplatin and paclitaxel treatment.[13] We also identified a subset of ovarian cancer-initiating cells that exhibited an enhanced ability to form spheres in response to chemotherapy drugs.[10] Increased resistance to paclitaxel and cisplatin is associated with ovarian CSCs.[14] Various therapeutic interventions can eliminate CSCs.[15]

The importance of telomerase in tumorigenesis is fully recognized.[16] Telomerase activity and dysfunctional telomeres have been demonstrated in human ovarian carcinoma.[17] Telomerase is not present in the normal ovarian surface epithelium and premalignant lesions, but is upregulated in 90-97% of ovarian carcinomas.[18] To the best of our knowledge, no report has elucidated the role of deoxyribonucleic acid (DNA) topoisomerase I inhibitors in cancer-initiating cells. In our study, a microarray analysis demonstrated elevated expression of topoisomerase-related genes in chemoresistant ovarian cancer-initiating cells. Our results of *in vitro* and human-tissue studies confirmed that topotecan inhibits the growth of CP70 SPS cells. This study demonstrated that topoisomerase I inhibitors may have a tumor-suppressive effect in ovarian cancer-initiating cells.

A previous report demonstrated that the *RET* finger protein (RFP) confers resistance to chemotherapeutic drugs in cancer cells. RFP was highly expressed in patients with ovarian cancer and correlated with chemotherapeutic resistance. The use of RNA interference to deplete RFP in ovarian cancer cell lines promoted carboplatin - or paclitaxel-induced apoptosis and decreased chemotherapeutic resistance.[19] Perturbed DNA topoisomerase I activity can affect fragile-site breakage at the *RET* oncogene.[20] The topoisomerase I inhibitor topotecan may stabilize the fragile-site breakage of the *RET* oncogene and render cancer cells more sensitive to chemotherapy.

CONCLUSION

We demonstrated that topoisomerase-related genes are highly expressed in chemoresistant ovarian cancer-initiating cells. A topoisomerase I inhibitor (topotecan) had a tumor-suppressive effect in chemoresistant ovarian cancer-initiating cells. The use of topotecan in the treatment of cancer-initiating cells could be an adjuvant therapy for the treatment of patients with chemoresistant ovarian cancer.

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DISCLOSURE

The authors declared this study has no conflicts in interest.

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