

# Endocrine therapy inhibits proliferation and migration, promotes apoptosis and suppresses survivin protein expression in colorectal cancer cells

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**Abstract.** The majority of colorectal cancers (CRCs) are hormone-dependent. Thus, endocrine therapy has become an attractive strategy to treat CRC. The aim of the present study was to investigate the inhibitory effect of combined tamoxifen (TAM) plus  $\beta$ -estradiol (E2) treatment on human DLD-1 CRC cells. The human DLD-1 CRC cell line was treated with different concentrations of TAM,  $\beta$ -estradiol, or a combination of these two agents. Cell viability was assessed using an MTT assay, while apoptosis was detected using flow cytometry analysis. Alterations in the RNA and protein levels of the apoptosis-associated factors cyclin D1 and survivin were measured in the treated DLD-1 cells using semi-quantitative polymerase chain reaction (sqPCR) and western blot analyses. Alterations in cellular migration ability were monitored using a Transwell migration assay. Treatment with TAM,  $\beta$ -estradiol and TAM plus  $\beta$ -estradiol inhibited DLD-1 cell viability. The flow cytometry results revealed that these drugs promoted cell apoptosis, and the Transwell migration assay results indicated that the reduction in cell migration was greater in the TAM+E2 treatment group when compared with each treatment alone. sqPCR and western blot analysis results demonstrated that

TAM, E2 and a combination of the two affected survivin expression based on the drug concentration and the treatment duration; however, they demonstrated no significant effect on cyclin D1 expression. In conclusion, treatment of DLD-1 cells with TAM,  $\beta$ -estradiol, or a combination of these two drugs, inhibited cell viability and migration, promoted cell apoptosis, and reduced the mRNA and protein expression levels of survivin in a dose- and time-dependent manner. These results provide novel experimental basis for hormonal adjuvant therapy for the treatment of CRC.

## Introduction

Colorectal cancer (CRC) is one of the most common malignant digestive tumors in China. In recent years, the global incidence of CRC has increased by 2% every year, while the incidence rate in China has increased by 4.2% each year (1,2). As patients are more likely to be asymptomatic during the early stages of the disease, the majority of patients are already at advanced stages at the time of diagnosis. Therefore, their prognosis is poor and risk of mortality is higher (3). The comprehensive treatment for CRC primarily involves surgical resection combined with a variety of therapeutic measures, such as chemotherapy and radiotherapy. Chemotherapy remains one of the most essential approaches to CRC treatment. However, although chemotherapeutic drugs, including irinotecan, oxaliplatin and fluoropyrimidines, increase the efficacy of advanced CRC treatment, the median survival time of patients remains <2 years (4). Therefore, investigating novel and effective strategies to treat CRC is particularly important.

During the 1960s, Elwood V. Jensen and colleagues were the first to experimentally confirm the existence of the estrogen receptor (ER) (5). In the early 1970s, physicians began to use the ER as an indicator for the use of endocrine therapy in patients with breast cancer (6). The efficacy of endocrine therapy for ER-positive cancers was greater than that for ER-negative cancers. Starting in the mid-1970s, the ER was used as a prognostic indicator for patients with breast cancer, and gradually became the most effective therapeutic target (7). In 1997, investigators discovered a novel ER, known

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**Abbreviations:** CRC, colorectal cancer; TAM, tamoxifen; ER, estrogen receptor; E2, 17 $\beta$ -estradiol; DEPC, diethyl pyrocarbonate

**Key words:** colorectal cancer, endocrine therapy, tamoxifen, phenyl tetrazosurvivin, cyclin D1

as ER $\beta$  (8,9). The distribution of this receptor is different to that of the classic ER $\alpha$ . In addition, this receptor demonstrates different specificities and affinities for ligands. ER $\beta$  is highly expressed in the gonads, uterus, colon and brain (10-12); however, its function in cancer has not yet been fully elucidated (13,14). Similar to breast cancer, previous studies have demonstrated that CRC is a hormone-dependent cancer. The majority of CRC tissues and cell lines do not express ER $\alpha$ , whereas they do express ER $\beta$  to a high level (15,16). Using the semi-quantitative polymerase chain reaction (sqPCR) method, Arai *et al* (16) demonstrated that five human CRC cell lines expressed ER $\beta$  and not ER $\alpha$ . As an anticancer hormonal therapy, tamoxifen (TAM) has been applied in hormone therapies targeting breast cancer. TAM is a chemically synthesized, nonsteroidal, anti-estrogen, antitumor drug. The precise mechanisms underlying the anticancer effects of TAM are currently unclear. TAM may compete with the intracellular ER to inhibit estradiol absorption in the body, thus inhibiting estrogen-dependent cancer growth (17). The main member of the estrogen family, 17 $\beta$ -estradiol (E2), is a corticosteroid hormone that is primarily synthesized by ovarian follicles, the corpus luteum and the placenta during pregnancy (18). Epidemiological studies have demonstrated that the incidence of CRC in females is lower than that in males (19,20), and additional studies have indicated that patients undergoing hormone replacement therapy, usually in the form of estrogen supplements, were less prone to suffer from CRC (21,22). Therefore, the aim of the present study was to investigate the anticancer effects of combined TAM and E2 treatment on human DLD-1 CRC cells, in order to provide a theoretical and experimental basis for the clinical treatment of CRC using these agents.

## Materials and methods

**Cell culture.** DLD-1 cells (Sun Yat-sen University Cancer Centre, Guangdong, China) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 units/ml penicillin and 100 mg/ml streptomycin (both Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Effects of TAM and/or E2 on cell growth.** DLD-1 cells were dissociated with trypsin and were re-suspended in culture medium containing 10% FBS to produce a single-cell suspension. The cells were seeded onto 96-well plates at 5x10<sup>3</sup> cells/well with 100  $\mu$ l in each well. Following incubation at 37°C in 5% CO<sub>2</sub> for 24 h, the cells were treated with varying concentrations of the drugs. The concentrations applied in the group treated with E2 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) only were 0.015625x10<sup>-3</sup>, 0.03125x10<sup>-3</sup>, 0.0625x10<sup>-3</sup>, 0.125x10<sup>-3</sup>, 0.25x10<sup>-3</sup>, 0.5x10<sup>-3</sup> and 1x10<sup>-3</sup> M. The concentrations applied in the group treated with TAM (Yangtze River Pharmaceutical Group Co., Ltd., Taizhou, Jiangsu, China) only were 0.015625x10<sup>-4</sup>, 0.03125x10<sup>-4</sup>, 0.0625x10<sup>-4</sup>, 0.125x10<sup>-4</sup>, 0.25x10<sup>-4</sup>, 0.5x10<sup>-4</sup> and 1x10<sup>-4</sup> M. The concentrations in the combined-treatment group were those of the lowest to highest corresponding concentrations in the TAM and E2 groups combined. Each well was treated with

100  $\mu$ l of drug. The blank control group was treated with dimethyl sulfoxide (DMSO). A total of three replicates for each treatment were included. Following incubation for 24, 48 and 72 h, 0.05% MTT (Guangzhou Whiga Technology Co., Ltd., Guangzhou, Guangdong, China; 20  $\mu$ l/well) was added, and the cells were incubated for a further 4 h at 37°C. The supernatant was then discarded and 150  $\mu$ l of DMSO was added to each well. Following vigorous mixing for 10 min, the absorbance of each well was measured at wavelengths of 490 and 655 nm using a microplate reader. The rate of cell viability inhibition was then calculated using the following equation based on optical density (OD):  $1 - [\text{OD}_{\text{treatment group}} / \text{OD}_{\text{control group}}]$ .

**Detection of cell apoptosis by flow cytometry analysis.** DLD-1 cells (~5x10<sup>5</sup>) were seeded onto 60-mm tissue culture plates and cultured for 24 h. The culture medium was then discarded and fresh culture medium containing TAM or E2 was added. DMSO was used as a control. The experimental design was based on the MTT assay results. The concentration of drug that exhibited a 30% reduction in cell viability (IC<sub>30</sub>), which demonstrated a relatively low inhibitory effect, and the IC<sub>70</sub>, which demonstrated a greater inhibitory effect, were used as a basis for grouping. Cells were treated with the following: DMSO (control); 0.0625x10<sup>-3</sup> M E2; 0.5x10<sup>-3</sup> M E2; 0.0625x10<sup>-4</sup> M TAM; 0.25x10<sup>-4</sup> M TAM; 0.0625x10<sup>-3</sup> M E2 + 0.0625x10<sup>-4</sup> M TAM; 0.5x10<sup>-3</sup> M E2 + 0.25x10<sup>-4</sup> M TAM. The cells were cultured for 24, 48 or 72 h, then the supernatant was collected and adherent cells were dissociated with trypsin by centrifuging at 500 x g for 5 min and 4°C for downstream analysis. The cells were then washed with phosphate-buffered saline (PBS), centrifuged again at 500 x g for 5 min and 4°C and double-stained using the Annexin-V-FLUOS Staining kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions in the dark for 5 min. Apoptosis of the cells was measured using a flow cytometry instrument (Beckman Coulter, Inc., Brea, CA, US).

**sqPCR analysis.** DLD-1 cells (~2.5x10<sup>5</sup>) were seeded onto 6-well plates and incubated for 24 h for complete attachment. Cells were then treated with E2 and TAM, and total cellular RNA was extracted using TRIzol® reagent (cat. no. 15596; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit and DreamTaq DNA Polymerase (both Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) according to the manufacturer's instructions. Reverse transcription of RNA was first performed using 1  $\mu$ g total RNA, 1  $\mu$ l random primers and diethyl pyrocarbonate (DEPC)-treated water to a final volume of 12  $\mu$ l. Following incubation at 65°C for 5 min, 4  $\mu$ l Reaction Buffer (5X), 200 units Ribolock RNase inhibitor, 2 mM dNTP Mix and 200 units Revert Aid Moloney murine leukemia virus reverse transcriptase were added. Samples (20  $\mu$ l) was mixed gently and centrifuged at 500 x g for 5 min and 4°C. The RT reaction conditions were as follows: 25°C for 5 min, 42°C for 60 min, and reaction termination at 70°C for 5 min. For amplification of cyclin D1, survivin and  $\beta$ -actin (the internal control) cDNA sequences, a PCR reaction mixture containing 2  $\mu$ l DreamTaq Buffer (10X), 0.2 mM dNTP Mix, 0.05  $\mu$ M primers, 1  $\mu$ l cDNA, 0.2  $\mu$ l DreamTaq DNA Polymerase

and 13.8  $\mu$ l DEPC-treated water to a final volume of 20  $\mu$ l was used. PCR was performed in a S1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction mixture was first heated to 95°C for 5 min, then amplification was performed for 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, which was followed by a final extension step at 72°C for 7 min. The primer sequences were as follows: Cyclin D1, forward, 5'-GTCACCTAGCAAGCTGCCGAACC-3', and reverse, 5'-ACGACAGACAAAGCGTCCCTCAA-3'; survivin, forward, 5'-TCTGGCGTAAGATGATGG-3', and reverse, 5'-GAAATAAGTGGGTCTGAAGTG-3';  $\beta$ -actin, forward, 5'-TTAGTTGCGTTACACCCTTTC-3', and reverse, 5'-AACCGACTGCTGTCACCTTC-3'. The PCR products were separated on a 2% agarose gel and visualized using 1% ethidium bromide staining and ultraviolet illumination. The expected sizes of the amplification products were 222 bp for cyclin D1, 363 bp for survivin and 164 bp for  $\beta$ -actin. Target gene expression levels were semi-quantified based on band intensities using the Bio-Rad GelDoc XR instrument and Quantify One software version 4.6.9 (both Bio-Rad Laboratories, Inc.). The following equations were used: Band Intensity = mean OD x band area; and Relative Quantification of mRNA = band intensity<sub>target gene</sub>/band intensity<sub>internal reference</sub>.

**Western blot analysis.** DLD-1 cells ( $\sim 1 \times 10^6$ ) were seeded onto 100-mm<sup>2</sup> tissue culture plates and incubated for 24 h for complete attachment. The cells were treated according to the aforementioned experimental groupings and were harvested following dissociation with trypsin. Cells were washed twice with PBS, centrifuged at 500 x g for 5 min and 4°C, and 60  $\mu$ l ProteoJET Mammalian Cell Lysis Reagent (Fermentas; Thermo Fisher Scientific, Inc.) was added. The cells were vortexed for 10 sec and lysed for 30 min with occasional vortexing. Following centrifugation at 16,000 x g for 30 min and 4°C, the supernatant was transferred to a fresh Eppendorf tube and stored at -80°C. The protein concentration was determined using a Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equal quantities of protein (20  $\mu$ g) were loaded and separated by 10% SDS-PAGE. The proteins were then electrotransferred onto a polyvinylidene fluoride membrane. The membrane was blocked with Tris-buffered saline solution containing 1% Tween-20 (TBS-T) buffer and 5% skim milk for 1 h at room temperature, followed by incubation with the following primary antibodies at 4°C overnight: Anti-cyclin D1 (cat. no. 2926; dilution, 1:1,000; CST Biological Reagents Company Limited, Shanghai, China), anti-survivin (cat. no. ab8228; dilution, 1:1,000; Abcam, Cambridge, UK) and anti- $\beta$ -actin (cat. no. ms-1295; dilution, 1:5,000; Thermo Fisher Scientific, Inc.). The membrane was then washed using TBS-T and incubated with secondary antibodies [Amersham ECL sheep anti-mouse IgG, horseradish peroxidase (HRP)-linked whole Ab; cat. no. NA931; dilution, 1:5,000; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA; and the Amersham ECL donkey anti-rabbit IgG, HRP-linked whole Ab; cat. no. NA934; dilution, 1:5,000; GE Healthcare Bio-Sciences] at room temperature for 1 h. The membrane was subsequently washed three times with TBS-T, and proteins were detected using an enhanced chemiluminescence reagent

kit (cat. no. WBKLS0100; EMD Millipore, Billerica, MA, US) according to the manufacturer's instructions. Then the membrane was exposed using X-ray film in a dark room. The X-ray film was scanned, and protein expression was quantified by densitometric analysis using Image J v1.46r software (National Institutes of Health, Bethesda, MD, USA). Three experimental repeats were performed.

**Detection of cell migration ability using the Transwell assay.** Cells were treated for 24 h according to the aforementioned experimental groupings. The cells were then dissociated with trypsin, harvested, and counted. The upper chambers of the Transwell plates (Corning Incorporated, Corning, NY, USA) containing an 8.0- $\mu$ m membrane, were seeded with  $5 \times 10^5$  cells. Complete medium containing 20% FBS was then added to the lower chambers, and the Transwell plates were placed in an incubator at 37°C for 24 h. Non-migrated cells in the upper chamber were then removed with a cotton swab. Following washing twice with PBS, the cells were fixed with 75% ethanol for 20 min at room temperature, stained with 10% Giemsa stain for 10 min at room temperature, washed with running water, dried and cells in ten random high-magnification fields of view were counted under a light microscope. The average number of migrated cells in each group was then calculated.

**Statistical analysis.** Data were presented as a percentage or the mean. Statistical analyses were performed using SPSS software (version 21.0; IBM SPSS, Armonk, NY, USA). Group comparisons of normally distributed data were performed using Student's t-tests (for two samples) or one-way analysis of variance (for multiple comparisons using a Bonferroni post hoc test). All statistical tests used in this study were two-sided, and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**The inhibitory effects of TAM and E2 on the viability of DLD-1 CRC cells.** As shown in Fig. 1A, the rate of inhibition of DLD-1 cell viability was positively associated with TAM concentration following 24 h ( $P=0.018$ ), 48 h ( $P=0.016$ ) and 72 h ( $P=0.017$ ) of treatment. The rate of inhibition of DLD-1 cell viability was also positively associated with E2 concentration following 24 h ( $P=0.024$ ), 48 h ( $P=0.028$ ) and 72 h ( $P=0.021$ ) of treatment (Fig. 1B). In addition, as shown in Fig. 1C, the rate of inhibition of DLD-1 cell viability was positively associated with the concentration of combination-treatment (TAM+E2) following 24 h ( $P=0.018$ ), 48 h ( $P=0.021$ ) and 72 h ( $P=0.028$ ) of treatment. The IC<sub>50</sub> values for TAM, E2 and combined drug treatment (TAM+E2) for 24, 48 and 72 h were decreased, respectively (Fig. 1D). Furthermore, the combination-treatment group exhibited the greatest reduction in DLD-1 cell viability when compared with the single-drug-treatment groups ( $P < 0.05$ ). In addition, TAM treatment alone reduced the viability of DLD-1 cells to a greater extent than E2 treatment alone ( $P < 0.05$ ; Fig. 1D).

**Induction of DLD-1 cell apoptosis by TAM and E2.** The results of the quantitative annexin-V/propidium iodide double-staining assay revealed that TAM, E2 and TAM+E2



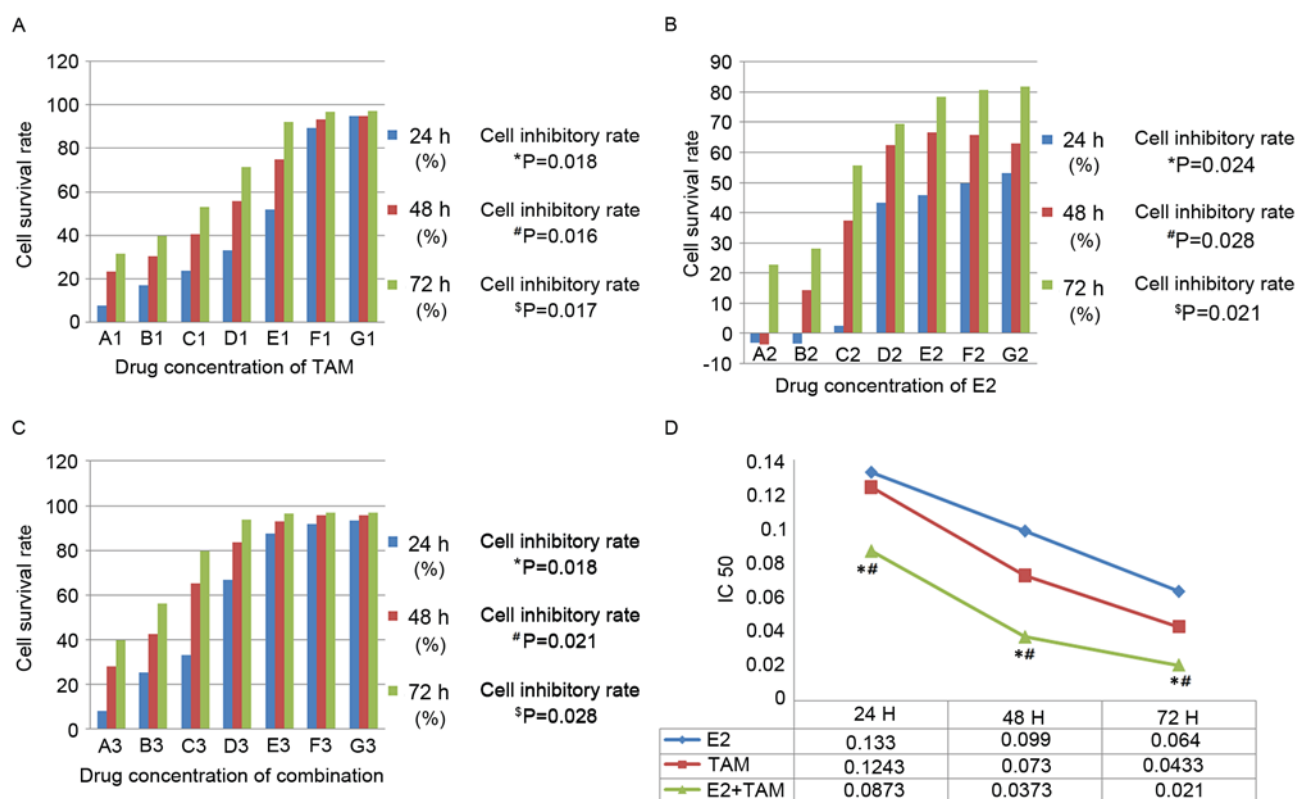


Figure 1. Inhibitory effects of TAM, E2 or TAM plus E2 treatment on cell viability, as determined using an MTT assay. (A) TAM only treatment: A1,  $0.015625 \times 10^{-4}$  M; B1,  $0.03125 \times 10^{-4}$  M; C1,  $0.0625 \times 10^{-4}$  M; D1,  $0.125 \times 10^{-4}$  M; E1,  $0.25 \times 10^{-4}$  M; F1,  $0.5 \times 10^{-4}$  M; G1,  $1 \times 10^{-4}$  M. The effect of cell viability inhibition was positively associated with TAM concentration ( $^*P < 0.05$  at 24 h;  $^*P < 0.05$  at 48 h;  $^*P < 0.05$  at 72 h; comparisons were made between all concentrations at each time point). (B) E2 only treatment: A2,  $0.015625 \times 10^{-3}$  M; B2,  $0.03125 \times 10^{-3}$  M; C2,  $0.0625 \times 10^{-3}$  M; D2,  $0.125 \times 10^{-3}$  M; E2,  $0.25 \times 10^{-3}$  M; F2,  $0.5 \times 10^{-3}$  M; G2,  $1 \times 10^{-3}$  M. The effect of cell viability inhibition was positively associated with E2 concentration ( $^*P < 0.05$  at 24 h;  $^*P < 0.05$  at 48 h;  $^*P < 0.05$  at 72 h; comparisons were made between all concentrations at each time point). (C) Combination-treatment group: A3, A1+A2; B3, B1+B2; C3, C1+C2; D3, D1+D2; E3, E1+E2; F3, F1+F2; G3, G1+G2. The effect of cell viability inhibition was positively associated with combination-treatment concentration ( $^*P < 0.05$  at 24 h;  $^*P < 0.05$  at 48 h;  $^*P < 0.05$  at 72 h; comparisons were made between all concentrations at each time point). (D) The IC<sub>50</sub> results indicate that the inhibitory effects in these three treatment groups increased with an increase in treatment duration. The combination-treatment group exhibited the highest rate of inhibition.  $^*P < 0.05$  vs. TAM;  $^*P < 0.05$  vs. E2. TAM, tamoxifen; E2, 17AM, tamoxifen; IC<sub>50</sub>, half maximal inhibitory concentration.

significantly induced DLD-1 cell apoptosis when compared to the control group (TAM:  $P < 0.05$ ; E2:  $P < 0.05$ ; TAM+E2:  $P < 0.05$ ). In addition, apoptosis rates were positively associated with the treatment duration and drug concentration (Fig. 2A). The rate of apoptosis was low following treatment with TAM and E2 for 24 h, while the rate of apoptosis in the drug-combination treatment group reached 15% following 24 h (Fig. 2A). Following 48 h of treatment, the TAM and E2-treated groups exhibited increased apoptosis. The rate of apoptosis in  $0.5 \times 10^{-3}$  M E2-treated cells was 23.1%, and in  $0.25 \times 10^{-4}$  M TAM-treated cells the apoptosis rate was 22% (Fig. 2). The rate of apoptosis increased following 72 h, whereby  $0.0625 \times 10^{-3}$  M E2 and  $0.5 \times 10^{-3}$  M E2-treated groups demonstrated apoptosis rates of 34.7 and 51.6%, respectively, and the rates of apoptosis for the  $0.0625 \times 10^{-4}$  M TAM and  $0.25 \times 10^{-4}$  M TAM-treated groups were 16.4 and 54.9%, respectively. In addition, the rate of apoptosis for the drug-combination treatment group reached 32.3% following 72 h (Fig. 2A).

**Effects of TAM and E2 on survivin and cyclin D1 mRNA expression as determined by sqPCR.** DLD-1 cells were treated with  $0.015625 \times 10^{-4}$ ,  $0.03125 \times 10^{-4}$ ,  $0.0625 \times 10^{-4}$  and  $0.125 \times 10^{-4}$  M TAM,  $0.015625 \times 10^{-3}$ ,  $0.03125 \times 10^{-3}$ ,  $0.0625 \times 10^{-3}$ ,  $0.125 \times 10^{-3}$ ,

$0.25 \times 10^{-3}$ ,  $0.5 \times 10^{-3}$  and  $1 \times 10^{-3}$  M E2, or a combination of these two drugs for 24, 48 and 72 h. The results indicated that the cyclin D1 mRNA expression levels were not significantly altered, while the expression of survivin mRNA was decreased; the degree of which was positively associated with drug concentration and treatment duration. The combined treatment markedly decreased the expression of survivin mRNA when compared with each treatment alone (Fig. 3).

**Effect of TAM and E2 on survivin and cyclin D1 protein expression levels in DLD-1 cells as determined by western blot analysis.** Following treatment of DLD-1 cells with  $0.015625 \times 10^{-4}$ ,  $0.03125 \times 10^{-4}$ ,  $0.0625 \times 10^{-4}$  and  $0.125 \times 10^{-4}$  M TAM for 24, 48 or 72 h, the level of cyclin D1 protein expression was not significantly altered, while survivin protein expression levels were decreased; the degree of which was positively associated with drug concentration and treatment duration (Fig. 4A). Following treatment with  $0.015625 \times 10^{-3}$ ,  $0.03125 \times 10^{-3}$ ,  $0.0625 \times 10^{-3}$ ,  $0.125 \times 10^{-3}$ ,  $0.25 \times 10^{-3}$ ,  $0.5 \times 10^{-3}$  and  $1 \times 10^{-3}$  M E2, the level of cyclin D1 protein expression was not significantly altered. In addition, the level of survivin protein expression was not significantly altered following 24 h of E2 treatment, however, it was observed to increase with increasing

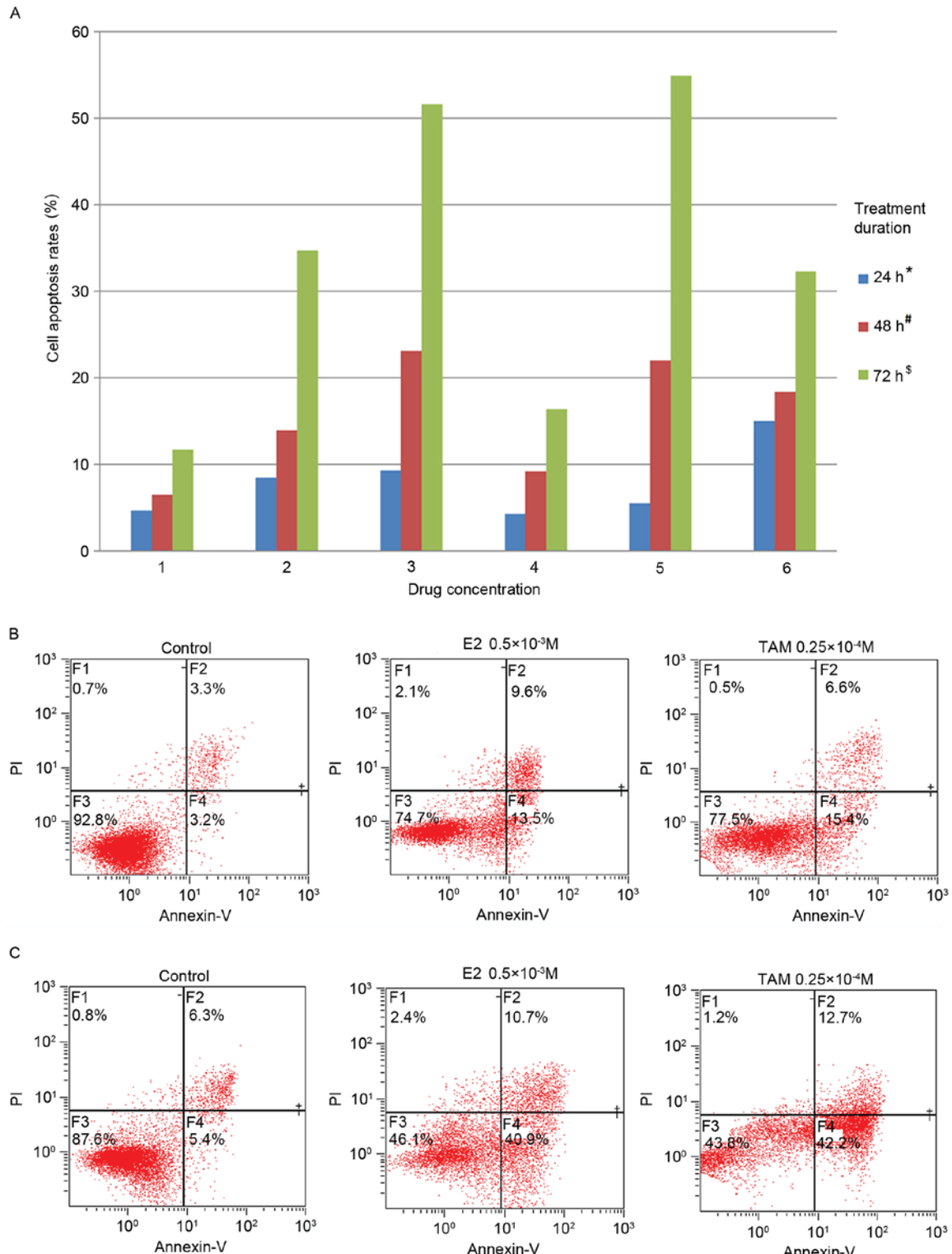


Figure 2. Induction of DLD-1 cell apoptosis by TAM and E2. (A) Cell apoptosis rates following treatment with TAM and/or E2. 1, control; 2,  $0.0625 \times 10^{-3}$  M E2; 3,  $0.5 \times 10^{-3}$  M E2; 4,  $0.0625 \times 10^{-4}$  M TAM; 5,  $0.25 \times 10^{-4}$  M TAM; 6,  $0.0625 \times 10^{-3}$  M E2 +  $0.0625 \times 10^{-4}$  M TAM. With increasing concentrations of drug and treatment duration, the rate of apoptosis increased in 24 h ( $P < 0.05$ ), 48 h ( $P < 0.05$ ) and 72 h ( $P < 0.05$ ; comparisons were made between all concentrations at each time point). Following drug treatment for 72 h, high concentrations of E2 and TAM effectively induced DLD-1 cell apoptosis, and the rate of apoptosis reached 51.6 and 54.9%, respectively. (B) Apoptosis induced by  $0.5 \times 10^{-3}$  M E2 or  $0.25 \times 10^{-4}$  M TAM for 48 h. (C) Apoptosis induced by  $0.5 \times 10^{-3}$  M E2 or  $0.25 \times 10^{-4}$  M TAM for 72 h. TAM, tamoxifen; E2, 17 $\beta$ -estradiol; PI, propidium iodide.

drug concentrations at 48 and 72 h of treatment (Fig. 4B). Higher levels of survivin protein expression were observed at high and low drug concentrations, and lower levels of expression were observed at medium drug concentrations (Fig. 4B).

In the combination-drug treatment group, the level of cyclin D1 protein expression was not significantly altered, while expression of the survivin protein decreased in a concentration and time-dependent manner (Fig. 4C).

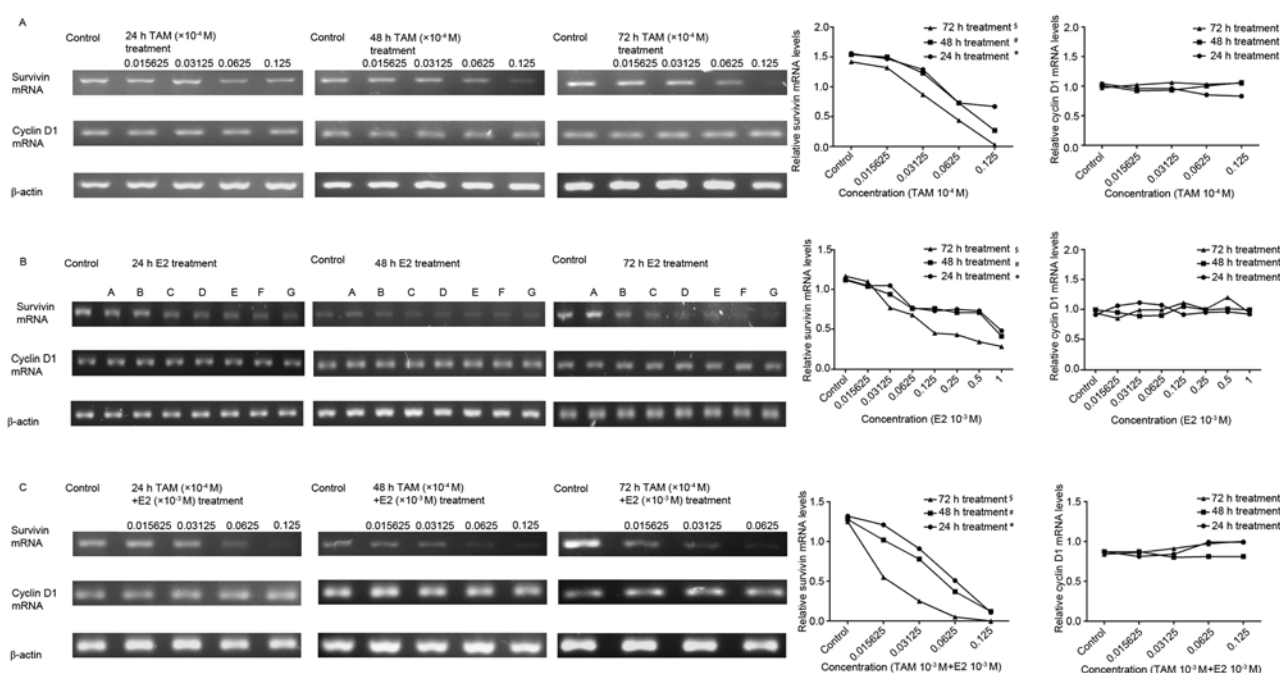


Figure 3. Effect of TAM and/or E2 treatment on survivin and cyclin D1 mRNA expression levels, as determined by sqPCR analysis. Gray-scale band densities of the reverse transcription-polymerase chain reaction results in each treatment group were compared and analyzed using ImageJ software. (A) Following treatment of DLD-1 cells with TAM, cyclin D1 mRNA expression levels were not significantly altered, while relative survivin mRNA expression levels were significantly decreased following treatment with increasing concentrations of TAM at 24 h ( $P<0.05$ ), 48 h ( $P<0.05$ ) and 72 h ( $P<0.05$ ; comparisons were made between all concentrations at each time point). (B) The expression of survivin and cyclin D1 following treatment of DLD-1 cells with E2. A,  $0.015625 \times 10^{-3}$  M; B,  $0.03125 \times 10^{-3}$  M; C,  $0.0625 \times 10^{-3}$  M; D,  $0.125 \times 10^{-3}$  M; E,  $0.25 \times 10^{-3}$  M; F,  $0.5 \times 10^{-3}$  M; G,  $1 \times 10^{-3}$  M. Following treatment, cyclin D1 mRNA expression levels were not significantly altered, while relative survivin mRNA expression levels were significantly decreased with increasing concentrations of E2 at 24 h ( $P<0.05$ ), 48 h ( $P<0.05$ ) and 72 h ( $P<0.05$ ; comparisons were made between all concentrations at each time point). (C) Expression of survivin and cyclin D1 following treatment of DLD-1 cells with TAM + E2. Cyclin D1 mRNA expression levels were not significantly altered, while relative survivin mRNA expression levels were significantly decreased with increasing drug concentrations at 24 h ( $P<0.05$ ), 48 h ( $P<0.05$ ) and 72 h ( $P<0.05$ ; comparisons were made between all concentrations at each time point). The results are presented as the expression level of survivin relative to that of the  $\beta$ -actin internal control. TAM, tamoxifen; E2, 17 $\beta$ -estradiol.

**Effects of TAM and/or E2 treatment on the migration capability of DLD-1 cells.** The results of the Transwell migration assay demonstrated that TAM and/or E2 treatment demonstrated significant inhibitory effects on the migration capabilities of DLD-1 cells (Fig. 5). DLD-1 cells were treated with  $0.015625 \times 10^{-3}$ ,  $0.03125 \times 10^{-3}$ ,  $0.0625 \times 10^{-3}$ ,  $0.125 \times 10^{-3}$ ,  $0.25 \times 10^{-3}$ ,  $0.5 \times 10^{-3}$  and  $1 \times 10^{-3}$  M E2 and the average number of cells that traversed the membrane in ten random high magnification fields of view was  $436 \pm 11.1$ ,  $330 \pm 9.7$ ,  $226 \pm 8.4$ ,  $154 \pm 3.9$ ,  $54 \pm 3.1$ ,  $36 \pm 2.3$  and  $20 \pm 1.6$ , respectively (Fig. 5A). Following treatment with  $0.015625 \times 10^{-4}$ ,  $0.03125 \times 10^{-4}$ ,  $0.0625 \times 10^{-4}$ ,  $0.125 \times 10^{-4}$  and  $0.25 \times 10^{-4}$  M TAM, the average numbers of cells that traversed the membrane were  $217 \pm 9.5$ ,  $215 \pm 8.2$ ,  $200 \pm 7.6$ ,  $65 \pm 3.4$  and  $16 \pm 1.4$ , respectively. Following TAM plus E2 treatment, the calculated average numbers of migrated cells were  $225 \pm 7.0$ ,  $165 \pm 5.5$ ,  $106 \pm 5.1$  and  $11 \pm 1.0$  at increasing concentrations, respectively (Fig. 5). Treatment with TAM, E2 and the combination-treatment significantly inhibited the migration capabilities of DLD-1 cells in a dose-dependent manner. The inhibitory effect of the combined treatment on cell migration was greater than each treatment alone ( $P<0.05$ ; Fig. 5).

## Discussion

Previous studies have demonstrated that estrogen serves a role in the development of CRC (23,24). The incidence rates of breast cancer are increased in postmenopausal women treated

with hormone replacement therapy, while the incidence rate of CRC is significantly decreased (25,26). The expression of the ER $\alpha$  subtype is low in the gastrointestinal tract and in gastrointestinal tract tumors. Therefore, it was hypothesized that the decreased incidence of CRC in these individuals may be associated with the ER $\beta$  subtype (27). Paruthiyil *et al* (28) demonstrated that E2 promoted the proliferation of ER $\alpha$ -positive breast cancer cell lines, and inhibited the proliferation of ER $\beta$ -positive breast cancer cell lines. Li *et al* (29) confirmed that low concentrations of E2 stimulated the proliferation of human colon carcinoma-derived caco-2 cells, while Arai *et al* (16) revealed that estrogen did not affect the proliferation of five human CRC cell lines with ER $\beta$  expression and no ER $\alpha$  expression, however, estrogen did affect the proliferation of ER $\alpha$ -expressing cell lines. Therefore, it was hypothesized that the expression of ER $\alpha$  is very important for the function of estrogen. However, it is possible that the expression levels of ER $\beta$  were low in these cell lines. Hendrickse *et al* (30) demonstrated that the level of the ER in HT-29, Colo320 and Lovo cells was lower than 12 fmol/mg protein, and the transcriptional activity of ER $\beta$  was lower than that of ER $\alpha$ . Therefore, one of the aims of the present study was to determine the effect of different concentrations of estrogen on the viability of CRC cells. The present study used DLD-1 cells, which express the ER $\beta$  subtype only. The results revealed that a low concentration of E2 promoted DLD-1 cell viability following a short duration of treatment. With an increase in treatment duration

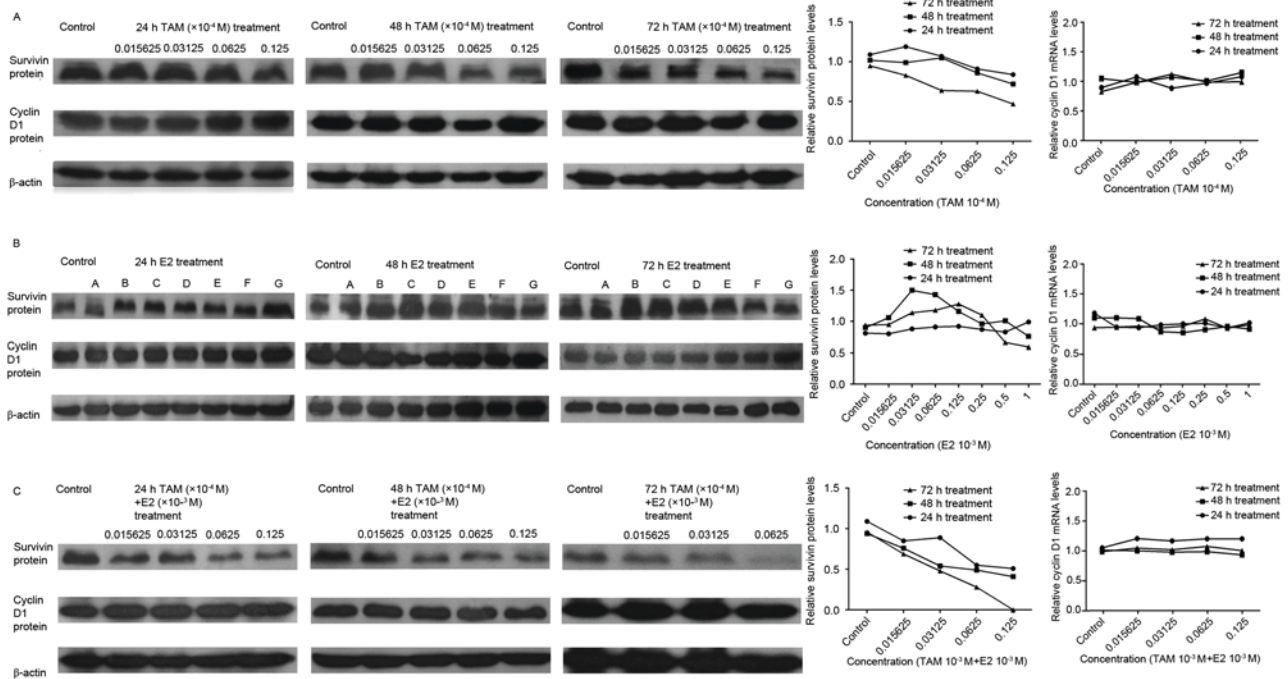


Figure 4. Effect of TAM and/or E2 treatment on survivin and cyclin D1 protein expression levels, as determined by western blotting. Gray-scale band densities were compared and analyzed using the ImageJ software. (A) DLD-1 cells following treatment with TAM. Cyclin D1 protein expression levels were not significantly altered, while relative survivin protein expression levels exhibited decreased with increasing drug concentrations. (B) DLD-1 cells following treatment with E2. A, 0.015625 $\times 10^{-3}$  M; B, 0.03125 $\times 10^{-3}$  M; C, 0.0625 $\times 10^{-3}$  M; D, 0.125 $\times 10^{-3}$  M; E, 0.25 $\times 10^{-3}$  M; F, 0.5 $\times 10^{-3}$  M; G, 1 $\times 10^{-3}$  M. Following treatment, cyclin D1 protein expression levels were not significantly altered. Survivin protein expression levels were not significantly altered following 24 h incubation, whereas treatment with  $>0.03125 \times 10^{-3}$  M E2 at 48 h and  $>0.125 \times 10^{-3}$  M E2 at 72 h was associated with a reduction in survivin protein expression levels. (C) DLD-1 cells following treatment with TAM + E2. Cyclin D1 protein expression levels were not significantly altered, while relative survivin protein expression levels decreased with increasing drug concentrations. Results are presented as the expression of survivin relative to the  $\beta$ -actin internal control. TAM, tamoxifen; E2, 17 $\beta$ -estradiol.

and concentration, E2 exhibited inhibitory effects on DLD-1 cell viability. The authors hypothesize that this result may be associated with the lower transcriptional activity of the ER $\beta$  subtype following exposure to low concentrations of estrogen.

Fox *et al* (31) proposed that ER $\beta$  may be suitable as a therapeutic target for TAM as the affinity of ER $\beta$  for TAM is higher than for E2. In addition, Miller *et al* (32) observed that TAM treatment was associated with an increase in ER $\alpha$  and a decrease in ER $\beta$  expression. Arai *et al* (16) demonstrated that TAM inhibited the proliferation of MCF-7, HT-29 and Colo320 cells. The results of the present study indicated that TAM inhibited the viability of DLD-1 cells, and that this effect was positively associated with drug concentration and treatment duration. TAM belongs to a class of drugs known as selective ER modulators. TAM competes with estrogen for binding to the ER. However, the effects of TAM are complex (33,34). Whether TAM activates or inhibits the ER following binding depends on the target tissue type. Krishnan *et al* (35) demonstrated that TAM binds to the ER $\beta$  subtype to effectively antagonize the function of estrogen and downregulate ER $\beta$  expression. In the present study, treatment with TAM plus E2 demonstrated inhibitory effects on the viability of DLD-1 cells. This effect was positively associated with the concentration of drug and duration of treatment, and was stronger than that of the single-drug treatments. These results indicate that the antiestrogenic function of TAM via binding to the ER $\beta$  may not be the only mechanism involved in the inhibition of cell proliferation by TAM.

The results of the present study demonstrated that TAM and E2 induce DLD-1 apoptosis. The apoptosis rate was positively associated with treatment duration and drug concentration. Induction of apoptosis following TAM plus E2 treatment was greater than that of the single-drug treatments. Therefore, TAM and E2 may demonstrate synergistic effects in the regulation of apoptosis. In previous breast cancer studies, Hou *et al* (36) revealed that ER $\beta$  promoted the development and metastasis of cancer. In the present study, the Transwell assay results indicated that TAM, E2 and the combined drug treatment demonstrated significant inhibitory effects on the migration capabilities of the DLD-1 cells. In addition, combined treatment had a greater effect on the cell migration capabilities of DLD-1 cells. Therefore, the authors speculated that TAM and E2 may exhibit synergistic effects in the downregulation of ER $\beta$  expression, thereby inhibiting proliferation, infiltration to the surrounding tissues and distal metastasis of CRC.

Previous studies have demonstrated that CRC is associated with a variety of genes including k-ras, c-Myc, B-cell lymphoma-2, p53, survivin and cyclin D1 (37-41). Among these genes, survivin is a member of the inhibitors of apoptosis protein family. The survivin gene is localized on human chromosome 17q25 and is associated with the apoptosis and proliferation of cells. Downregulation of survivin demonstrates antitumor effects, which have a therapeutic value (42). Overexpression of cyclin D1, one of the cell cycle regulators, is a hallmark of a number of primary human tumors (43). Cyclin D1 expression is very important for the diagnosis and



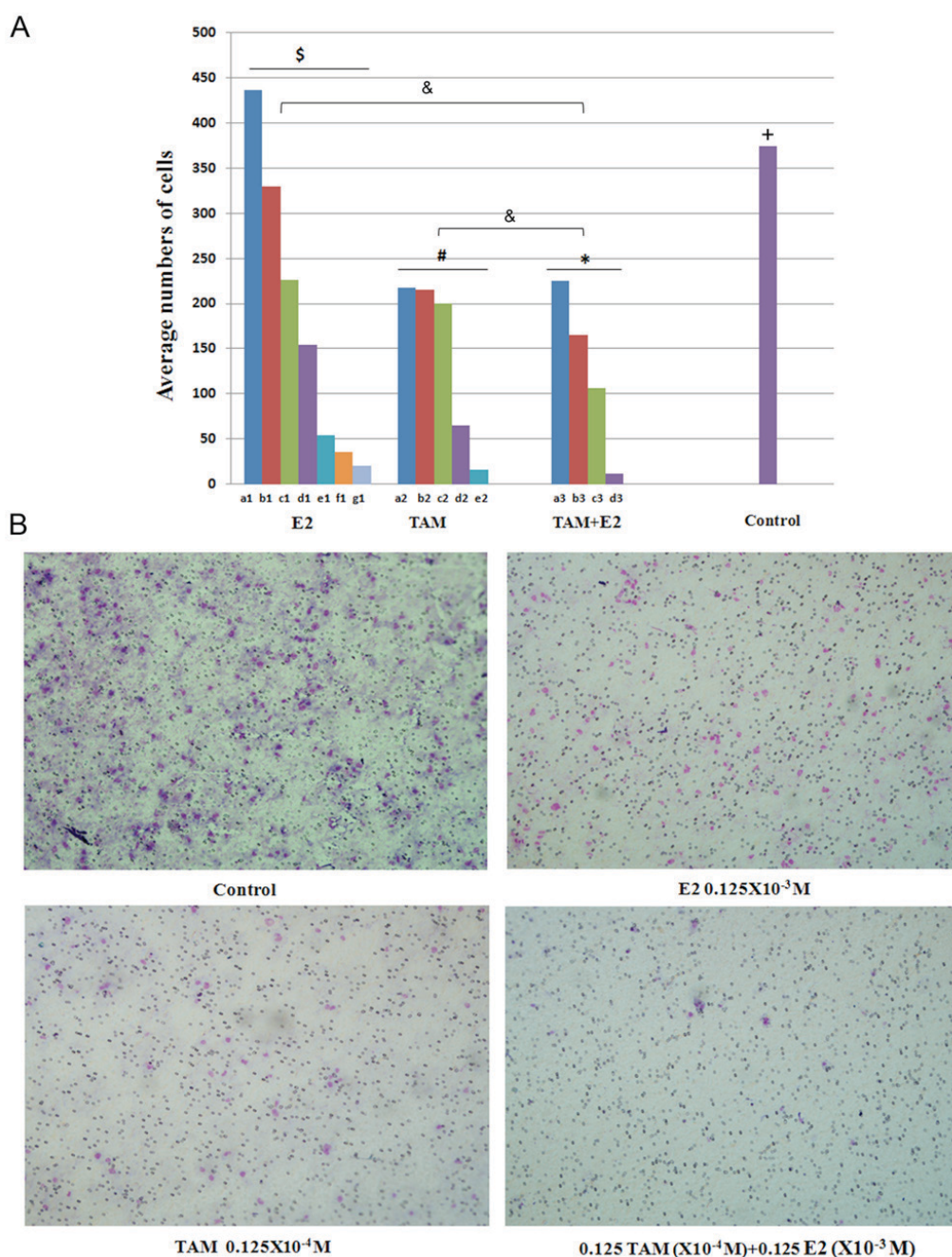


Figure 5. Effect of TAM and/or E2 treatment on the migration of DLD-1 cells. (A) The average numbers of cells that traversed the Transwell membrane in 10 high magnification fields of view selected at random. E2 significantly inhibited the migration capabilities of DLD-1 cells in a dose-dependent manner ( $^{\$}P<0.01$ , comparisons were made between all concentrations in the E2 group); a1,  $0.015625 \times 10^{-4}$  M E2; b1,  $0.03125 \times 10^{-4}$  M E2; c1,  $0.0625 \times 10^{-4}$  M E2; d1,  $0.125 \times 10^{-4}$  M E2; e1,  $0.25 \times 10^{-4}$  M E2; f1,  $0.5 \times 10^{-4}$  M E2; g1,  $1 \times 10^{-4}$  M E2. TAM significantly inhibited the migration capabilities of DLD-1 cells in a dose-dependent manner ( $^{\#}P<0.05$ , comparisons were made between all concentrations in the TAM group); a2,  $0.015625 \times 10^{-3}$  M TAM; b2,  $0.03125 \times 10^{-3}$  M TAM; c2,  $0.0625 \times 10^{-3}$  M TAM; d2,  $0.125 \times 10^{-3}$  M TAM; e2,  $0.25 \times 10^{-3}$  M TAM. Combination-treatment significantly inhibited the migration capabilities of DLD-1 cells in a dose-dependent manner ( $^*P<0.05$ , comparisons were made between all concentrations in the TAM+E2 group); a3, a1+a2; b3, b1+b2; c3, c1+c2; d3, d1+d2. The inhibitory effect of combined treatment on cell migration was significantly greater than that observed in each individual treatment alone [ $^{+}P<0.05$ , TAM+E2 vs. the individual treatment groups (E2 and TAM treatment alone)]. Cell migration capabilities in the control group were significantly greater when compared to the E2, TAM and combined treatment, respectively ( $^{+}P<0.05$ , control vs. each treatment group). (B) Representative light microscope images (magnification, x40) were used to evaluate the effect of E2, TAM and E2+TAM combined drug treatments on cell migration. TAM, tamoxifen; E2, 17M, tamoxifen.

prognosis of tumors (44). The results of the present study indicated that TAM possesses apoptosis-promoting functions. Therefore, the authors investigated whether TAM and E2 may affect the expression of survivin and cyclin D1. Li *et al* (29) previously demonstrated that TAM may inhibit the expression of the survivin gene to relieve the inhibitory effect of survivin on caspase-3 and therefore increase caspase-3 activity, which

leads to apoptosis induction in breast cancer cells. In addition, a previous study demonstrated that TAM decreases the number of cells in S phase by decreasing the ratio of cells in the G<sub>2</sub>/M phases of the cell cycle, thus decreasing the expression of survivin (45). Previous studies have demonstrated via immunohistochemical analyses, that the normal colorectal mucosa does not express survivin (46,47). With the transition from



normal colorectal mucosa to low-grade dysplastic adenoma and highly dysplastic adenoma/CRC, the positive rate of survivin expression consequently increased. Wang *et al* (41) transfected a recombinant adenovirus containing survivin into SW480 cells, which led to a significant decrease in survivin mRNA expression. Once survivin was silenced, the percentage of apoptotic cells was observed to increase. It has therefore been postulated that silencing of survivin expression may inhibit cell growth and induce apoptosis in CRC cells. The results of the present study demonstrated that survivin protein expression decreased to some extent following treatment of DLD-1 cells with TAM; the degree of which was positively associated with drug concentration and treatment duration. Therefore, the authors hypothesized that the downregulation of survivin expression induced by TAM occurs in a time- and dose-dependent manner. TAM downregulated survivin expression in DLD-1 cells and induced CRC cell apoptosis. It was speculated that the mechanism underlying the induction of apoptosis by TAM may have been associated with survivin expression. In addition, following treatment of DLD-1 cells with different concentrations of E2, the survivin protein expression levels were not significantly altered in the 24 h treatment group; however, it increased with increasing drug concentrations in the 48 and 72 h groups. Higher levels of survivin protein expression following treatment with medium concentrations of E2 were observed, whereas lower levels of expression were observed following treatment with high and low E2 concentrations. However, following treatment of cells with E2, survivin mRNA expression levels were reduced. The extent of this decrease was positively associated with the concentration of drug and duration of treatment. He *et al* (48) demonstrated that estrogen promotes the G<sub>2</sub> to S phase transition in ovarian cancer cell lines. In addition, with increasing doses of estrogen, the number of cells in G<sub>1</sub> phase was not significantly altered; however, the number of cells transitioning from G<sub>2</sub> to S phase increased. The apoptosis-inhibition factor survivin is primarily expressed in the G<sub>2</sub> phase. He *et al* (48) demonstrated that, when E2 concentration increased, survivin expression was decreased accordingly. These results are consistent with the results of the present study regarding survivin mRNA levels following E2 treatment, however this is not consistent with the survivin protein expression levels. A possible explanation for these conflicting results may be that the expression of the ER is different among different cell lines. Whether different survivin expression levels are induced by the expression of different ER subtypes will be the focus of future studies. Hwang *et al* (49) and an additional study (50) previously demonstrated that TAM downregulated cyclin D1 expression *in vitro* in ER-positive MCF-7 human breast cancer cells and rat breast cancer cells to inhibit tumor development. However, the present study indicated that following drug treatment in three different groups, the expression levels of cyclin D1 mRNA and protein were not altered. It is possible that, following the interaction of TAM with ER $\alpha$ , transcription is interrupted and the expression of cyclin D1 mRNA and protein are downregulated, thus blocking the G<sub>1</sub>/S transition. However, as the DLD-1 cells employed in the present study expressed ER $\beta$  alone, TAM may have elicited little effect on cyclin D1 protein expression.

In conclusion, the results of the present study demonstrated that TAM effectively inhibited the viability and migration ability of DLD-1 colorectal cells and promoted apoptosis. A high concentration of E2 demonstrated inhibitory effects on the viability of the DLD-1 cells, and TAM and E2 may synergistically inhibit cell viability. The inhibitory effect of TAM plus E2 treatment was greater than that of each agent alone. In addition, the anticancer effects of TAM and E2 may be associated with the downregulation of survivin expression. These results provide a novel experimental basis for hormonal adjuvant therapy in the treatment of CRC. Based on these results, the authors aim to conduct *in vivo* animal studies to provide an experimental basis for final randomized controlled clinical trials.

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