

## Research Article

# Bladder Cancer Chemotherapy Resistant Cell Harbors Stem-Like Characteristics Construction and Characterization of T24 Resistance Strain of Bladder Cancer

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## Abstract

**Objective:** Establish a human drug-resistant bladder cancer cell line (T24/MMC), which are integral to the initiation, high recurrence and chemo-resistance of bladder cancer. BCSCs are heterogeneous and originate from multiple cell types, including urothelial stem cells and differentiated cell types, including basal, intermediate stratum and umbrella cells, then investigate its characteristics.

**Methods:** Human bladder cancer cell line T24 was established using gradually increased concentration of mitomycin (MMC) in culture. Basic characteristics of both the parental cell line T24 and the drug-resistant cell line (T24/MMC) were analyzed and compared, including the ability of proliferation, colony formation, mobility and invasion, sphere forming ability.

**Results:** Compared with T24, the (T24/MMC) cell grows quickly, and the ability of proliferation, colony formation, mobility and invasion, stemness gene, sphere forming ability increased.

**Conclusion:** Gradually increased concentration of MMC can induce drug-resistant bladder cancer cell line, and it was enriched of cancer stem cells.

**Keywords:** Bladder cancer; T24; Drug-resistant cancer cell line; Cancer stem cells

## Introduction

With an estimated 81,190 new diagnoses and 17,240 deaths in 2017 urothelial carcinoma of the bladder cancer (UCB) is a leading cause of cancer related mortality in the western world [1]. Radical Cystectomy (RC) and Lymphadenectomy with or without Perioperative Systemic Chemotherapy is the gold standard treatment for patients with muscle invasive and a subgroup of patients with high-risk non-muscle invasive UCB, respectively [2]. For patients with metastatic UCB, systemic chemotherapy is the therapeutic fundament [3]. Despite improved staging techniques, enhanced perioperative recovery support as well as an disease-free survival benefit of 9% to 34% with perioperative chemotherapy, up to 50% of patients treated on curative intent develop metastasis and die from UCB due to micro metastatic disease undetectable prior to definitive therapy and/or unresponsive to modern systemic treatment respectively [4]. Indeed, in general, UCB

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is considered a chemo sensitive disease, and systemic platinum-based combination chemotherapy is the standard of care in the neoadjuvant, adjuvant and salvage therapy setting [5]. Despite response rates about 50% to cisplatin based chemotherapy, a non-negligible number of patients do not benefit from perioperative or salvage therapy [6]. In addition, a significant number of patients are unfit for cisplatin-based chemotherapy, and optimal systemic treatment remains unsettled [3]. Chemotherapy resistance is the main cause of recurrence. The mechanism of chemotherapy resistance is currently unclear. In this study, we constructed a drug-resistant strain of bladder transitional cell carcinoma T24, and analyzed its characteristics. The report is as follows.

## Materials and Methods

### Experimental materials

The human metastatic cell bladder cancer cell line T24 was purchased from the China National Culture Collection Center of Wuhan University. RPMI 1640 medium (Gibco), Fetal Bovine Serum (FBS, Hangzhou Sijiqing Bioengineering Materials Co. Ltd.), CO<sub>2</sub> incubator (ESCO, USA), Transwell chamber (Corning), low adhesion 6-well plate (Corning), DMEM/F12 (Gibco), mitomycin MMC, produced by Zhejiang Haizheng Pharmaceutical Co. Ltd., and other reagents are analytical purity chemically.

### Cell culture

Incubate in RPMI 1640 medium containing 10% fetal bovine serum and 100 µ/mL penicillin and 100 µg/mL streptomycin by a conventional method at 37°C, 5% CO<sub>2</sub> and 100% humidity in an incubator every 3 d Pass it once. Experiment with cells in the logarithmic growth phase.

## Establishment of chemotherapy resistant strains

Cell proliferation was induced by a continuous increase in the concentration gradient of mitomycin [7]. T24 cells in logarithmic growth phase were observed under light microscope until the cells were covered to 70% of the bottom area of cell culture flask. The medium was changed to 10  $\mu\text{g/mL}$  MMC, 10% FBS RPMI 1640 medium; according to the proliferation of viable cells, the concentration of the drug is gradually increased; this is repeated until the cells can stably and well grow in the RPMI 1640 medium containing 0.2 mg/mL MMC, and can be continuously passaged.

## MTT

T24 and T24/MMC cells with good growth were obtained, and 2000 cells per well were inoculated into 96-well plates, 200  $\mu\text{L}$  medium/well, 6 replicate wells, cultured for 1 d, and then observed continuously for 4 d. MTT assay The absorbance value (A value) of each well is plotted against the growth curve.

## Plate colony formation test

T24 and T24/MMC were inoculated into 6-well plates at 200 cells/well, and cultured in an incubator for 14 days. The cells were immersed and washed in PBS for 2 times, fixed at room temperature for 15 min in methanol, and stained with Giemsa for 15 min. The number of clones containing more than 50 cells was counted under a microscope. Then calculate the clone formation rate according to the formula:

Clonal formation rate (%) = number of clones/number of cells inoculated  $\times$  100%

## Migration and invasion test

The 1640 blood-free culture medium was diluted 1:3 in a matrigel gel-coated upper chamber, 40  $\mu\text{L}$  per well, and incubated for 4 h at room temperature. The cells were digested, serum-free medium was used to homogenize the cell pellet, and 100  $\mu\text{L}$  of the cells containing  $1 \times 10^5$  cells were seeded in the upper chamber. Add 500  $\mu\text{L}$  of complete medium containing 10% FBS to the lower chamber, culture for 24 h to 48 h, remove the culture solution, wipe off the cells in the upper chamber with a cotton swab, fix the cell in the methanol for 15 min, wash 2 times with 1 $\times$ PBS, and stain the cell with the crystal violet dye solution for 15 min. After washing 3 times with 1 $\times$ PBS, the cells transferred to the lower layer of the microporous membrane were counted under a microscope, and each sample was counted for 10 fields of view.

The migration experiment was the same as the cell invasion experiment, but there was no need to coat the matrigel gel in the upper chamber.

## Microsphere formation test

The monolayer adherent T24 cells were digested with 0.25% trypsin containing 0.02% EDTA, and the digestion was terminated by adding RPMI 1640 medium containing 10% fetal bovine serum [8]. The supernatant was discarded after centrifugation and washed 3 times with PBS. Adding serum-free stem cell medium (serum-free DMEM and F12 mixed in a ratio of 1:1, add 20 ng/mL EGF, 10 ng/mL bFGF, 2% B27, 5  $\mu\text{g/mL}$  insulin, 0.5% BSA). Single cell suspensions were seeded at 500 cells per well in low adhesion 6-well culture plates and routinely cultured. The culture was terminated by the 10<sup>th</sup> day, and the positive microspheres were counted under a 100-fold optical microscope. The diameter of more than 50  $\mu\text{m}$  was defined

as a positive microsphere, and the microsphere formation rate = the number of positive microspheres/500  $\times$  100%.

## Statistical methods

Statistical analysis was performed on the data using the SPSS 13.0 statistical software. The t-test between rows and groups,  $P < 0.05$  is the difference between the statistical significance.

## Results

### Growth curve

Compared with T24 cells, T24/MMC cells have enhanced growth ability, and the cell growth curve is shown in Figure 1.

### Cell plate colony forming ability

Plate cloning experiments reflect the colony forming ability of individual cells, and T24/MMC resistant cells have stronger clonality than T24 cells Figure 2.

### Migration and invasion test

T24/MMC cells showed enhanced migration and invasion compared to T24 cells, see Figure 3 and 4.

### Microsphere formation test

The ability of microsphere formation reflects the self-renewal ability of cancer stem cells. Compared with T24 cells, T24/MMC cells have enhanced globular ability, as shown in Figure 5.

## Discussion

The Cancer Stem Cell (CSC) model is a relatively new concept in cancer biology. Unlike the more traditional theories of clonal selection, where tumors are thought to develop genetic and phenotypic heterogeneity following repeated rounds of mutation and selection, the CSC concept relies on a notion of an intrinsic hierarchy among tumor cells for cancer treatment. However, tumors can recur and develop chemo resistance [9]. Solid tumors possess a rare population of cancer stem cells (CSCs), which have similar characteristics to normal stem cells and exhibit self-renewal, asymmetric cell division, and resistance to toxic agents [10]. CSCs display surface markers, including CD44 and CD133, in various cancer types [11]. CD44 and CD133 have been identified as surface biomarkers for cancer cells resistant to chemotherapeutic drugs [12-14]. CSC like cells are enriched following short-term single treatment of chemotherapy, suggesting that malignant cells may also be enriched by cisplatin treatment [14].

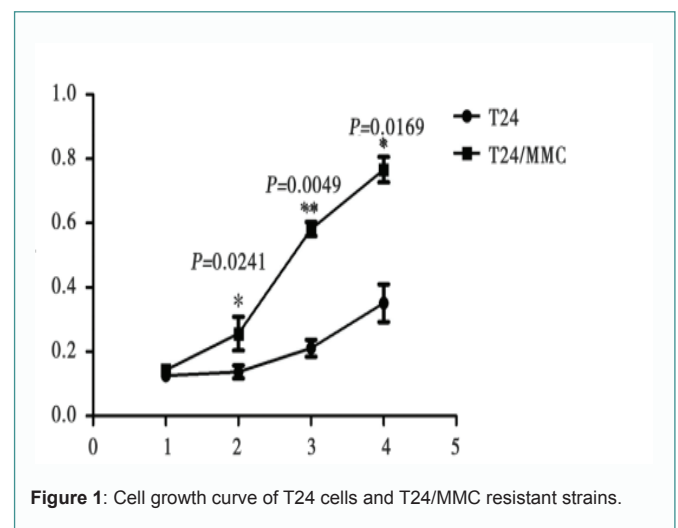
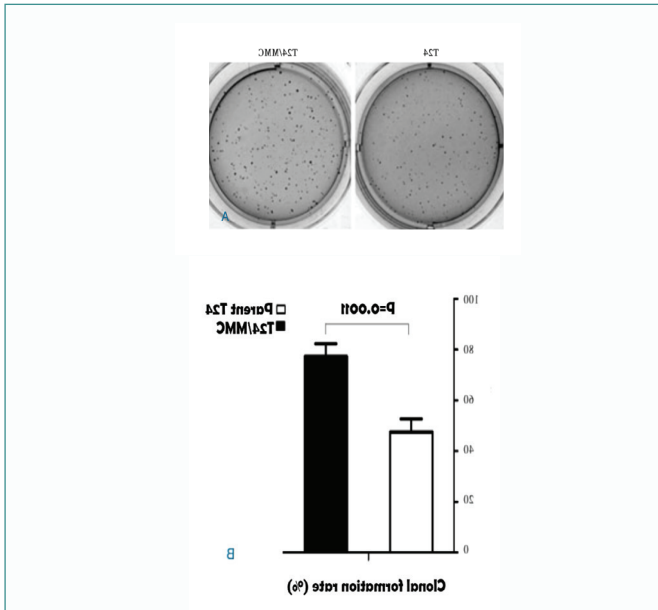
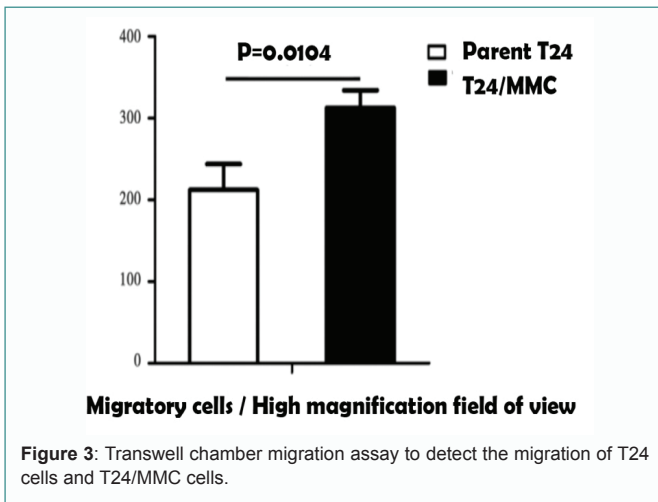


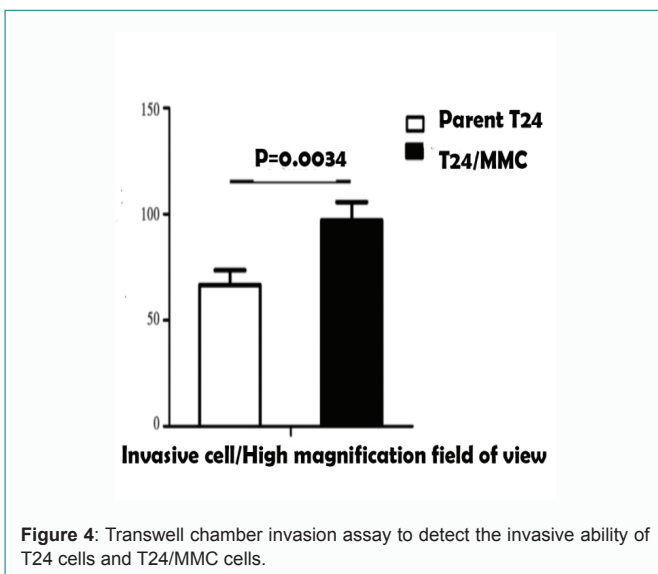
Figure 1: Cell growth curve of T24 cells and T24/MMC resistant strains.



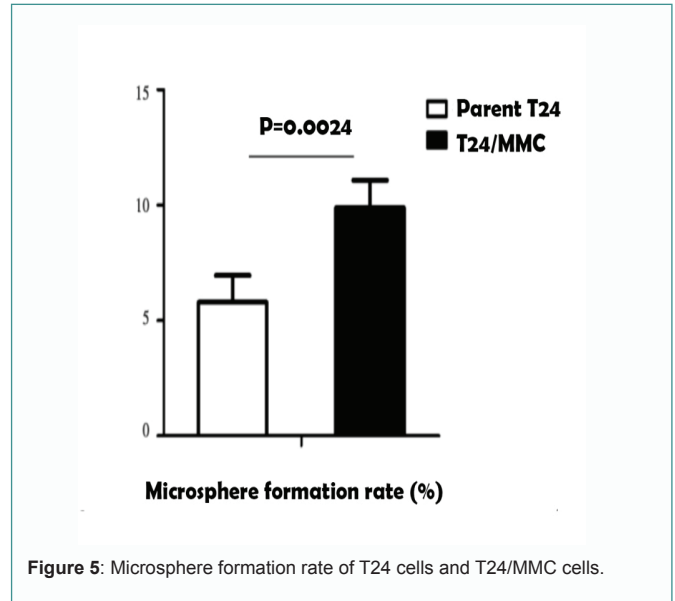
**Figure 2:** T24 cells and T24 / MMC cell plate colony forming ability. A. Plate cell clone formation. B. Statistical results of cell clone formation rate.



**Figure 3:** Transwell chamber migration assay to detect the migration of T24 cells and T24/MMC cells.



**Figure 4:** Transwell chamber invasion assay to detect the invasive ability of T24 cells and T24/MMC cells.



**Figure 5:** Microsphere formation rate of T24 cells and T24/MMC cells.

Mitomycin is a commonly used drug for the treatment of bladder cancer. In this study, the human metastatic cell bladder cancer cell line T24 was used to induce T24/MMC resistant strains *in vitro* by increasing concentration gradient, which can construct resistance strain of bladder cancer. The research on drug mechanism provides theoretical basis and has certain practical significance.

In the process of establishing drug-resistant strains, the growth of bladder cancer T24 cells in MMC medium was alleviated, and the growth rate of T24/MMC cells in MMC medium gradually returned to normal with the increase of passage times and the prolongation of culture time. When cultured in the normal medium after drug withdrawal, the proliferation rate of the cells was faster than that of the parental T24 cells with time prolonging. In the colony formation experiment, it was found that T24/MMC had stronger clonal formation ability than the parental cells, indicating that the self-renewal ability of T24/MMC was enhanced. In the migration and invasion experiments, the migration and invasive ability of T24/MMC cells were increased compared with the parental T24 cells. Serum-free suspension pellet culture is a common method for isolating cancer stem cells. In serum-free pellet culture experiments, T24/MMC cells begin to form a ball on the third day, and the ball formation time is more than that of the parental T24 cells. Short, higher globalization rate, tighter balloon and larger volume, indicating that T24/MMC cells are rich in cancer stem cells. Therefore, in this study, it was found that the bladder cancer resistant strain T24/MMC showed an accelerated proliferation rate after withdrawal, and the colony formation rate, migration and invasion ability, and the ability to become globular, which may be related to drug-resistant strain T24/MMC. enriched with cancer stem cells.

Many tumor-resistant strains have been found to have tumor stem cell characteristics [15-17], but not all tumor-resistant strains are cancer stem cells. Yan et al. [18] found that prostate cancer Du145 resistant strains compared with their parental cells, their proliferation, colony formation and tumorigenic ability of nude mice decreased, and there were no CD44+ cells in DU145 resistant strains. Similarly, colon cancer DLD1 resistant strains also showed decreased tumorigenic ability in nude mice compared with parental cells.

In the study of bladder cancer stem cells, Ning et al. [19] first used flow cytometry to sort out side population cells (SP cells) from bladder cancer T24, and confirmed that these SP cells have the characteristics of cancer stem cells, and their growth rate, clone formation, resistance to radiotherapy and a variety of chemotherapy drugs have increased significantly. Falso et al. [20] used Acetaldehyde Dehydrogenase (ALDH1) to conduct cancer stem cell sorting in three bladder cancer cell lines, in which ALDH1 high cells were found to be resistant to cisplatin in T24 and 5637, and ALDH1 high cells. The ability to clone, differentiate, migrate and invade has increased. These studies indicate the presence of cancer stems in bladder cancer.

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