


CpG Oligodeoxynucleotide Promotes Apoptosis of Human Bladder Cancer T24 Cells Via Inhibition of the Antiapoptotic Factors

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Abstract

Objective: Unmethylated cytosine-phosphorothioate-guanine oligodeoxynucleotide, a synthetic oligodeoxynucleotide, has been used as an adjuvant in clinic and in the antitumor activity. However, the antitumor mechanism of cytosine-phosphorothioate-guanine oligodeoxynucleotide against human bladder cancer is unknown. The purpose of this study is to evaluate the cytotoxicity and molecular mechanism of anticancer effect of cytosine-phosphorothioate-guanine oligodeoxynucleotide on T24 cells (a human bladder cancer cell line). **Methods:** The cytotoxic activity of cytosine-phosphorothioate-guanine oligodeoxynucleotide was examined by cell viability assay in the presence and absence of 5-fluorouracil, respectively. Apoptosis and cell-cycle phase distribution were detected by flow cytometry analysis. To investigate the molecular mechanisms of cytosine-phosphorothioate-guanine oligodeoxynucleotide cytotoxicity, the expression of antiapoptotic factors (B-cell lymphoma-2 and Survivin, β -actin as control) in RNA, and protein level was assayed by quantitative real-time polymerase chain reaction and automated capillary Western blot. **Results:** The inhibition ratio of T24 cells treated with both cytosine-phosphorothioate-guanine oligodeoxynucleotide and 5-fluorouracil was higher than those treated with either cytosine-phosphorothioate-guanine oligodeoxynucleotide or 5-fluorouracil alone. In the combination group (cytosine-phosphorothioate-guanine oligodeoxynucleotide and 5-fluorouracil), the apoptosis rate was significantly increased, and more cells were arrested at "S" and "G2/M" phases compared to those in cytosine-phosphorothioate-guanine oligodeoxynucleotide or 5-fluorouracil alone. Furthermore, the expression of antiapoptotic factors was decreased by cytosine-phosphorothioate-guanine oligodeoxynucleotide alone or combined with 5-fluorouracil. **Conclusion:** Cytosine-phosphorothioate-guanine oligodeoxynucleotide promoted apoptosis and enhanced the chemosensitivity of 5-fluorouracil in T24 cells. Cytosine-phosphorothioate-guanine oligodeoxynucleotide downregulated the expression of antiapoptotic factors and inhibited cell-cycle phase by arresting more cells at "S" and "G2/M" phases. This study indicated the potential ability of cytosine-phosphorothioate-guanine oligodeoxynucleotide as a candidate drug for human bladder cancer.

Keywords

CpG-ODN, human bladder cancer cells, apoptosis, cell cycle arrest, chemosensitizer

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Abbreviations

5-FU, 5-fluorouracil; BCG, Bacillus Calmette-Guérin; Bcl-2, B-cell lymphoma-2; cDNA, complementary DNA; CpG-ODN, cytosine-phosphorothioate-guanine oligodeoxynucleotide; DOX, doxorubicin; gDNA, genomic DNA; IAPs, inhibitor of apoptotic proteins; M-VAP, methotrexate + vincristine + adriamycin + cisplatin; OD, optical density; PCR, polymerase chain reaction; PI, propidium iodide; RT, real time.

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Introduction

Bladder cancer is one of the most common malignant tumors of the urinary system in adults, which is estimated to be the ninth most incident cancer worldwide.¹ Around 70% to 80% of bladder cancer are nonmuscle invasive bladder cancer, which results in metastasis and death.² Similar to other neoplastic diseases, bladder cancer could be treated with conservative or operative methods based on the actual situation of patients.³ Chemotherapeutic drugs have significant side effects to human body.⁴ Therefore, many immunologic adjuvants, such as Bacillus Calmette-Guérin (BCG) and Cytosine-phosphorothioate-guanine oligodeoxynucleotide (CpG-ODN), have been investigated to combat the side effect of chemotherapeutic drugs.⁵

Bacillus Calmette-Guérin vaccine is commonly used for postoperative perfusion of human bladder cancer.⁶ However, significant side effects (bladder urinary tract stimulation) of BCG limited its routine clinical application. Cytosine-phosphorothioate-guanine ODN is one of the synthetic ODNs containing a high frequency of unmethylated CpG motifs.⁷ Previous studies have shown antitumor effects on CpG-ODN via inducing tumor cells to release cytokines and enhancing immune response.⁸ For example, CpG-ODN inhibited the viability of HepG2 (a kind of human hepatoma cells) by decreasing the expression of Survivin.⁹ Moreover, CpG-ODN could function as a T-helper-1-promoting adjuvant and activate dendritic cells.¹⁰ As an adjuvant for the induction of antitumor immune responses, CpG-ODN might also be a potential chemosensitizer with slight side effects.¹¹ Clinical studies have also demonstrated that chemotherapy combined with CpG-ODN therapy increased both curative effect and the chemotherapeutic tolerance of patients.¹² Although the beneficial effects of CpG-ODN have been demonstrated, the direct cytotoxicity and potential mechanism of CpG-ODN against human bladder cancer cells are unclear. The purpose of this study is to investigate the effect of CpG-ODN on T24 cells (a human bladder cancer cell line) and its potential mechanism.

Materials and Methods

Reagents

MTS (CellTiter 96 Aqueous One Solution Cell Proliferation assay) was purchased from Promega, Madison, Wisconsin (cat no.: G3582). Trizol reagent was purchased from Invitrogen, Waltham, Massachusetts (cat no.: 15596026). PrimeScript RT reagent kit with genomic DNA (gDNA) Eraser (Perfect Real Time) and SYBR Premix Ex TaqII (Tli RNaseH Plus)

were purchased from TaKaRa, Dalian, Liaoning, People's Republic of China (cat nos.: RR047A and RR420A). Propidium iodide (PI) and RNase A were purchased from Sigma, St. Louis, Missouri (cat nos.: P4170 and R6148). Annexin V/FITC kit and Hoechst Staining kit (Hoechst 33258) were purchased from Beyotime, Shanghai, People's Republic of China (cat nos.: C1063 and C0003). 5-Fluorouracil (5-FU) was purchased from Sigma (cat no.: F6627). Doxorubicin (DOX) hydrochloride was purchased from Sangon, Shanghai, People's Republic of China (cat no.: A603456). RIPA assay was purchased from Beyotime, Jiangsu, People's Republic of China (cat no.: P0013B). Bicinchoninic acid protein assay kit was purchased from Keygen Biotech, Nanjing, Jiangsu, People's Republic of China (cat no.: KGP903). Primary antibodies of B-cell lymphoma-2 (Bcl-2) and Survivin were purchased from Proteintech, Wuhan, Hubei, People's Republic of China (cat nos.: 12789-1-AP and 10508-1-AP), and β -actin were purchased from Cell Signaling Technology, Boston, Massachusetts (cat no.: 8457). All the secondary antibodies were anti-rabbit and purchased from ProteinSimple, San Jose, California (cat no.: 042-206).

Human bladder cancer T24 cells were purchased from Laboratory Animal Center Cell Library of Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China. All the primers and CpG-ODN were synthesized by Sangon. Sequences of primers and CpG-ODN are presented in Table 1.

Cell Culture

T24 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum in 37°C and 5% CO₂ with humidified atmosphere. Routine passages were carried out.

Cell Viability Assay

To evaluate the cytotoxicity of CpG-ODN on T24 cells, MTS assay was carried out. T24 cells were seeded in 96-well plates at the density of 5×10^3 cells/well overnight. Cells were treated with CpG-ODN of 6 different concentrations (0, 0.25, 1.25, 2.5, 12.5, and 25 μ M, respectively) for 24 and 48 hours, respectively. Cell viability was assessed using MTS assay (Promega) according to the protocol. Optical density (OD) of the samples was measured at 490 nm on a microplate reader (Bio-Rad). The viability (%) was calculated as follows: $\text{viability (\%)} = \frac{[(\text{OD}_{\text{treated}} - \text{OD}_{\text{empty}})/(\text{OD}_{\text{medium}} - \text{OD}_{\text{empty}})] \times 100\%}{1 - \text{Viability}} \times 100\%$. The inhibition rate was calculated as follows: $\text{inhibition rate (\%)} = (1 - \text{Viability}) \times 100\%$.

Table 1. All the Primers and CpG-ODN Were Synthesized by Sangon.^a

Factor	Sequences
Bcl-2	Forward: 5'-CTTCGCCGAGATGTCCAGCCA-3' Reverse: 5'-CGCTCTCCACACACATGACCC-3'
Survivin	Forward: 5'-GACCACCGCATCTCTACATTC-3' Reverse: 5'-AAGTCTGGCTCGTTCTCAGTG-3'
β-Actin	Forward: 5'-TGACGTGGACATCCGCAAAG-3' Reverse: 5'-CTGGAAGGTGCACAGAGAGG-3'
CpG-ODN	5'-AACGTTGT <u>CGTCGACGTCGTCGTCAGGC</u> CTGACGTTATCGATGGCGTTGT <u>CGTCAA</u> CGTTGTCGTTAACGTT-3'

Abbreviations: Bcl-2, B-cell lymphoma-2; CpG-ODN, cytosine-phosphorothioate-guanine oligodeoxynucleotide.

^aCytosine-phosphorothioate-guanine-ODN contains a total of 72 bases and CG dinucleotides were indicated with underline. All oligodeoxynucleotides used in the experiments were in phosphorothioate backbone. The oligodeoxynucleotides and primers were dissolved with sterilized double distilled water.

To further examine the combination effect of chemotherapeutic drugs + CpG-ODN on T24 cells, MTS was carried out. According to the cell inhibition result of CpG-ODN treatment on T24 cells, we chose a proper concentration (2.5 μM) and a proper time (48 hours) for further study. Two chemotherapeutic agents 5-FU and DOX were selected, and the treatments on T24 cells are as follows: (1) 5-FU alone with the concentration of 0, 2.5, 5, 7.5, 15, and 25 μg/mL for 48 hours, respectively; (2) DOX alone with the concentration of 0, 2.5, 5, 7.5, 15, and 25 μg/mL for 48 hours, respectively; (3) CpG-ODN + 5-FU: CpG-ODN (2.5 μM) + 5-FU (2.5, 5, 7.5, 15, and 25 μg/mL, respectively) for 48 hours; and (4) CpG-ODN + DOX: CpG-ODN (2.5 μM) + DOX (2.5, 5, 7.5, 15, and 25 μg/mL, respectively) for 48 hours.

Cell Morphology

To examine the T24 cell morphology, Hoechst 33258 was carried out. T24 cells were seeded in 12-well plates at density of 5×10^4 cells/well overnight. The cells were then treated with CpG-ODN (2.5 and 5 μM), 5-FU (7.5 μg/mL), and CpG-ODN (2.5 and 5 μM) + 5-FU (7.5 μg/mL) for 48 hours, respectively. After the treatment, cells were stained by Hoechst 33258 at a concentration of 0.5 μg/well, and the nuclei with Hoechst 33258 stained were observed under ultraviolet excitation (352/461 nm) and photographed with fluorescence microscopy (Olympus (Version Bx63)).

Apoptosis Assay

To measure the apoptosis of T24 cells with CpG-ODN (0.25 and 2.5 μM), 5-FU (7.5 μg/mL), or CpG-ODN + 5-FU treatments, Annexin V/FITC kit was used according to the manufacturer's instruction. T24 cells with density of 1×10^5 cells/well were plated in 6-well plates. After overnight culture, the medium was replaced by fresh medium containing different concentrations of CpG-ODN (0.25 and 2.5 μM), 5-FU (7.5 μg/mL), and CpG-ODN (0.25 and 2.5 μM) + 5-FU (7.5 μg/mL) for 48 hours. A COULTER Epics xL flow cytometer (Beckman Coulter, California) was used to detect

apoptotic cells. Data were analyzed by Flowjo software (Version 7.5.2).

Cell-Cycle Phase Distribution Assay

Similar to apoptosis assay, T24 cells were seeded in 6-well plates at a density of 1×10^5 cells/well and cultured for overnight. The cells were treated with different concentrations of CpG-ODN (0.25 μM), 5-FU (2.5 μg/mL), or CpG-ODN (0.25 μM) + 5-FU (2.5 μg/mL) for 48 hours. Both adherent and floating cells were collected and fixed in ice-cold ethanol (70% in ddH₂O) at 4°C overnight. After fixation, the cells were centrifuged to remove ethanol and washed by phosphate-buffered saline. After stained with 0.02% PI for 30 minutes at 4°C in the dark, cell-cycle distribution was examined using a COULTER Epics xL flow cytometer (Beckman Coulter). Data were analyzed using Flowjo software.

Quantitative Real-Time Polymerase Chain Reaction assay

The expression of antitumor factors (Bcl-2 and Survivin) was assayed by real-time polymerase chain reaction (RT-PCR) with β-actin as control. T24 cells at a density of 1×10^5 cells/well were plated in 6-well plates overnight and were treated with various concentrations of CpG-ODN (0.25, 2.5, and 25 μM), 5-FU (2.5, 7.5, and 25 μg/mL) or CpG-ODN (2.5 μM) + 5-FU (2.5, 7.5, and 25 μg/mL) for 2, 4, and 8 hours, respectively. Total RNA was extracted using a Trizol reagent and then reversely transcribed into complementary DNA (cDNA) using PrimeScript RT reagent kit with gDNA Eraser. The transcribed cDNA template was mixed with SYBR Premix Ex Taq II (Tli RNaseH Plus) to amplify these genes using a quantitative PCR instrument (Bio-Rad, California, USA, product type: iQ5). Quantitative RT-PCR was performed by a 2-step cycling conditions: predenature at 95°C for 300 seconds (1 cycle), followed by 95°C for 30 seconds, 60°C for 20 seconds, 72°C for 45 seconds for 40 cycles, and finally, an additional extension at 72°C for 7 minutes. Dissociation curve analysis was performed to examine if there was any bimodal dissociation curve or abnormal amplification plot. The gene-expression data were analyzed with $2^{-\Delta\Delta C_t}$ method.

Automated Capillary Western Blot

Protein level of antitumor factors (Bcl-2 and Survivin, with β-actin as control) were detected by automated capillary Western blot, an automated capillary-based size sorting system (ProteinSimple) following the protocol. T24 cells at a density of 1×10^6 cells/well were plated in 6-well plates overnight and were treated with various concentrations of CpG-ODN (2.5 μM), 5-FU (7.5 μg/mL), or CpG-ODN (2.5 μM) + 5-FU (7.5 μg/mL) for 24 hours, respectively. Briefly, 8 μL of diluted protein lysate was mixed with 2 μL of $5 \times$ fluorescent master mix and heated at 95°C for 5 minutes. The samples, blocking reagent, wash buffer, primary antibodies (1:100), secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in a manufacturer provided microplate.

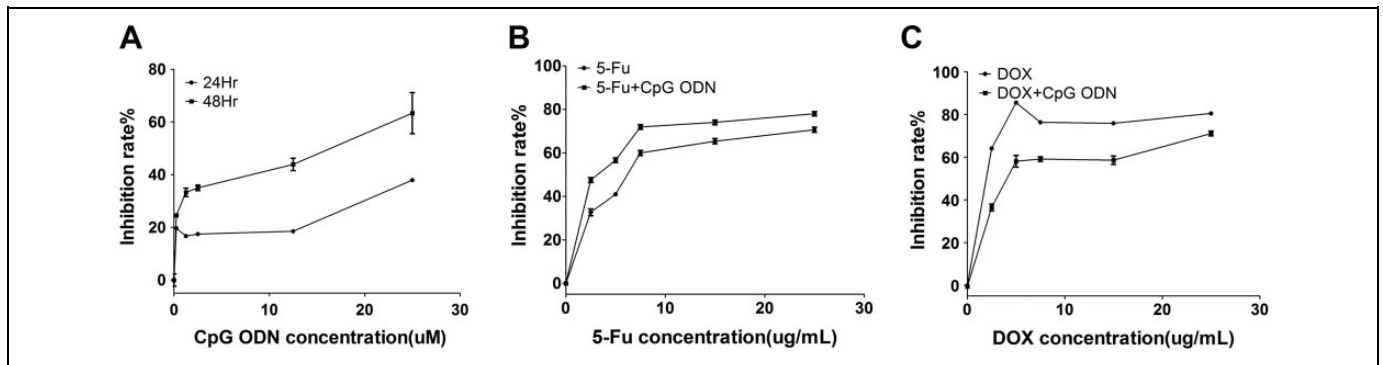


Figure 1. Effect of CpG-ODN, CpG-ODN + 5-FU, and CpG-ODN + DOX on cell viability *in vitro*. A, The inhibitory ratio of T24 cells treated with various concentrations of CpG-ODN for either 24 or 48 hours in MTS assay. Data were presented as percentages related to controls (medium). B, The inhibitory ratio of T24 cells treated with various concentrations of 5-FU in the presence or absence of 2.5 μ M CpG-ODN for 48 hours. C, The inhibitory ratio of T24 cells treated with different concentrations of DOX in the presence or absence of 2.5 μ M CpG-ODN for 48 hours. All results were expressed as means (standard deviation) from 3 independent experiments. CpG-ODN indicates cytosine-phosphorothioate-guanine oligodeoxynucleotides; DOX, doxorubicin; 5-FU, 5-fluorouracil.

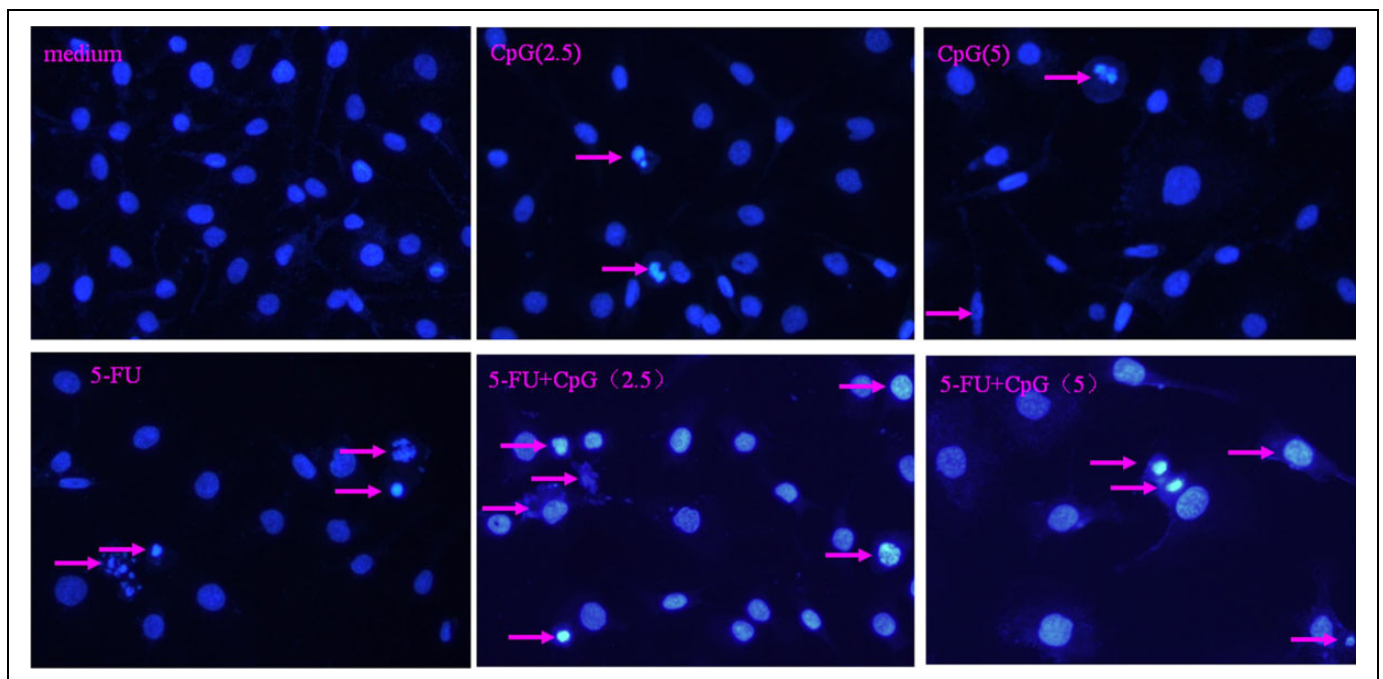


Figure 2. Effect of CpG-ODN, 5-FU, and CpG-ODN + 5-FU on T24 cell morphology. Cells were incubated with CpG-ODN and/or 5-FU at various concentrations for 48 hours. In this figure, 5-FU, CpG (2.5), and CpG (5), respectively, represented the concentration of 5-FU, CpG (2.5), and CpG (5) which were 2.5 μ g/mL, 2.5 μ M, and 5 μ M. CpG-ODN indicates cytosine-phosphorothioate-guanine oligodeoxynucleotides; 5-FU, 5-fluorouracil.

The plate was loaded into the instrument and protein was drawn into individual capillaries on a 25-capillary cassette provided by the manufacturer. Protein separation and the resulting chemiluminescent signal were performed automatically on the individual capillaries using default settings. The data were analyzed using Compass software (ProteinSimple).

Statistical Analysis

The data were presented as mean (standard deviation). One-way analysis of variance was used to analyze the differences among the

treated and the control groups, followed by Tukey multiple comparisons. Differences with the $P < .05$ were considered as significance.

Results

Cytosine-Phosphorothioate-Guanine ODN Inhibited the Viability of T24 Cells

Cytosine-phosphorothioate-guanine ODN significantly reduced the viability of T24 cells with dose- and time-dependency. With higher concentrations and long treatment time, the inhibition of CpG-ODN on the viability of T24 cells increased significantly

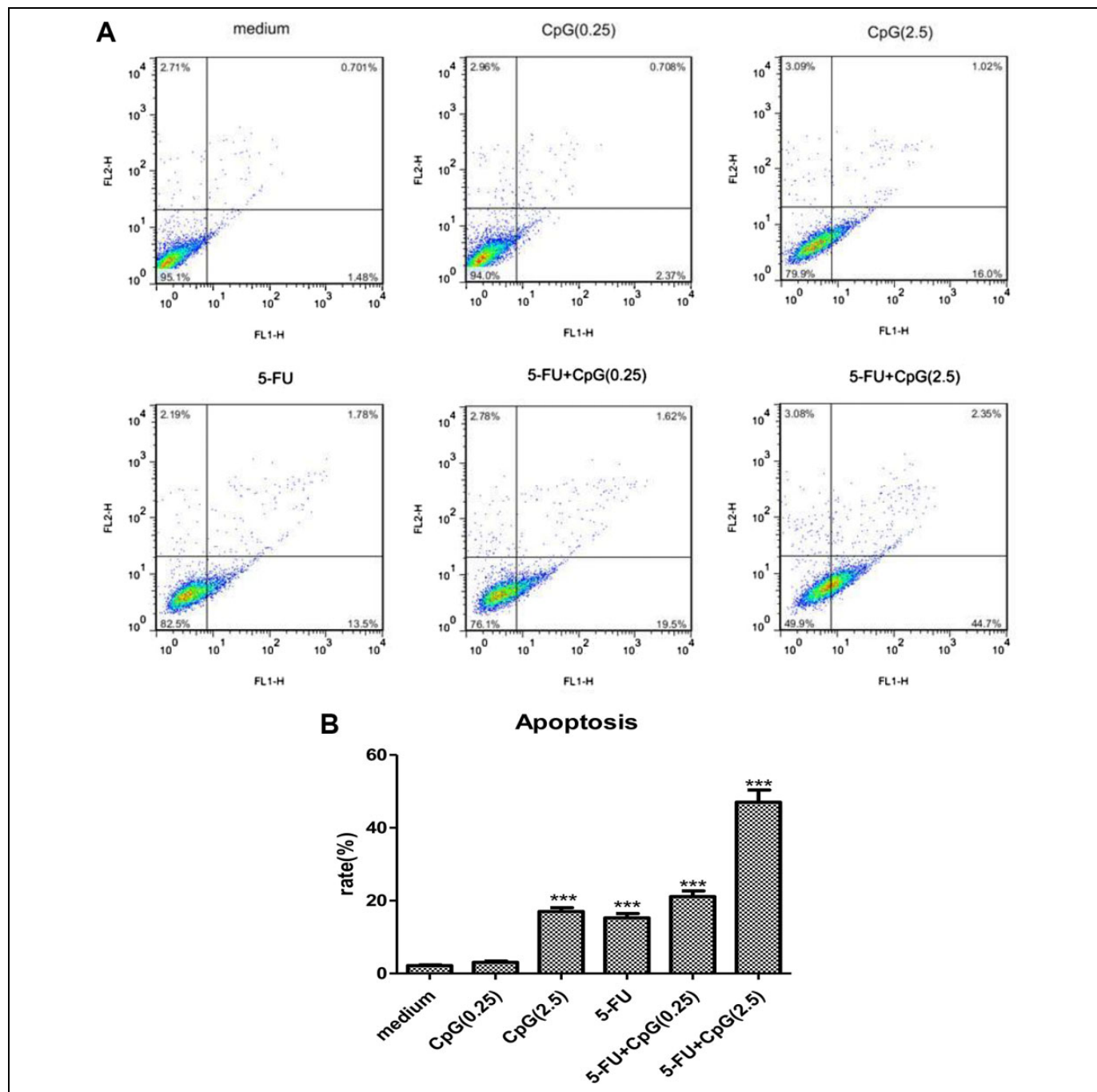


Figure 3. Effect of CpG-ODN, 5-FU, and CpG-ODN + 5-FU on apoptosis of T24 cells. A, The cells were treated with indicated concentrations of CpG-ODN, 5-FU, and CpG-ODN + 5-FU for 48 hours. The induction of apoptosis was determined by Annexin V-FITC/PI staining and flow cytometry. x-Axis represented FITC, and y-axis was PI. FITC⁻/PI⁻, FITC⁺/PI⁻, FITC⁺/PI⁺, and FITC⁻/PI⁺ were classified as living, early apoptotic, late apoptotic, and necrotic cells, respectively. B, The percentage of apoptotic cells from flow cytometry as shown in (A). Results were showed by means (standard deviation) from 3 replicates. *** $P < .001$ versus medium. ### $P < .001$ versus 5-FU. In this figure, 5-FU, CpG (0.25), and CpG (2.5) represent the concentrations of 5-FU, CpG-ODN, and CpG-ODN which were 2.5 $\mu\text{g}/\text{mL}$, 0.25 μM , and 2.5 μM , respectively. CpG-ODN indicates cytosine-phosphorothioate-guanine oligodeoxynucleotides; PI, propidium iodide; 5-FU, 5-fluorouracil.

(Figure 1A). Compared with 5-FU alone, cell viability was significantly reduced with the treatment of CpG-ODN + 5-FU ($P < .05$, Figure 1B). On the contrary, no significant difference of cell viability was observed between treatment with DOX alone and CpG-ODN + DOX (Figure 1C).

Cytosine-Phosphorothioate-Guanine ODN Induced Morphological Changes in T24 Cells

Cells treated with CpG-ODN or CpG-ODN + 5-FU showed obvious staining chromatin pyknosis (Figure 2). Chromatin pyknosis and drug concentrations showed positive correlation.

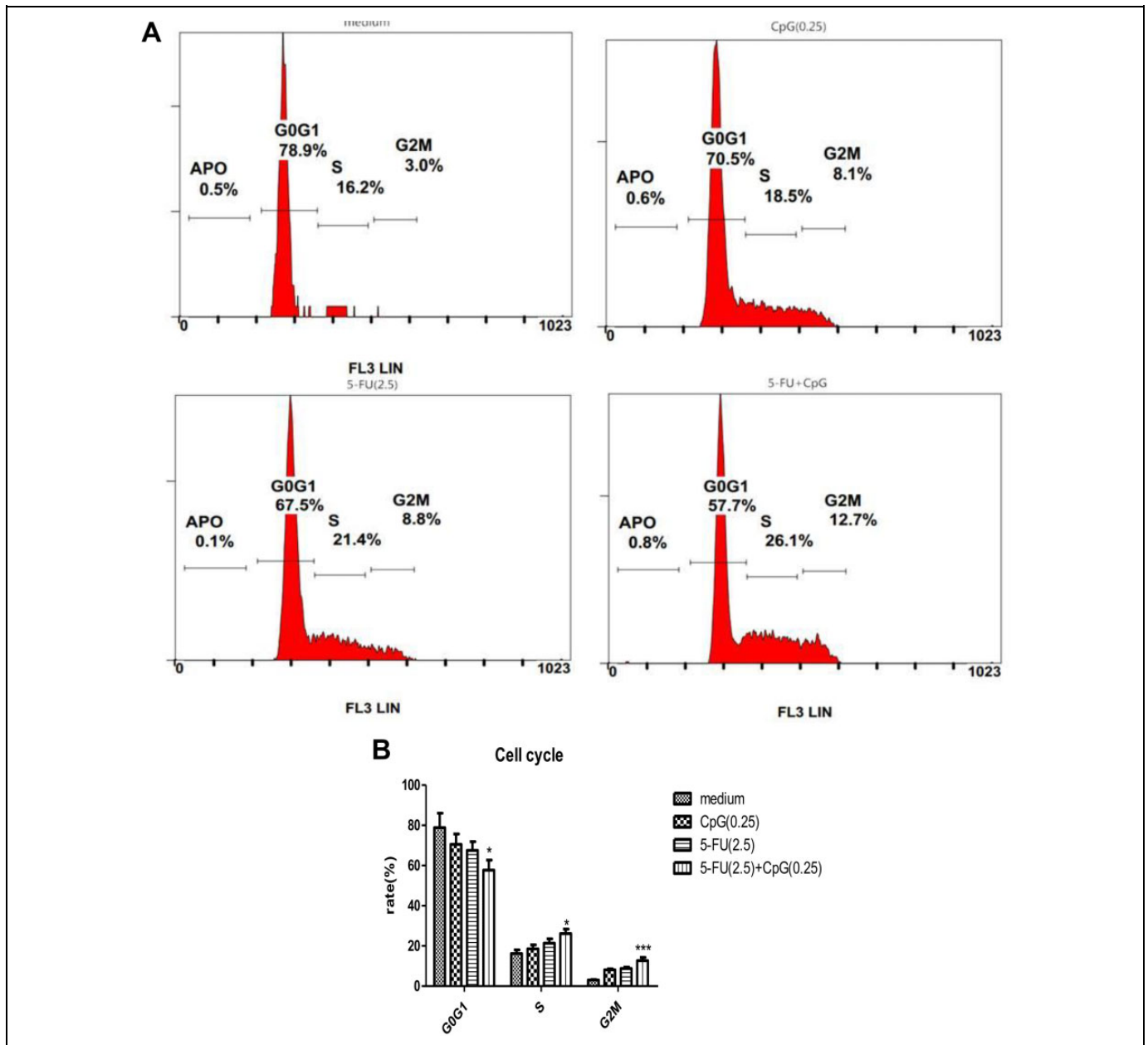


Figure 4. Effect of CpG-ODN, 5-FU, and CpG-ODN + 5-FU on cell cycle of T24 cells. A, Cells were incubated with CpG-ODN, 5-FU, and CpG-ODN + 5-FU at indicated concentrations for 48 hours. Cell-cycle distribution was determined by flow cytometry after PI staining. B, The percentage of cells in different phases was expressed as means (standard deviation) of 3 independent experiments. * $P < .05$, *** $P < .001$ versus medium. In this figure, 5-FU and CpG (0.25) represented that the concentration of 5-FU and CpG-ODN was 2.5 $\mu\text{g}/\text{mL}$ and 0.25 μM , respectively. CpG-ODN indicates cytosine-phosphorothioate-guanine oligodeoxynucleotides; PI, propidium iodide; 5-FU, 5-fluorouracil.

These results demonstrated that CpG-ODN or CpG-ODN + 5-FU significantly induced morphological changes of T24 cells.

Cytosine-Phosphorothioate-Guanine ODN Increased Apoptosis Rate and Arrested More Cells at the S and G2/M Phases

Cytosine-phosphorothioate-guanine ODN at 2.5 μM significantly increased the proportion of cell apoptosis ($P < .001$). Synergistic effect on cell apoptosis of CpG-ODN + 5-FU was

significantly higher than CpG-ODN or 5-FU alone (Figure 3A and B). Both CpG-ODN and/or 5-FU increased cells proportion in S and G2/M phases. However, CpG-ODN + 5-FU reached significance (Figure 4A and B).

Cytosine-Phosphorothioate-Guanine ODN Inhibited the Expression of Antiapoptotic Factors

Cytosine-phosphorothioate-guanine ODN decreased the expression of Bcl-2 in both RNA and protein levels (Figures 5

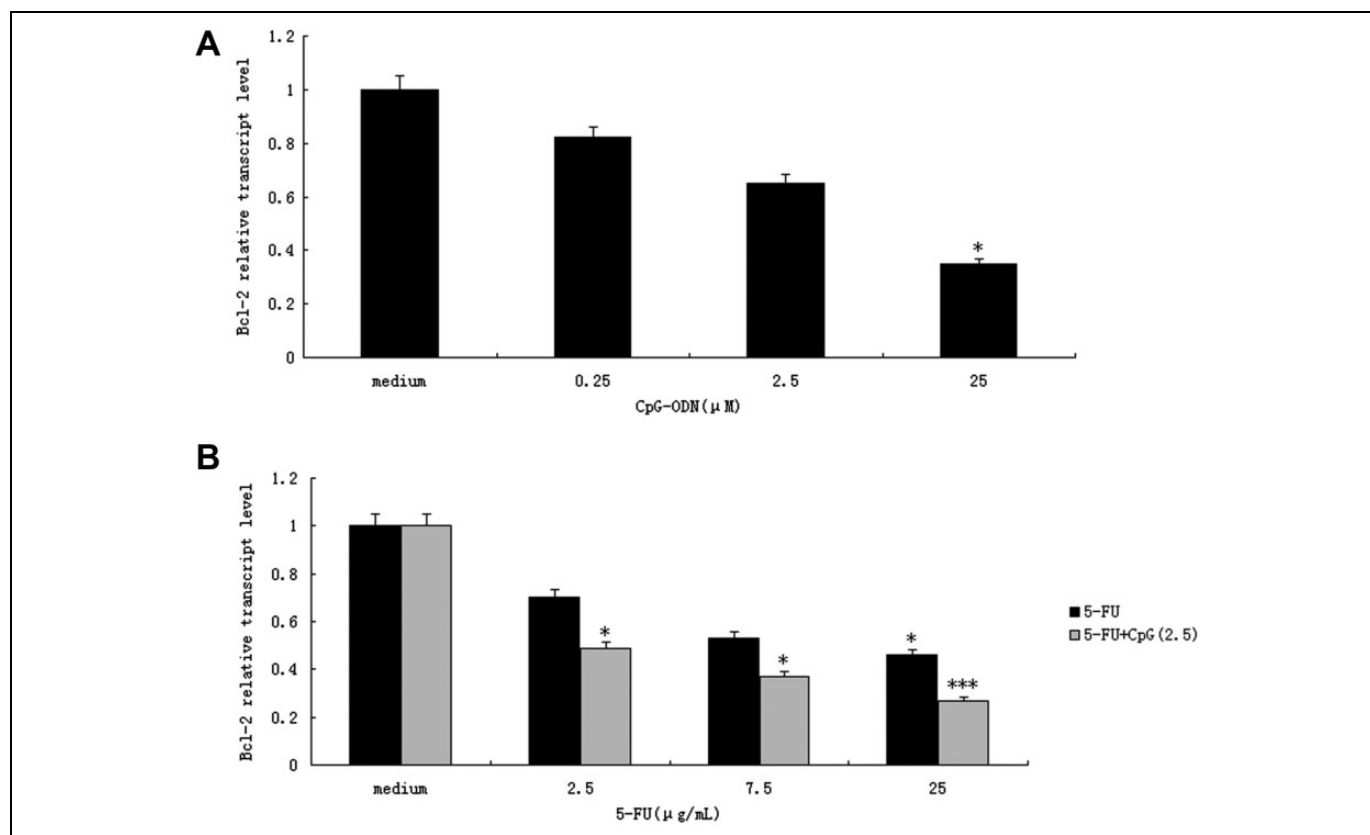


Figure 5. Effect of CpG-ODN and 5-FU alone or in combination on the expression of Bcl-2 in T24 cells. A, The expression of Bcl-2 in T24 cells treated with different concentrations of CpG-ODN. B, The expression of Bcl-2 in T24 cells treated with different concentrations of 5-FU in the presence and absence of 2.5 μM CpG-ODN for 6 hours. Results were showed as means (standard deviation) from 3 replicates (* $P < .05$, *** $P < .001$ vs medium). Bcl-2 indicates B-cell lymphoma-2; CpG-ODN, cytosine-phosphorothioate-guanine oligodeoxynucleotides; 5-FU, 5-fluorouracil.

and 6B). When CpG-ODN was combined with 5-FU, Bcl-2 was decreased significantly at RNA level, rather than 5-FU alone (Figure 5). Treatments of CpG-ODN + 5-FU and 5-FU obviously decreased the protein level of Bcl-2, with no difference between the groups (Figure 6B). Furthermore, CpG-ODN significantly decreased the RNA level of Survivin with time and concentration dependence (Figure 7). However, downregulation of Survivin in protein level induced by CpG-ODN treatment showed no significance (Figure 6C). Similar with the treatment of 5-FU alone, when CpG-ODN was combined with 5-FU, protein expression of Survivin was decreased significantly, and no significance was observed between the groups (Figure 6C).

Discussion

Cytosine-phosphorothioate-guanine ODN is an ODN drug and composed of different DNA bases. Studies proved that CpG-ODN could enhance the antitumor effects of chemotherapies on some malignant tumors.¹³ For example, CpG-ODN 7909 induced the activation of immunomodulatory and the death of chronic lymphocytic leukemia cells.¹⁴ Cytosine-Phosphorothioate-Guanine-ODN 1826 inhibited metastatic

colorectal adenocarcinoma in mouse hepatic tumor model.¹⁵ However, reports about the role of CpG-ODN in human bladder cancer are rare. Though CpG-ODN may be used as a candidate in the treatment of bladder cancer, chemotherapy is still the key method. The main chemotherapy for metastatic bladder cancer is methotrexate + vincristine + adriamycin + cisplatin (M-VAP), gemcitabine + cisplatin, and methotrexate + vincristine + cisplatin. Currently, M-VAP method is most used in clinical trials.¹⁶ Doxorubicin is a broad-spectrum antitumor drug and independent of cell-cycle specificity and also works in part by interfering with the DNA function. The main mechanism of DOX is embedding molecule into the DNA base pairs, inhibiting the synthesis of DNA and RNA, and leading to cancer cells death.¹⁷ 5-Fluorouracil is another widely used anti-neoplastic drug.¹⁸ Its mechanism is to inhibit thymine synthase, thereby reducing DNA synthesis. In this study, we assayed the potential role of CpG-ODN against human bladder cancer and the immunoenhancement to chemosensitivity with T24 cell line, by comparing the antitumor activity of CpG-ODN with those of DOX and 5-FU directly, or with those of the combination of CpG-ODN and DOX/5-FU, respectively.

We found that CpG-ODN alone effectively reduced cell viability and induced apoptosis at S and G2/M phases in T24

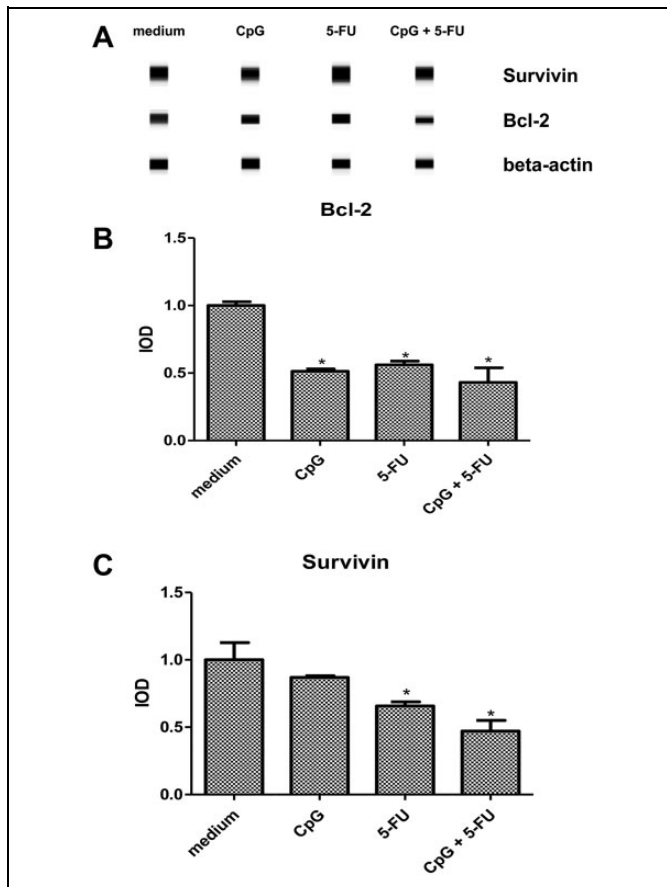


Figure 6. Effect of CpG-ODN and 5-FU alone or in combination on the protein expression of Bcl-2 and Survivin in T24 cells. A, The WES graph of Bcl-2 and Survivin in T24 cells treated with CpG-ODN, 5-FU, and combined CpG-ODN and 5-FU for 24 hours. B, The protein expression of Bcl-2 in T24 cells treated with CpG-ODN, 5-FU, and combined CpG-ODN and 5-FU for 24 hours. C, The protein expression of Survivin in T24 cells treated with CpG-ODN, 5-FU, and combined CpG-ODN and 5-FU for 24 hours. Results were showed as means (standard deviation) from 3 replicates (* $P < .05$ vs medium). Bcl-2 indicates B-cell lymphoma-2; CpG-ODN, cytosine-phosphorothioate-guanine oligodeoxynucleotides; WES, automated capillary Western blot; 5-FU, 5-fluorouracil.

cell line (Figures 1A, 3, and 4). This indicated that the mechanism of chemosensitization of CpG-ODN may be related to the induction of cell-cycle arrest at S and G2/M phases, and inducing apoptosis. Moreover, CpG-ODN induced the cells apoptosis and enhance chemosensitivity of 5-FU by decreasing the expression of some antiapoptotic factors (Figures 1B, 3-6). Further study showed that CpG-ODN could improve chemosensitivity of 5-FU (Figure 1B), rather than DOX (Figure 1C). The inhibition of CpG-ODN + 5-FU on T24 cells was higher than that single-drug treatment groups (Figure 1B). In contrast, compared with the single-drug groups, the combination of CpG-ODN and DOX showed the lowest inhibitory effect on T24 cells compared with single-drug groups (Figure 1C). This result may be caused by the formation of a new complexes of CpG-ODN and DOX and thus reduce their antitumor activity. Nevertheless, the target of 5-FU is an enzyme that does not cooperate with CpG-ODN structurally¹⁹; thus, CpG-ODN may have a synergistic effect with 5-FU. These results suggested that CpG-ODN might be antagonistic to chemotherapy if the drug acted on the nucleotide base pairs. Therefore, CpG-ODN could be used as chemosensitivity enhancer with some drugs.

Cell-cycle arrest is regarded to be another important apoptotic characteristic induced by antitumor drugs.²⁰ 5-Fluorouracil is a cell-cycle-specific factor and arrests cells at S phase, whereas CpG-ODN does not have this specificity.²¹ In this study, CpG-ODN combined with 5-FU arrested more cells at S and G2/M phases than single medicine groups (Figure 4). The results suggested that the chemosensitization of CpG-ODN was associated to cell-cycle arrest at S phase and the combination of CpG-ODN with 5-FU promoted cells apoptosis by inducing the cell-cycle distribution. However, further study is needed to assay the exact molecular mechanism.

B-cell lymphoma-2 is an antiapoptotic factor that links to many human cancers and its overexpression leads to the resistance of radiotherapy and chemotherapy.^{22,23} The balance of Bcl-2 plays a crucial role in regulating and executing a series of apoptotic pathways.²⁴ Survivin, an inhibitor of apoptotic proteins (IAPs), produced antiapoptotic effect through

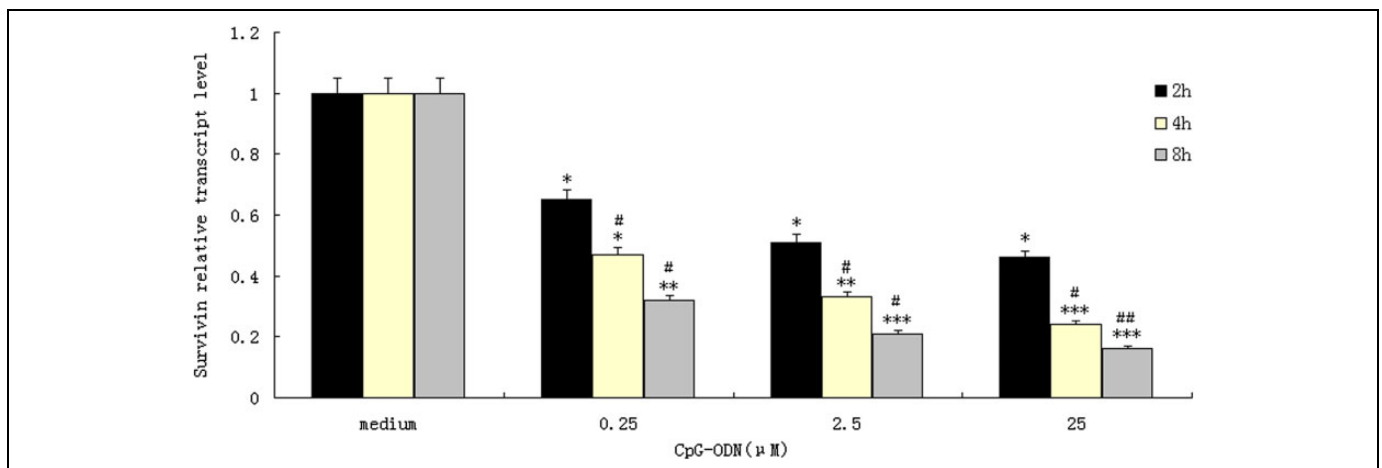


Figure 7. The expression of Survivin in T24 cells treated with different concentrations of CpG-ODN for 3 periods. Results were showed as means (standard deviation) from 3 replicates (* $P < .05$, ** $P < .01$, *** $P < .001$ vs medium, # $P < .05$, ### $P < .01$ vs 2 hour-group). CpG-ODN indicates cytosine-phosphorothioate-guanine oligodeoxynucleotides.

complex signaling pathways.²⁵ Previous studies have demonstrated that the overexpression of IAPs inhibited the apoptosis of cancer and resistance to chemotherapy or radiotherapy.²⁶ Therefore, the expression of these antiapoptotic factors is often used to detect the efficacy of antitumor drugs. In this study, we found that CpG-ODN and 5-FU alone reduced the expression of Bcl-2 in T24 cells, which showed a dose-dependence (Figure 5). Moreover, the combination of CpG-ODN and 5-FU significantly reduced the expression of Bcl-2 compared with other groups (Figures 5 and 6B). Furthermore, CpG-ODN inhibited the expression of Survivin in a time-dependent manner. Automated capillary Western blot assay supported the results to quantitative RT-PCR (Figure 7). In addition, the expression of Bcl-2 and Survivin were down-regulated by CpG-ODN + 5-FU. These results indicated the anticancer effect and immunoenhancement on chemotherapeutics of CpG-ODN.

Conclusion

Our study showed that CpG-ODN could promote the apoptosis of T24 cells and improve the therapeutic effect of 5-FU by arresting more cells in S and G2/M phases and inhibiting some antiapoptotic factors (Bcl-2 and Survivin). The viability inhibition of T24 cell indicated the direct cytotoxicity of CpG-ODN on bladder cancer cells. The change of cell morphology demonstrated that CpG-ODN could enhance the chemosensitivity of 5-FU by increasing cell apoptosis without activating the immune system. Thus, CpG-ODN may be a potential candidate for human bladder cancer.

Authors' Note

Minjie Meng is also affiliated with Guangdong Pharmaceutical University.


Declaration of Conflicting Interests

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