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Effects of Concurrent Chemoradiotherapy on the Metastasis and the Mesenchymal Transition of Bladder Urothelial Carcinoma Cells

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Abstract. *Background/Aim: Cancer stem cells (CSCs) have been suggested playing a crucial role in the tumorigenesis and tumor progression. Clinically, concurrent chemoradiotherapy (CCRT) after transurethral resection of the bladder is the widely accepted treatment option for high-grade bladder urothelial carcinoma (UC); however, a proportion of bladder UC patients still suffer from recurrence and metastasis. In the present study, we investigated the stemness properties of bladder UC cells with respect to various disease stages. The metastatic capability and epithelial–mesenchymal transition (EMT) of the parental cells and the CSC cells of bladder UC, after chemotherapy with cisplatin alone or CCRT were also studied, respectively. Materials and Methods: The aldehyde dehydrogenase (ALDH)-positive cells were analyzed by a flow cytometer. The inhibitory effects of radiation in combination with cisplatin on the cell viability, migration, invasion and EMT characteristics were also examined. Results: We found that the proportion of ALDH$^+$-CSCs of bladder UC cells and the disease grading were independent. Furthermore, cisplatin alone significantly ($p<0.05$) enhanced the migration of both grade-III T24 cells and advanced-stage HT1197 cells, while CCRT treatment significantly ($p<0.05$) inhibited the T24 cell migration capability, compared to the cisplatin alone group. Interestingly, we found that the cell invasion capability was obviously increased upon the treatment with CCRT in both T24 and HT1197 CSCs. Furthermore, cisplatin played a promoting role in EMT whether in the presence or absence of irradiation. Conclusion: CSCs as well as EMT signaling might contribute to the resistance and metastasis of one-shot CCRT in malignant bladder cancer.*

Urothelial carcinoma (UC) of bladder is one of the most fatal and gender-specific malignancies worldwide (1). Up to 25-30 % of the patients are primarily diagnosed with high-grade muscle-invasive bladder cancer (MIBC). Owing to the development of treatment, trimodal therapy (TMT) and transurethral resection of the bladder followed by concurrent

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chemoradiotherapy (CCRT) provide comparable overall survival outcomes to those of radical cystectomy (2, 3). However, a recent systematic review and meta-analysis suggested that neoadjuvant chemotherapy followed by radical surgery had no survival benefit for the patients with stage IB2-IIB cervical cancer as compared with CCRT, but was associated with some side effects (4). In bladder cancer, it is suggested that neoadjuvant chemotherapy with radical cystectomy (RC) lead to improved survival as compared to RC (5). In conclusion, the recurrence due to chemoradiotherapy failure is the major obstacle in the treatment of malignancies.

Cancer stem cells (CSCs) or tumor-initiating cells (TICs), a small subpopulation present in the heterogeneous cancer cell population, are defined as undifferentiated and quiescent cells that are able to form tumor tissues (6). Isolation of CSCs predominantly depends on the membrane transporter function (chemical efflux, such as ATP-binding cassette (ABC) transporters), cellular surface marker specificity (transmembrane glycoprotein, such as CD133) and enzyme activity (such as aldehyde dehydrogenase, ALDH) (7). For CSCs culture, ALDH-based isolation was considered more convenient and causes less damage to the cells (8, 9).

Epithelial–mesenchymal transition (EMT) is defined as the remodeling of tumor cells to transform from the epithelial phenotype to the mesenchymal phenotype that possesses the invasive and metastatic potential (10). Recently, it has been suggested that ionizing radiation (IR) paradoxically promotes metastasis and invasion of the cancer cells by inducing the EMT (11). Furthermore, it was also shown that chemotherapy-induced EMT was involved in the metastasis of lung adenocarcinoma (12); however, there is lack of evidence regarding the chemoradiotherapy-mediated drug resistance and metastasis of bladder UC cancer.

In this study, we hypothesized that cancer stemness might have positive correlation to the chemo- or radio- resistance. We isolated the CSCs from bladder UC cancer cells and investigated the effects of CCRT on the migration and invasion of bladder UC parental cells and CSC cells. Furthermore, the effects of CCRT on the transformation of cancer type were also investigated.

Materials and Methods

Cell lines, reagents and chemicals. T24 human bladder carcinoma (BCRC No.60062), BFTC905 human bladder papillary transitional cell carcinoma (BCRC No.60068) and HT1197 human bladder carcinoma (BCRC No.60059) cell lines were bought from Bioresources collection and Research Center (Hsin Chu, Taiwan, ROC). Basal medium McCoy's 5a (for T24 cells), RPMI1640 (for BFTC905 cells), minimum essential medium (Eagle) with Earle's BSS (for HT1197 cells), Alamar Blue™ Cell Viability Reagent, L-glutamate, fetal bovine serum and TrypLE Express Enzyme solution, were purchased from Gibco (ThermoFisher Scientific Inc., Waltham, MA, USA). ALDEFLUOR™ Kit was bought from Stemcell™ Technologies (Seattle, WA, USA). Oris™ Cell Migration Kit and Cultrex® 96 Well 3D Spheroid BME Cell Invasion Kit were purchased from Platypus Techonologies (Madison, WI, USA) and Trevigen (Minneapolis, MN, USA), respectively. Other high-quality reagents were all purchased from local certified companies.

Culture of human bladder cancer cells. The T24, BFTC905 and HT1197 human bladder carcinoma cell lines were cultured in complete media (90% basal medium containing 1.5 g/l sodium bicarbonate + 10-15% fetal bovine serum) in a humidified cell culture incubator at 37˚C and 5% CO_2, respectively. The cells were detached by using TrypLE Express Enzyme solution for cell passage.

Identification of stemness bladder UC cells. The assay was followed by the manufacturer's instructions. First, added ALDEFLUOR™ DEAB Reagent to the control tube, and the parental UC cells (1×10^6) were harvested and added ALDEFLUOR™ Reagent and well mixed. Finally, transferred half of cell suspension to the DEAB control tube. After 30 min of incubation at 37˚C in the dark, the cells were then centrifuged at 300 × *g* for 10 min at 25˚C. The supernatant was then aspirated directly and the cell pellet was re-suspended and gently mixed with PBS. Finally, the cells were analyzed by a flow cytometer (CantoII™, BD Biosciences, San Jose, CA, USA). The data from over 20,000 cells were recorded for analysis. Furthermore, the $ALDH^{bright}$ cells were sorted by a flowcytometic cell sorter (FX500, Sony Biotechnology, Tokyo, Japan). The harvested $ALDH^+$-CSCs were maintained in serum-free medium with basal medium-containing basic fibroblast growth factor (bFGF, 10 ng/ml), leukemia inhibitory factor (LIF, 10 ng/ml) and epidermal growth factor (EGF, 20 ng/ml) and incubated in a low-adherent petri dish in a humidified cell culture incubator at 37˚C and 5% CO_2, respectively as described previously (13).

Cytotoxic assay of radio-/chemo-therapy against bladder UC cells. The UC cells were inoculated in 96-well and treated according to regimens in group A (cisplatin (0-0.4 μg/ml) alone for 48 h) and group B (pretreated cisplatin (0-0.4 μg/ml) for 6 h, followed by X-ray radiation (CellRad System, Faxitron Bioptics, Tuson, AZ, USA) at a total dosage (Gray, Gy) of 10 Gy (at 129.7 kV and 5.0 mA and the total duration was about five min), and then directly incubated for another 18 h. After the treatment, cell viability was determined by Alamar blue assay. In short, the cells were washed by PBS. Then, the cells were placed in Alamar™ Blue contained basal medium to be incubated for another 3 h. The absorbance (AB) value was measured at 590 nm with an excitation wavelength of 544 nm using an ELISA reader (SpectraMax M2e, Molecular Devices, San Jose, CA, USA). The cell viability was presented as: (ABtreat-ABcontrol)/ABcontrol*100%.

Cell migration assay. The bladder UC cells were seeded in a stopper-containing 96-well plate coated with type I collagen. Briefly, after equilibration of plate at room temperature for about 1 h, the stopper was placed in and completely sealed to the plate. The migration detection mask was then applied to the bottom of the plate. The various UC cells suspension (1×10^4 cell/well) was added into each well of the plate and then was incubated overnight in the cell culture incubator. After incubation, the stoppers were removed carefully by using the removal tool. The medium was refreshed with

cisplatin-containing complete medium, which was followed by the 10 Gy irradiation (6 h after the cisplatin treatment) or not. After the total 24 h of incubation, cell migration capability was monitored by an inverted microscope. The data were analyzed by Image J software and were shown as the relative cell migration capacity (percentage of control).

Cell invasion assay. The CSCs were maintained in serum-free medium with basal medium-containing growth factors as above described. At day 3 of spheroid formation, one spheroid was transferred to a chilled flat-bottomed 96-well plate coated with 50 μl per well of Cultrex® BME (Trevigen) Matrix and 100 μl of CSC cell culture medium with or without cisplatin. After 24 h of incubation, a duplicate plate was treated with irradiation at a dose of 10 Gy and then directly incubated for further 48 h. The cell invasion capability was monitored by an inverted microscope and analyzed by Image J software. The data were shown as the relative cell invasion (percentage of control).

Western blot analysis. The treated bladder UC cells were harvested and lysed in cold RIPA lysis and extraction buffer containing inhibitor cocktails for protease and phosphatase (Merck KGaA, Darmstadt, Germany), respectively. Protein concentrations were measured by Bradford protein assay. The protein lysate was separated in gradient SDS-PAGE and electro-blotted to PVDF (Merck) membrane. The membranes were then blocked and incubated with indicated primary and then hybridized with specific secondary horseradish peroxidase (HRP)-linked antibodies including E-cadherin, N-cadherin and β-actin. Signal intensity was measured by chemiluminescence (Millipore) and visualized by ChemiDoc™ Imaging System (BIO-RAD). Equal protein loading was confirmed by quantifying β actin. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis. All data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and were showed as the mean±SE of the mean, and from at least three independent experiments. Differences between two groups were analyzed using one-way ANOVA, followed by a post hoc analysis. A p-value <0.05 was considered statistically significant.

Results

Identification of ALDH-positive population in bladder UC cells and the chemoresistance. The ALDH-expressing populations in various bladder UC cell lines were presented in Figure 1A. As compared with the DEAB-negative control, higher fluorescent intensity suggested that the cells have higher ALDH activity. We found that BFTC905 (grade III) and T24 (grade III) cells had the greatest level of $ALDH^{bright}$ cells; however, grade-IV HT1197 cells showed a relative lower level of $ALDH^{bright}$ population. Furthermore, we found that HT1197 cells comprised the lowest level of $ALDH^{high}$ cells but demonstrated the highest resistance to cisplatin with an IC_{50} value of 2±0.25 μg/ml, compared to T24 (1.1±0.41 μg/ml) and BFTC905 (0.7±0.16 μg/ml) for 48 h of incubation, respectively (Figure 2).

Effects of CCRT on the migration capability of bladder UC cells. To evaluate the effects of irradiation in combination with cisplatin on the cell migration, bladder UC cells were treated with indicated concentrations (0-0.4 μg/ml) of cisplatin for 6 h, which was followed by the treatment of 10 Gy irradiation, and then the migration capability was evaluated after a total 24 h of incubation. In T24 cells, cisplatin induced cell migration in a dose-dependent manner (Figure 3). Furthermore, irradiation significantly (p<0.05) induced cell migration, while irradiation in combination with cisplatin significantly (p<0.05) inhibited the cell migration capability as compared to the cisplatin alone group, while there was no difference between CCRT and irradiation alone groups (Figure 3B and C). In contrast, we found that cisplatin obviously induced an increase in cell migration of grade-IV HT1197 cells in the presence or absence of irradiation treatment, and there was no difference between CCRT and cisplatin alone groups (Figure 4).

Effects of CCRT on the invasion capability of bladder UC stem cells. We further investigated whether chemoradiotherapy affects the invasion of CSCs. The T24 and HT1197 cells were treated with indicated concentrations (0-0.2 μg/ml) of cisplatin for 24 h first, which was then followed by the treatment of 10 Gy irradiation. The cells were then incubated for further two days and the cell invasion capability was evaluated. As shown in Figure 5, cisplatin did not have dramatic influence on the invasion of T24 CSCs; however, cisplatin in combination with irradiation significantly (p<0.05) enhanced the invasion capability. We also found that irradiation alone significantly inhibited the CSC invasion, while in the presence of cisplatin, the CSC invasion capability was obviously restored (Figure 5C). Similarly, the invasion capability of HT1197 CSCs was significantly (p<0.05) induced by the treatment of cisplatin in combination with irradiation (Figure 6).

Effects of CCRT on the EMT of bladder UC cells. Finally, we investigated the effect of CCRT on the transformation of bladder cancer cells. The expression of EMT markers in cells treated with cisplatin (0.2 μg/ml), 10 Gy irradiation, or CCRT was evaluated after 24 h of incubation. Western blot analysis indicated that the expression of the epithelial marker E-cadherin was significantly decreased (p<0.05), while the mesenchymal marker (N-cadherin) was increased (p<0.05) after cisplatin treatment in both T24 and HT1197 cells. The same results were observed after treatment with irradiation and cisplatin. However, transient irradiation alone did not induce any changes in the expression of these markers (Figure 7).

Discussion

Urothelial cancer ranks 13th in terms of yearly mortality from cancer that is the fourth most common cancer in men in the USA and is the ninth most common cancer worldwide

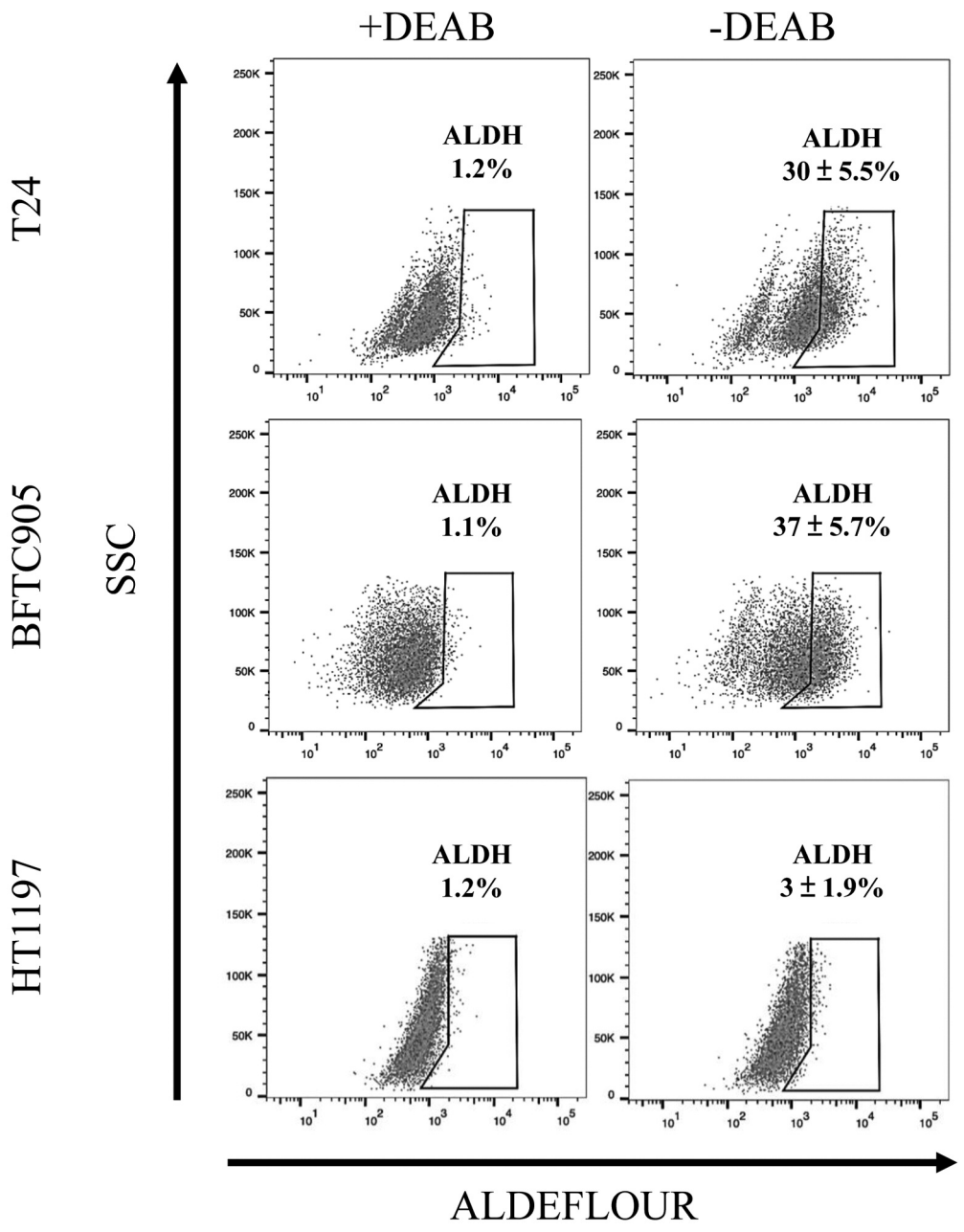

Figure 1. *Cancer stem cell identification. The T24, BFTC905 and HT1197 bladder UC cells were harvested and stained with ALDEFLUOR™ Reagent in the presence or absence of DEAB ALDH-Inhibitor Reagent. The cells were analyzed by a flow cytometer and the data from over 20,000 cells were recorded for analysis. SSC, side scatter. ALDEFLOUR, FL1 channel of flow cytometry.*

(14). Although the incidence rate in industrialized nations is three-fold greater versus low-resource countries, which mainly occurs in North America, Europe, and parts of Western Asia, the mortality rates are greater in developing regions (15). Radical cystectomy is considered the standard treatment for advanced UC (16). In concern of life quality, radiotherapy is used as an alternative, particularly in chemo-resistant patients; however, it is usually associated with a relatively high rate of incomplete response or local recurrence even distant metastasis (17).

In this study, we investigated the metastatic capability and EMT phenotype of the parental cells and the CSC cells of bladder UC, after chemotherapy with cisplatin alone or CCRT. We found that cisplatin exhibited cytotoxic effects

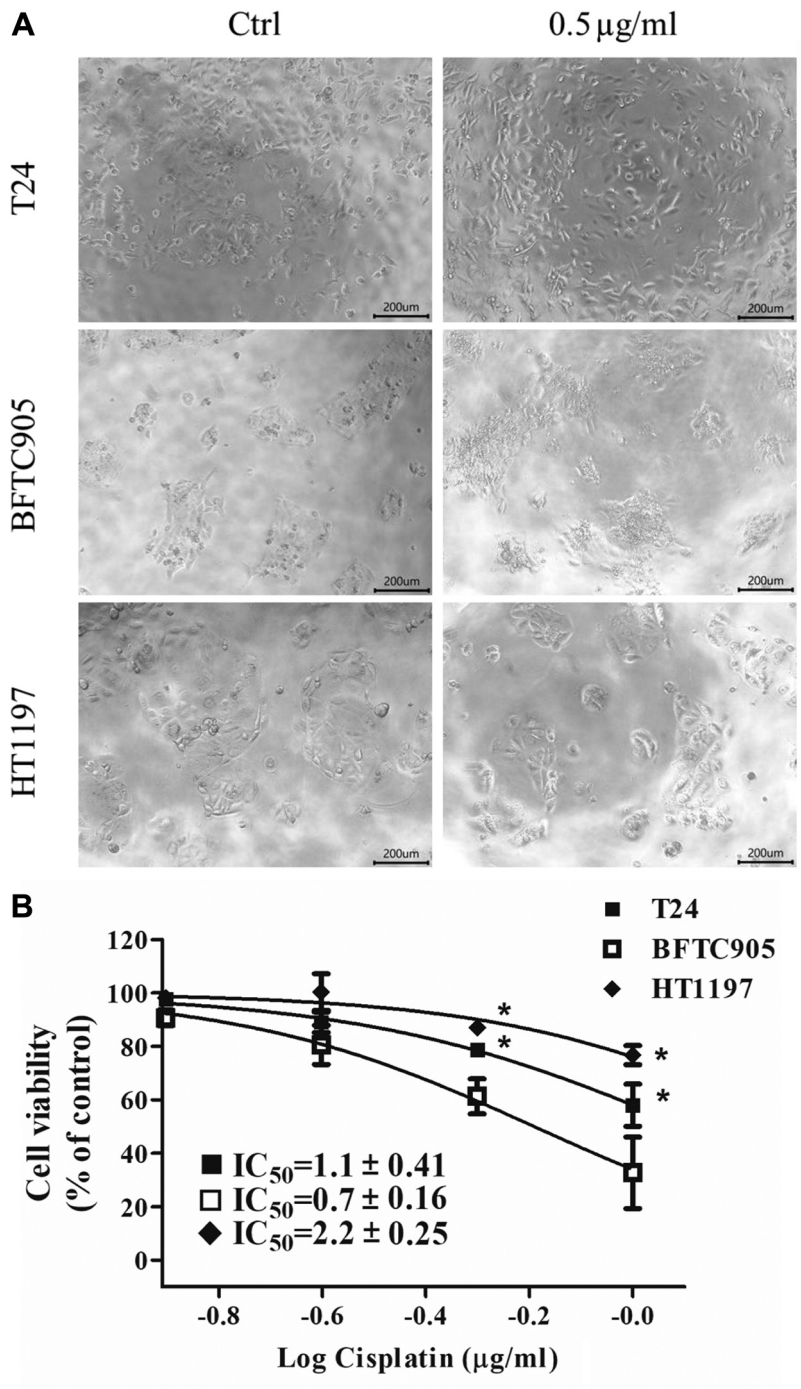

Figure 2. *Cytotoxic assay of cisplatin against bladder UC cells. The UC cells were inoculated in 96-well and treated with indicated concentrations of cisplatin (0-0.5 μg/ml) for 48 h. At the end of incubation, the cell viability was analyzed by Alamar blue assay. The morphology of bladder UC treated with or without cisplatin (A) and the cell viability (B) were evaluated. *p<0.05, compared to BFTC905 cells. The magnification was 40×.*

against parental bladder UC cells in a dose-dependent manner; however, cell migration capability was only inhibited in grade III T24 cells after CCRT and not in advanced UC cells. Furthermore, the $ALDH^+$-CSCs spheroid showed resistance against both chemo- and radiotherapy by the evidence of invasion assay. In addition, cisplatin alone and CCRT induced EMT in the studied bladder UC cell lines.

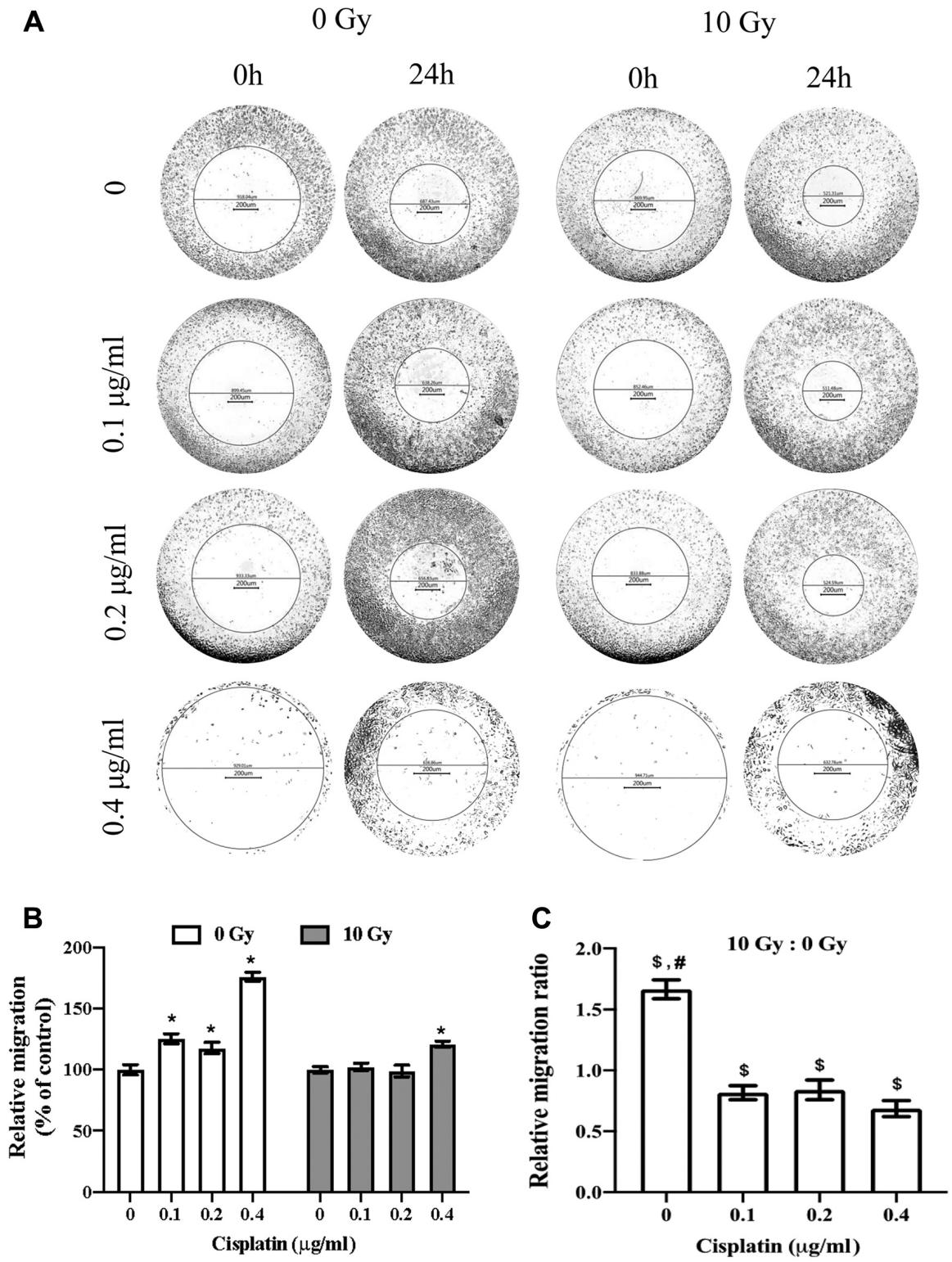

Figure 3. *Effects of chemo/radiotherapy on the migration of bladder T24 UC cells. The T24 cells were seeded in a stopper-containing 96 well plate coated with type I collagen. After 24 h of incubation, the stopper was removed and the medium was refreshed with cisplatin-containing (0-0.4 μg/ml) complete medium. Another duplicate plate was treated with 10 Gy irradiation after 6 h of cisplatin treatment. After the total 24 h of incubation, the cell migration capability was monitored by an inverted microscope (A) and analyzed (B and C). *p<0.05, compared to the control group; #p<0.05, compared to other groups; $p<0.05 between groups treated with or without irradiation.*

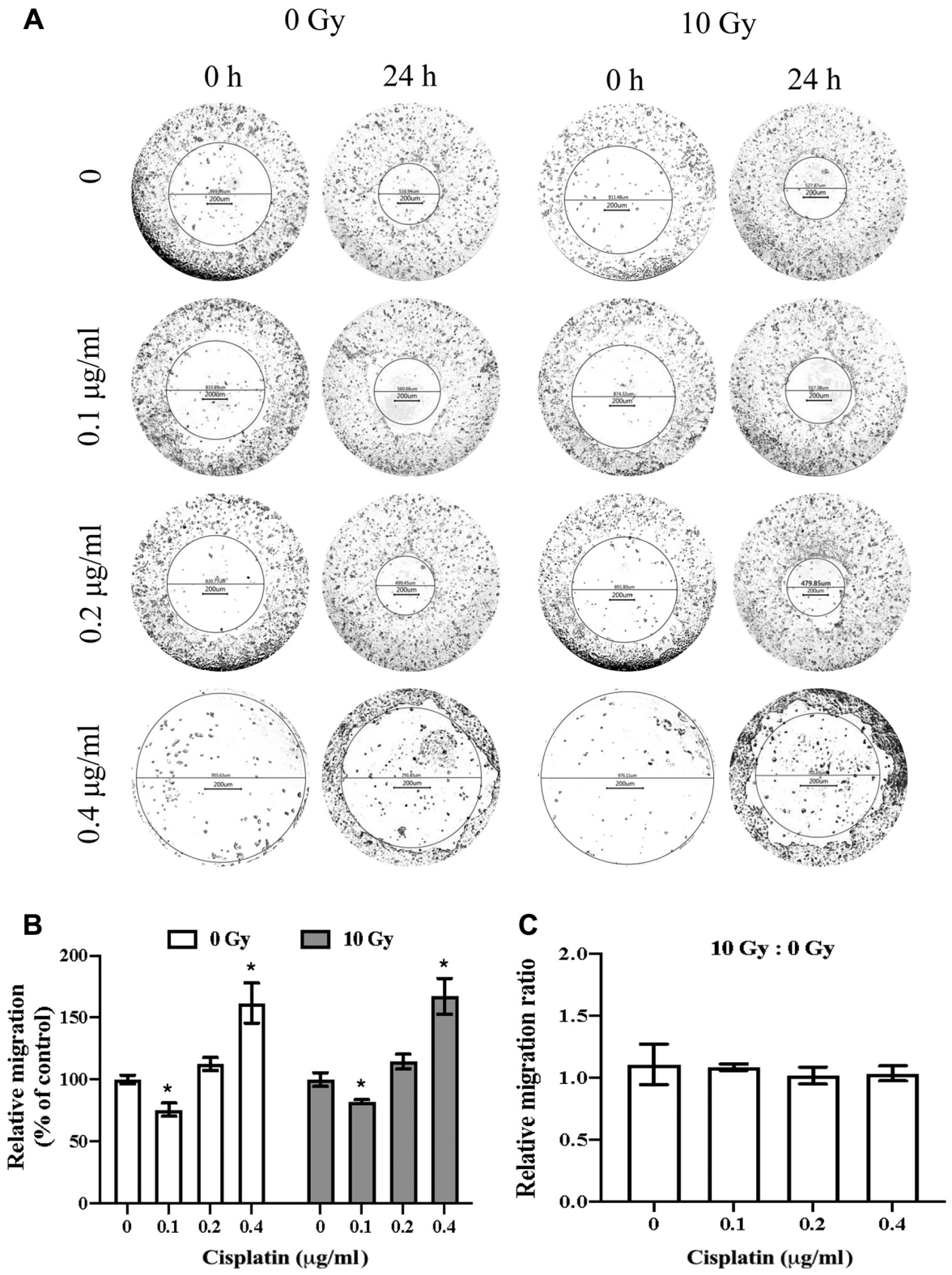

Figure 4. *Effects of chemo/radiotherapy on the migration of bladder HT1197 UC cells. The HT1197 cells were seeded in a stopper-containing 96-well plate coated with type I collagen. After 24 h of incubation, the stopper was removed and the medium was refreshed with cisplatin-containing (0-0.4 μg/ml) complete medium. Another duplicate plate was treated with 10 Gy irradiation after a 6 h of cisplatin treatment. After the total 24 h of incubation, the cell migration capability was monitored by an inverted microscope (A) and analyzed (B and C). *p<0.05 compared to the control group.*

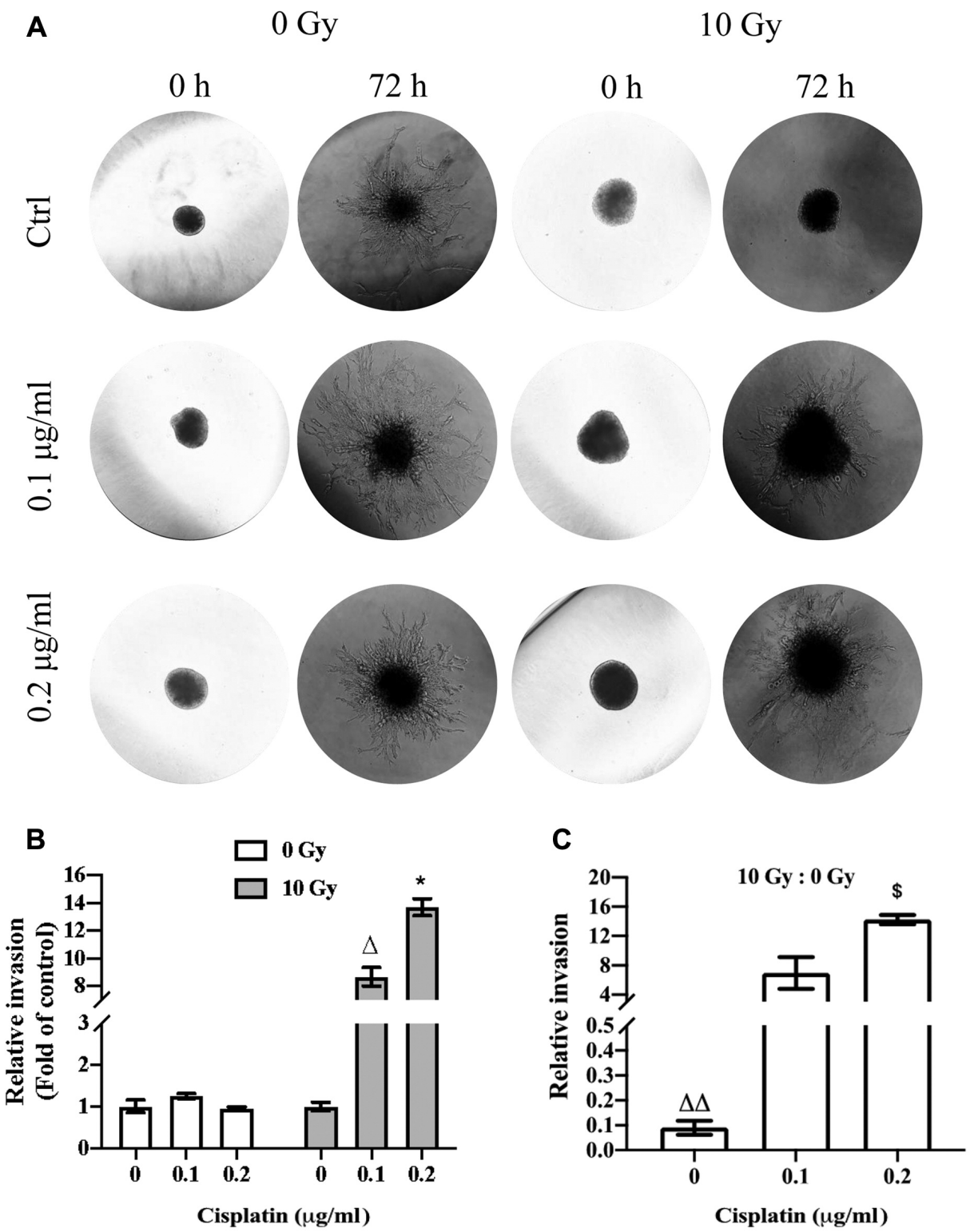

Figure 5. *Effects of chemo/radiotherapy on the invasion capability of bladder T24 UC stem cells. The T24 CSC spheroid was transferred to a 96-well plate coated with Cultrex® BME (Trevigen) Matrix and CSC cell culture medium with or without cisplatin. After 24 h of incubation, a duplicate plate was treated with irradiation at a dose of 10 Gy and then directly incubated for further 48 h. The cell invasion capability was monitored by an inverted microscope (A) and analyzed by Image J software (B). *p<0.05 compared to the control group; $^{\Delta}$p-value=0.0579, compared to the control group. $^{\$}$p<0.05 between groups treated with or without irradiation; $^{\Delta\Delta}$p-value=0.0516 between groups treated with or without irradiation.*

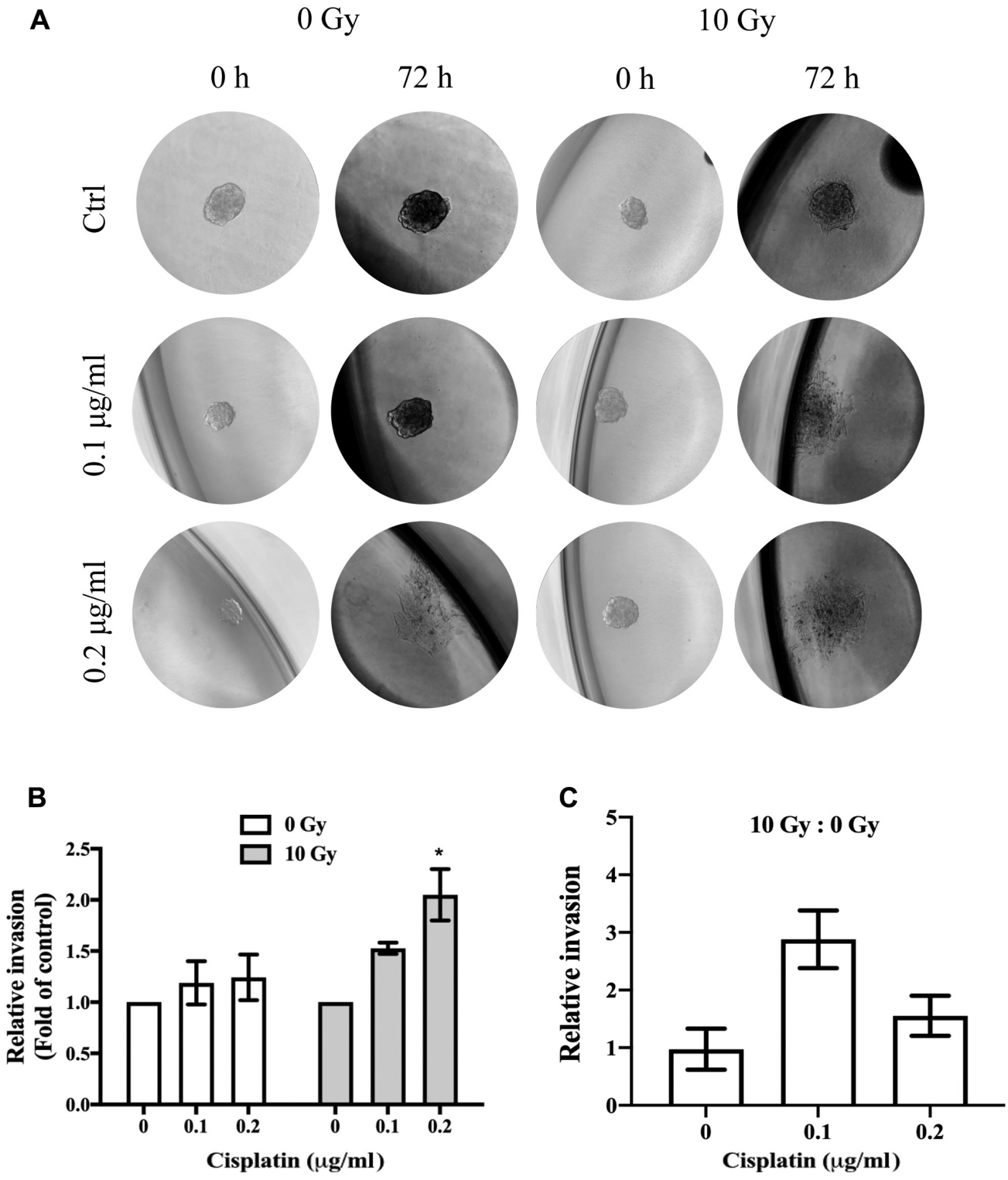

Figure 6. *Effects of chemo/radiotherapy on the invasion capability of bladder HT1197 UC stem cells. The HT1197 CSC spheroid was transferred to a 96-well plate coated with Cultrex® BME (Trevigen) Matrix and CSC cell culture medium with or without cisplatin. After 24 h of incubation, a duplicate plate was treated with irradiation at a dose of 10 Gy and then directly incubated for further 48 h. The cell invasion capability was monitored by an inverted microscope (A) and analyzed by Image J software (B). *p<0.05, compared to the control group.*

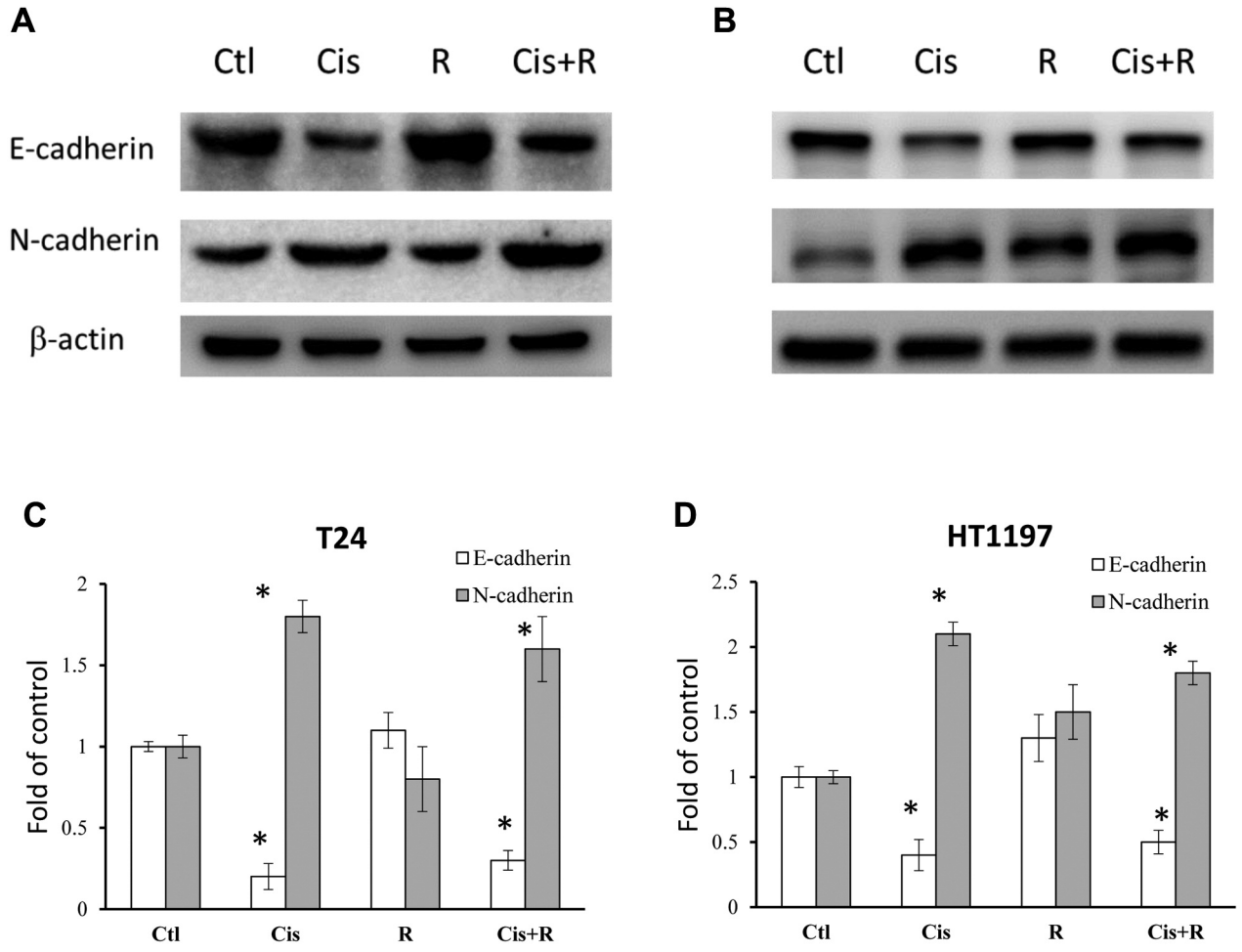

Figure 7. *Effects of chemo/radiotherapy on the epithelial-mesenchymal transition of bladder UC cells. The T24 (A and C) and HT1197 (B and D) cells were treated with cisplatin, 10 Gy of irradiation, or CCRT, respectively. After 24 h of incubation, the cells were harvested for protein analysis. *p<0.05, compared to the control (Ctl) group.*

It has been suggested that synchronous chemotherapy with fluorouracil and mitomycin C combined with radiotherapy significantly improved locoregional control of bladder cancer, as compared with radiotherapy alone, with no significant increase in adverse events (2). Neoadjuvant chemoradiotherapy has also been suggested to be the standard preoperative treatment strategy for locally advanced oesophageal squamous cell carcinoma (18). Interestingly, in the cervical cancer, cisplatin-based concurrent chemotherapy and external pelvic irradiation followed by brachytherapy has been the preferred treatment option for patients with stage IB2-IIB cervical cancer (19). Although most patients initially respond to this therapeutic approach, 22%-41% of patients still suffer recurrence (20). In the UC, neo-adjuvant cisplatin-based chemotherapy represents the standard treatment for patients with locoregionally advanced disease and is related to a 5-years overall-survival benefit of about 5% (14). Overall, recurrence and metastasis is still the unmet need for the cancer treatment.

Most bladder urothelial cancers belong to pure urothelial carcinoma (PUC), 10-53% of UC is composed of histologic variations of urothelial carcinoma (VUC), which has been suggested that may exhibit more aggressive behavior and poorer clinical outcomes (21). The World Health Organization has defined a subtype of VUC with mesenchymal-like spindle cells or sarcomatous elements, with or without epithelial markers (22). It is well-known that epithelial-mesenchymal transition (EMT) is the key inflection point of tumor cell to transform to be more drug-resistant with higher metastatic capability (10). In addition to chemo- and radio-resistance, it has been shown that higher EMT- and stroma-related gene expression is associated with lower response rates and shorter

progression-free and overall survival also in patients under immunotherapy (23). Previously, it has been suggested that EMT and cancer stem cell play critical roles in prostate cancer radioresistance (24). In this study, we found that transient irradiation alone treatment did not have any obvious effects on the transformation of cell phenotype; however, EMT transition was induced after treatment with cisplatin alone or CCRT. It has also been revealed that a cell subpopulation known as urothelial cancer stem cells (UroCSCs) in urothelial cell carcinoma (UCC) have self-renewal properties, ability to generate cellular tumor heterogeneity via differentiation, and are ultimately responsible for tumor growth and viability (25). Previously, it was suggested that $CD44^{+}$-CSC of UC cells had higher resistance against irradiation and CD44 could be a predictive biomarker for UC progression (26). Recently, it was also reported that ALDH1A1 plays a critical role in stemness and tumor growth (27). In the present study, we found that cisplatin could enhance the invasion of $ALDH^{high}$-CSC derived spheroids; thus, it may consequently promote distant metastasis and chemoresistance. Similar results were found in previous studies, in which ALDH-1-positive cells exhibited a radio-/chemo-resistant phenotype in cervical cancer (28, 29). Thus, therapeutics targeting CSCs would be a promising strategy to inhibit cancer progression and tumor relapse, and provide rationale in the treatment and clinical management of urothelial cancer.

Conclusion

In conclusion, the present study suggested that cancer stem cells and EMT signaling may play crucial roles in concurrent chemoradiotherapy-induced metastasis and resistance. Even though synchronous chemotherapy might have clinical benefit for radiotherapy, it still depends on cancer type, disease grade and medication. Furthermore, ALDH-based cancer stem cell isolation is still a reliable practice for research and clinical application that would cause less stress to cells, despite of no correlation between the amount of ALDH-bright cells and disease grade in urothelial cancer cells.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

SCC, DYC and PHS made contributions to the conception and design of the study and prepared the manuscript. YP, HYC and PHS performed the experiments and data analysis. CHH, HJS, SHH, SCW and CPC reviewed the literature and interpreted the results. YP, HYC and PHS revised the manuscript. All authors read and approved the final manuscript.

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