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The Role of Survivin Gene in the Chemoresistance of Ovarian Cancer Cell

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ABSTRACT

Objective: *To study the role of survivin in the chemoresistance of human ovarian cncer cell line CAOV3 induced by taxol and carboplatin.*
Methods: *After the secondary culture of ovarian cancer cell line CAOV3, inhibition of ovarian cancer cell growth induced by taxol and carboplatin in different concentration was detected by MTT assay. Alteration of survivin mRNA expression was evaluated by the reverse transcriptase polymerase chain reaction (RT-PCR) in human ovarian cancer cell line CAOV3 incubated in medium containing 20 mg/L taxol or 50 mg/L carboplatin for 1 day, 3 days, 5 days and 200 mg/L taxol or 500 mg/L carboplatin for 1 day. The apotosis of these cells in culture were detected by flow cytomertry.*
Results *: The cancer cell viability has a remarkable dose-dependent reduction in cancer cell viability in response to treatment with either taxol or carboplatin. The data from flow cytometry indicated that taxol or carboplatin induce apoptosis of human ovarian cancer line CAOV3 in a time-dependent and concentration-dependent manner. In addition, the concomitant expression of survivin gene was observed to be increased with increasing concentrations of both drugs in a time-depentant manner. The increase of survivin mRNA is more evident for taxol-treated cells.*
Conclusion *: This study indictes that survivin plays an important role in chemoresistance induced by taxol and carboplatin in human ovarian cancer cells in vitro, with a greater potency for taxol.*

Keywords: Survivin gene, Ovarian cancer, taxol, carboplatin, chemoresistance

INTRODUCTION

Apoptosis plays an important physiological role in several processes, ranging from embryonic development to maintenance of adult tissue homeostasis. Suppression of apoptosis contributes to carcinogenesis by several mechanisms, including aberrantly prolonging the cell life span, permitting growth dependent cell survival and allowing of cell cycle checkpoints[1-3]. Defects in apoptosis also play an important role in resistance to chemotherapy[4]. During epithelial cell homeostasis proliferation, migration, differentiation and apoptosis (programmed cell death) occur. Disturbances in these processed are associated with neoplastic transformation and progression. Several oncogenes such as p53 and the bcl-2 family are involved in regulation of cell apoptosis[5]. Survivin is a recently identified inhibitor of apoptosis (IAP)[6,7], which has been reported to directly inhibit caspase-3 and -7 activity[8] and regulates the cell cycle in the G2/M phase[9]. Unlike other IAP proteins, survivin is expressed during embryonic and fetal development, whereas it is completely down-regulated in normal adult tissues[10]. However, the timing and mechanism of reexpression of survivin during carcinogenesis is stil unknown at present. Because it is strongly expressed in embryonic organs and a variety of human tumors but is generally undetectable in differential normal tisues[7], survivin will become a selective target for treatment and diagnosis of carcinoma.

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Chemotherapy still played important role in the treatment of patients with ovarian carcinoma. Taxol and carboplatin are the first-line chemotherapautic agents for epithelial ovarian cancer, but the chemoresistance in the primary or recurrent tumors resulted in the treatment failure. The mechanism of chemoresistance of taxol and carboplatin in treating ovarian cancer was still unknown. To clarify the mechanism of chemoresistance of taxol and carboplatin, we examined the time and dose-dependent expression of survivin gene in ovarian cell lines induced by paclitaxel and carboplatin.

MATERIALS AND METHODS

Cell lines and culture
The established human ovarian cancer cell line CAOV3 was cultured in RPMI-1640 supplemented with 10% fetal bovine, 100KU/L penicillin and 0.1g/Lstreptomycin. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Ovarian cancer cell line CAOV3 was obtained from Cell Biology Laboratory of China Medical University.

TUMOR CELL INHIBITION ASSAYS

Cell proliferation assays were performed indirectly by modified MTT, based on enzymatic reduction of 3-(4,5-dimethl thiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) to form formazan crystal by mitochondria and cellular dehydrogenase enzymes[11]. Cells were seeded into 96-well flat-bottomed microplates with 100μl growth medium and allowed to attach and grow overnight. The medium was replaced with 100μl of growth medium containing taxol and carboplatin at concentration of 0.2 to $2x10^3$ mg/L. The plates were incubated in a humidified incubator in 5% CO_2 for 2 days at 37°C. Then 20μl MTT(SABC) solution (10mg/ml MTT in phosphate-buffered saline (PBS) stored at -20°C) was added and incubated for 4 hours at 37°C in 5% CO_2' The MTT-containing tissue culture fluid was removed from the wells, and the product was solubilized in 100 μl of DMSO for 5-10 min at room temperature. The plate was read at 492 nm in plate reader (SUNRISE Austria). Microplate reader zeroed on the reagent blanks to correlate the colored product with the number of cells, according to the following formula : cytotoxicity=((A492 of cell control)-(A492 of treated cell))/((A492 of cell control)-(A492 of vehicle control))x100%. All of the reported experiments were performed at least four times.

FLOW CYTOMETRY

According to the result of MTT assays, the human ovarian cancer cell line CAOV3 were incubated in medium containing 20 mg/L taxol or 50 mg/L carboplatin for 1 day, 3 days, 5 days and 200 mg/L taxol or 500 mg/L carboplatin for 1 day. After being washed with 1xPBS, $1x10^6$ cells were pelleted and 1 ml of ice-cold by potonic PI (50 μg/ml PI(Sigma), 20 μg/ml RNase(SABC) and 10 μl/ml Trinton X-100 in 0.1% sodium citrate solution) was added with subsequent vortexing. After 30 min at 4°C, flow cytometry was performed with Becton Dickinson FACAScan (Becton Dickinson Immuocytometry Systems, Scan Jose, CA). Cellquest V3.3 software (Becton Dikinson) was used for the acquisition and analysis of data.

SEMIQUANTITATIVE RT-PCR

(1) Total RNA Isolation The human ovarian cancer cell line CAOV3 were incubated in medium containing 20 mg/L taxol or 50 mg/L carboplatin for 1 day, 3 days, 5 days and 200 mg/L taxol or 500 mg/L carboplatin for 1 day. The cellular RNA was extracted from cultured cells according to the protocol of Trizol™ (Gibco).
(2) Reverse Transcription : Two μg RNA from each specimen was used for the synthesis of the first strand of cDNA. In each of the reactions, a 20 μl solution containing 5 mM $MgCl_2$, 1 x reverse transcription buffer, 1mM each dNTP, 1u/μl recombinant RNasin ribonuclease inhibitor, 15u/μl AMV reverse transcriptase, 0.5 μg $Oligo(dT)_{15}$ primer were incubated at 70°C for 10 min then incubated in a thermocycler at 42°C for 60 min. The reverse transcriptase enzymes were inactivated by heating the solution to 95°C for 5 min.
(3) Primer design and PCR The primers of the survivin for PCR were as follows : sense, 5□-GCA CTT TCT TCG CAG TTT CC-3□, PCR amplification was performed with 20 μl reactions using Taq polymerase (Promega). The PCR mixture was preheated to 94°C for 5 min. before cycling begins. The PCR was performed for 29 cycles consisting of the following steps: denaturation at 94°C for 45s, annealing at 59°C for 45s and extension at 72°C for 45s. After 29 cycles, extension was performed at 72°C for 10 min. β-actin primers were also amplified to examine the quality and quantity of the products by RT-PCR. Then, 10 μl of each PCR product was loaded on the 2% agarose gel in TAE buffer and then stained with ethidium bromide. The expression amount was determined using as auto-analysis system (Chemi Imager5500),(Relative amount of expression=density of survivin/density of β-action x 100)

Statistical analysis

Newman-Keuls test was used to evaluate the differential expresion of survivin. All analyses were performed with SPSS11.0 software.

RESULTS

Sensitivity of human ovarian cancer cell line CAOV3 to taxol and carboplatin.

The human ovarian cancer line CAOV3 was selected for the study, They were treated with different concentration (from 0.2mg/L to 2000mg/L) of taxol and carboplatin and cytotoxicity were determined by MTT assay. Result of MTT assays demonstrated a remarkable dose-dependent reduction in cancer cell viability in response to treatment with either taxol or carboplatin (Fig 1). For example, 48h of treatment at 2000mg/L concentration, taxol and carboplatin result in 94% and 97% reduction in CAOV3 cell viability, respectively.

Taxol and carboplatin induce apoptosis in human ovarian cancer cell line CAOV3.

We further investigated whether the cytotoxic effect was by apoptosis. For this purpose, cells incubated in medium with different concentrations of taxol or carboplatin were stained with propidium iodide at 1 day, 3 days, 5 days and evaluated by flow cytometry. As seen in the flow cytometric photographs in Fig.2, in contrast to the control, there was a clear shift in the degraded DNA in cells with 20mg/L taxol or 50 mg/L carboplatin

after 3 days and cells with 200 mg/L taxol after 24h Fig.3 presents the data related to the flow cytometry and indicates that taxol and carboplatin induced apoptosis of human ovarian cancer line CAOV3 in time-dependent and concentration-dependent manner.

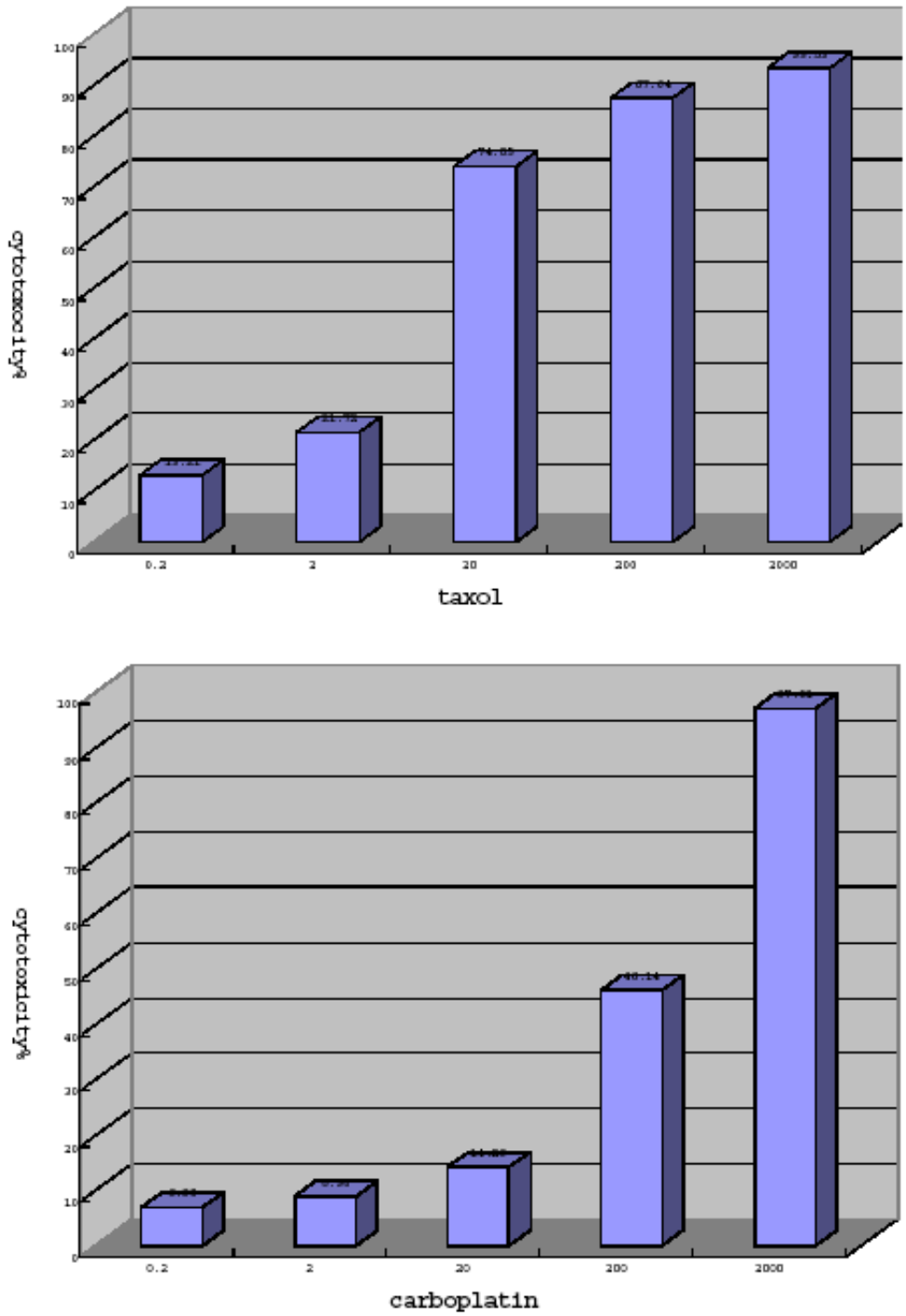

Fig.1. Cytotoxicity of human ovarian cancer cells treated with different concentrations of taxol or carboplatin. Cytotoxicity was assessed by MTT assay after a 72-h of culture. The data represent the mean of four different experiments.

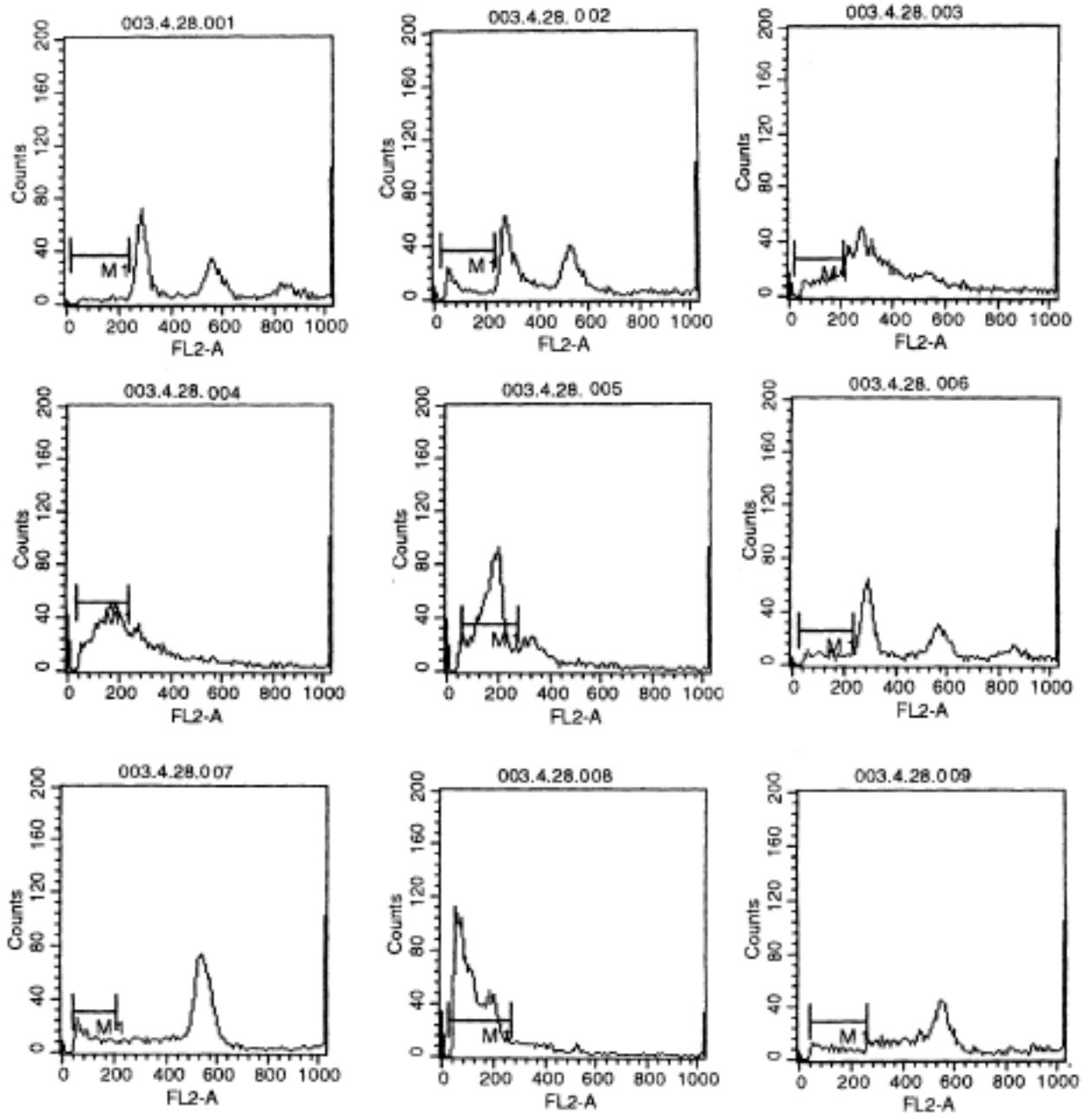

Fig.2. Taxol or carboplatin induces apoptosis in the human ovarian cancer Cell line CAOV-3. (1: control; 2,3,4: 20mg/L taxol for 1d,3d,5d; 5: 20mg/L taxol for 1d; 6,7,8: 50mg/L carboplatin for 1d,3d,5d; 9: 500mg/L for 1d.)

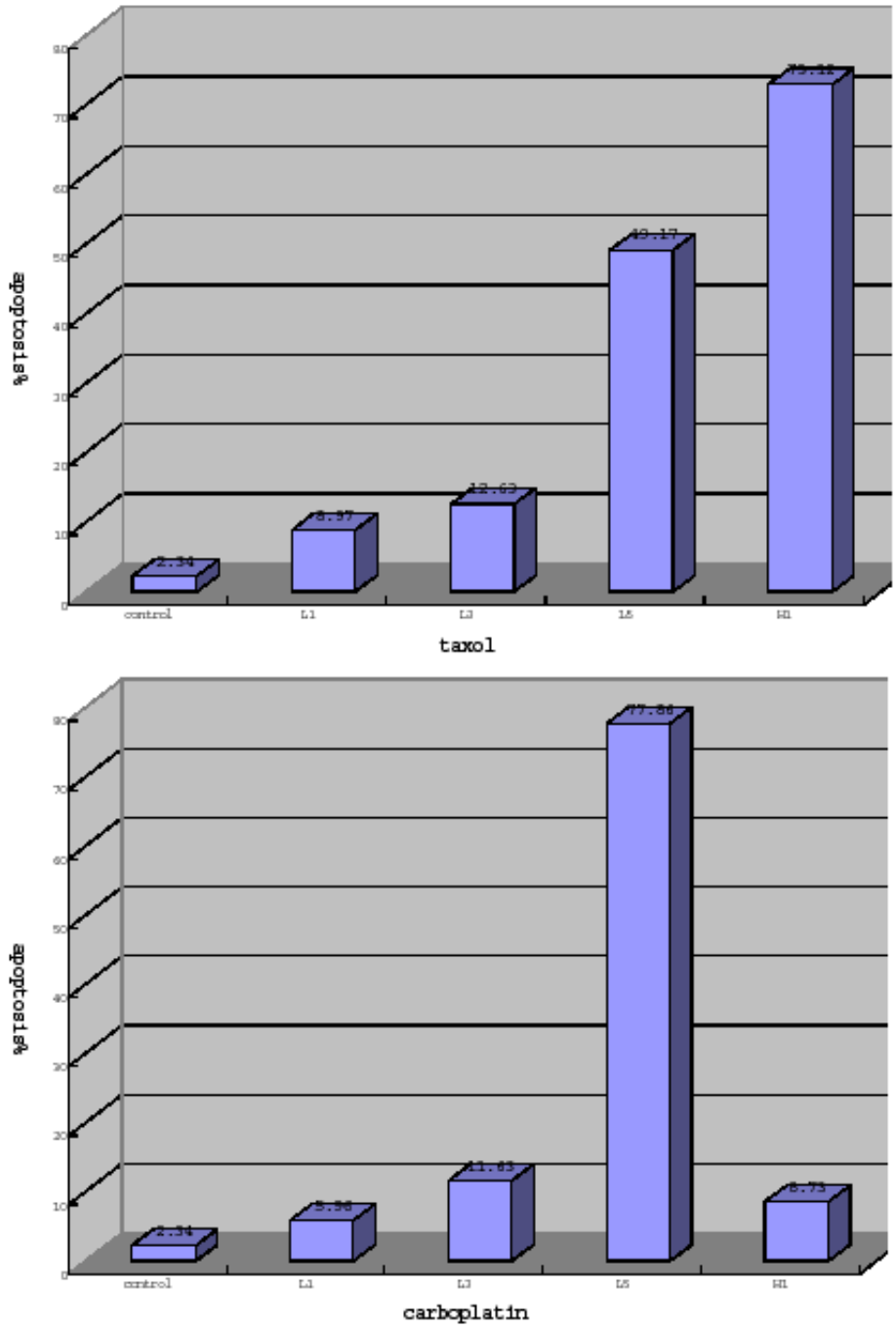

Fig.3. Apoptosis of human ovarian cancer cells with low concentrations of taxol and carboplatin for 1d, 3d 5d with high levels for 1d as assessed by flow cytometry

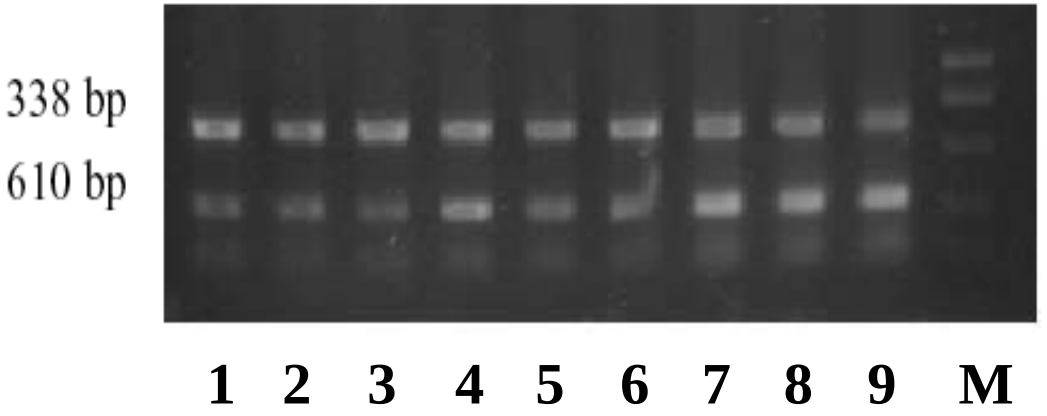

Fig.4. The expression of survivin in human ovarian cancer cells with different concentrations of taxol or carboplatin at different time intervals (1: control;2,3,4: : 2mg/L taxol for 1d,3d,5; 5:2mg/L taxol for 1d; 6,7,8: 50 mg/L carboplatin for 1d,3d,5d;9: 500 mg/L for 1d.)

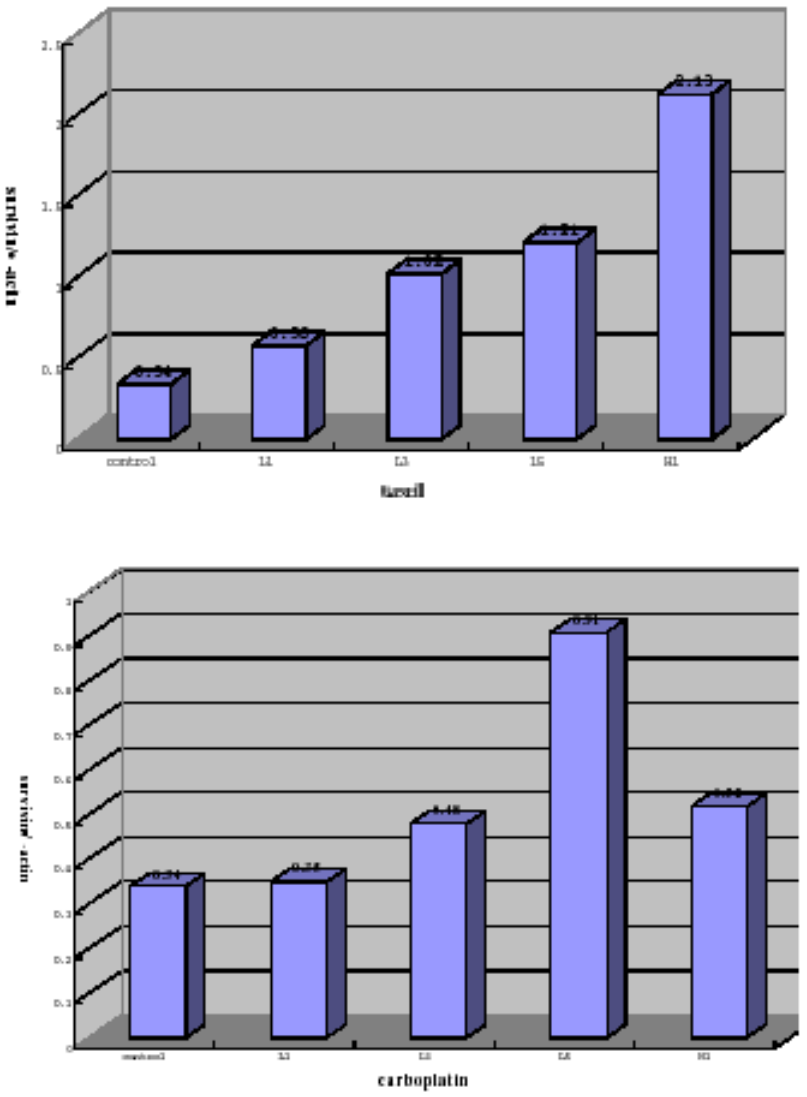

Fig.5. The intensity of survivin gene expression (survivin/•-actin) in human ovarian cancer cells with different concentrations of taxol or carboplatin

The alteration of survivin mRNA in human ovarian cancer cells with different concentrations of taxol and carboplatin after different times of incubation .

As a new member of IAP family, survivin may play an important role in apoptosis induced by taxol and carboplatin. We were therefore interested to examine the role of survivin gene in apoptosis induced by taxol and carboplatin in human ovarian cancer cells. The product of survivin amplified by primer is 438 bps band. Fig. 4 and 5 indicate the alteration of survivin in human ovarian cancer cells incubated in medium with 20mg/L taxol or 50 mg/L carboplatin after 1 day, 3 days, 5 days and cells with 200mg/L taxol or 500mg/L carboplatin after 24h. The data from the RT-PCR indicated the expression of survivin gene increased in time-dependent and concentration-dependent manner. The increase of survivin mRNA is evident for taxol. For example, the expression of survivin mRNA increased 3.74 times and 6.26 times than the control in human ovarian cancer with 20mg/L taxol for 3 days and 200mg/L taxol for 1 day.

DISCUSSION

Ovarian cancer is the leading cause of gynecologic cancer death. The overall 5-year survival rate for advanced ovarian cancer patients is still low (20-30%) and is due to chemoresistance in the primary or recurrent tumors, thus resulting in treatment failure. Defects in apoptosis play an important role in resistance to chemotherapy[4]. In a previous study[12], we have demonstrated that survivin gene was detected in 82.9% patients with ovarian cancer, but is undectectable in normal ovarian which indicate survivin play an important role in the genesis and development of ovarian cancer.

Survivin expression could therefore be associated with unfavourable clinical outcome in cancers.

Furthermore, survivin has been suggested to be involved in cell proliferation and it interacts with cyclin-dependent kinase 4 (CDK4) initiating S-phase entry[13]. These characteristics suggests that survivin is implicated in the regulation of cell cycle progression[14,15]. Unlike other IAP proteins, survivin contains a single baculovirus IAP repeat but no RING finger and has been reported to show maximal expression in the G2/M phase of cell cycle and is localized to the mitotic spindle[9]. These evidences favour the fact that this IAP molecule is directly involved in promoting escape from cell cycle control in the malignant cells.

Results of this study demonstrate the interaction between the alteration of survivin gene and apoptosis induced by taxol and carboplatin in human ovarian cancer cell line CAOV3. The result of MTT indicated a remarkable dose-dependent reduction in ovarian cancer cell viability in response to treatment with either taxol or carboplatin. In addition, the results of flow cytometry and RT-PCR demonstrated that apoptosis of ovarian cancer cells increased when the treated time prolonged and concentration enhanced, but the expression of survivin in survival cancer cells increased in time-dependent and concentration-dependent. The apoptosis of ovarian cancer cells did not change remarkably after treatment with low dose taxol for 1 day and 3 days, but the expression of survivin in viable ovarian cancer cells was 1.7 and 3 fold higher than that of control. The apoptosis of cancer cells treated with high dose carboplatin for 1 day did alter evidently when compared with cells treated with low dose carboplatin for 1 day, but the expression of survivin mRNA in ovarian cancer cells increased to 1.5 fold.

Survivin is a new member of IAP, its protein inhibits apoptosis induced by chemotherapy through direct banding, as other IAPs, or indirectly, requiring intermediate proteins to inhibit capases. Proposed models for indirect inhibition of capases imply that inhibitory protein, or that surviving somehow enhances the function of other IAPs, having a role opposite to SAMC. Moreover, since survivin is associated with microtubules and with the mitotic spindle it is likely that this protein can specifically contribute to cell response to microtubule-interacting agents as taxol[16].

It is noteworthy that survivin overexpression in tumors has been associated with increased aggressiveness and decreased patient survival in a number of different malignancies[9,10,13]. It has also been reported that the presence of survivin in tumor tissue could be used to identify patients at high risk for recurrence of bladder cancer. Overexpression of this IAP peptide was significantly associated with residual disease suggested that residual tumor cells in patients may have high growth potential when the positive rate of survivin is high in resected ovarian cancer tissues[17]. The expression of survivin may promote tumor cell proliferation, increase malignancy and result in unfavourable clinical outcome.

From this study, we conclude that survivin plays an important role in the chemoresistance induced by taxol and carboplatin in human ovarian cancer cells in vitro, especially for taxol. So targeting survivin is particularly attractive because of its relatively selective expression in tumor cells and its proven association with disease progression. Antisense oligonucleotide of survivin gene could enhance the sensitivity of ovarian cancer cells to chemotherapy in order to enhance the overall 5-year survival rate of patients with ovarian cancer.

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