Experiment time: 2025-04-27, 19:00 - 2025-04-30, 17:00

Flow Cytometric Analysis of Apoptosis (II)

I. Objective of the experiment

To assess and compare the apoptotic levels of T24 wild-type and low-, medium-, and high-drug-resistant T24-RC48 cell lines, both untreated and following treatment, using an Annexin V-FITC/PI apoptosis detection kit. This analysis aims to elucidate the apoptotic characteristics of the resistant variants and provide an experimental basis for understanding ADC resistance mechanisms in bladder cancer.

II. Experimental content

2.1 Experimental design

Sample grouping: Blank control group (untreated T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines);

Drug treatment group (T24 wild-type cell lines and low, medium and high drug resistant T24-RC48 cell lines were treated with videstizumab (RC48) for 48 hours respectively)

Number of repetitions: 3 samples per group.

2.2 Measurement principles

Annexin V-FITC is a fluorescently labeled membrane-associated protein that specifically binds to phosphatidylserine (PS) exposed on the outer leaflet of the plasma membrane in apoptotic cells. Propidium iodide (PI), a nucleic acid dye, penetrates the cell membranes of late-stage apoptotic and necrotic cells, staining the nucleus red. Flow cytometry analysis of both Annexin V-FITC and PI fluorescence signals enables precise differentiation between viable cells, early apoptotic cells, and late apoptotic/necrotic cells.

III. Materials and reagents

3.1 Materials

Human bladder transitional cell carcinoma cell line (T24)

Low, medium, and high drug-resistant cell lines T24-RC48

T25 cell culture flasks (Corning)

6-well plate (Corning Inc.)

15ml centrifuge tube (Axygen Company)

Micropipettes (Eppendorf)

Pipette tips (Axygen company)

3.2 Reagents

Annexin V-FITC/PI cell apoptosis kit (Lianke Biology, AT101)

McCoy's 5A medium (Shanghai Yuanpei Biotechnology Co., Ltd., China)

Fetal bovine serum (FBS, Gibco)

Trypsin (Gibco)

PBS buffer (Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Flow cytometer (BD, not the C6 model)

V. Experimental steps

5.1 Seed T24 wild-type and low-, medium-, and high-resistant T24-RC48 cell lines into 6-well plates, with six wells per cell line at a density of 1×10^5 cells per well.

5.2 After 24 hours, for each cell line, replace the medium in three wells with fresh complete medium (untreated control), and the medium in the duplicate well with fresh complete medium containing 200μg/mL Disitamab vedotin (RC48). Treat the cells for 48 hours.

5.3 Carefully collect the culture medium from each well into a centrifuge tube. Then, wash the adherent cells with PBS twice, and combine these washes with the previously collected medium.

5.4 Add Accutase solution to digest the cells and gently tap the culture bottle to detach the cells.

5.5 Collect the cell suspension per well and centrifuge at 1000rpm for 5 minutes. Discard the supernatant.

5.6 Wash cells twice with pre-colded PBS and discard the supernatant.

5.7 Dilute 5× Binding Buffer with double distilled water to 1× working buffer. Take 500μl 1×

Binding Buffer to resuspend the cells. Add 5μl Annexin V-FITC and 10μl PI to each tube. Vortex gently and incubate at room temperature in the dark for 5 minutes.

5.8 Harvest 1×10^6 untreated wild-type cells. Resuspend the cell pellet in 500μL of Apoptosis Positive Control Solution and incubate on ice for 30 minutes.

5.9 Centrifuge the cells, discard the supernatant, and resuspend in a small volume of ice-cold 1× Binding Buffer. Mix these induced apoptotic cells with an equal number of untreated, viable wild-type cells.

5.10 Adjust the total volume to 1.5 mL with ice-cold 1× Binding Buffer. Split this mixture equally into three tubes: one unstained control tube, one tube for Annexin V-FITC single stain (add 5μL), and one tube for PI single stain (add 10μL). Incubate all tubes at room temperature in the dark for 5 minutes.

5.11 Analyze the samples using a flow cytometer.

5.12 Use FlowJo software to analyze the flow cytometry data, calculate the apoptosis rate of each group of cells, and generate bar graphs representing the apoptosis rates to compare the apoptosis difference between different drug-resistant groups and wild-type cell lines.

VI. Experimental results

As shown in Figure 1, apoptosis rates increased across all cell groups under drug treatment. In the untreated group: low-resistance cells showed significantly lower apoptosis rates than wild-type cells ($p < 0.001$), moderate-resistance cells had significantly lower rates than low-resistance cells ($p < 0.001$), and high-resistance cells exhibited significantly lower rates than moderate-resistance cells ($p < 0.05$). In the treated group: low-resistance cells demonstrated significantly lower apoptosis rates than wild-type cells ($p < 0.0001$), moderate-resistance cells showed markedly reduced rates compared to low-resistance cells ($p < 0.0001$), while high-resistance cells displayed the lowest apoptosis rates among all groups ($p < 0.0001$).

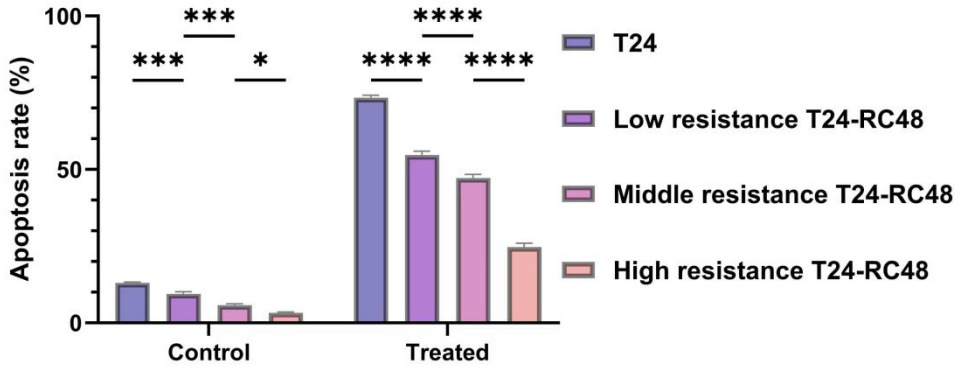

Figure 1 Comparison of apoptosis rate of bladder cancer cells with different resistance levels

As shown in Figure 2, the cell survival rates of all groups decreased under drug treatment conditions. In the untreated group: low-resistant cells showed significantly higher survival rates than wild-type cells ($p < 0.001$), moderate-resistant cells had significantly higher rates than low-resistant cells ($p < 0.001$), and highly-resistant cells demonstrated significantly higher rates than moderate-resistant cells ($p < 0.05$). In the treated group: low-resistant cells maintained significantly higher survival rates than wild-type cells ($p < 0.0001$), moderate-resistant cells showed significantly lower rates than highly-resistant cells ($p < 0.0001$), while highly-resistant cells exhibited markedly higher survival rates than moderate-resistant cells ($p < 0.0001$).

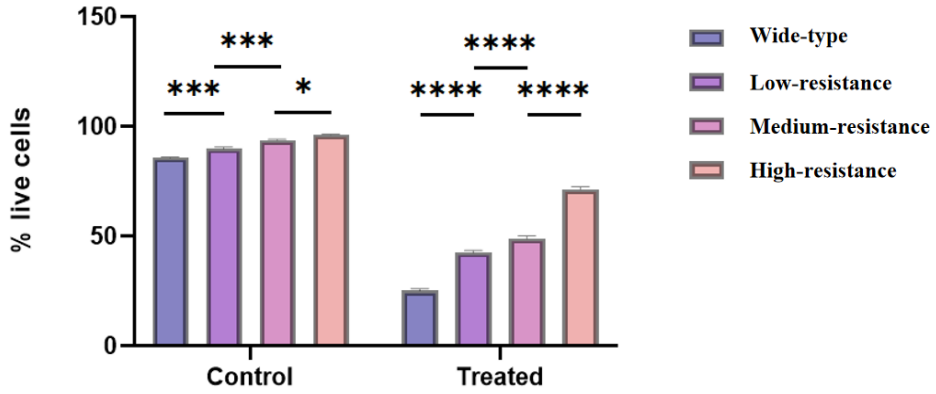

Figure 2 Comparison of survival rates of bladder cancer cells with different degrees of drug resistance

IX. Results analysis

This experiment incorporated the following optimizations based on the previous one:

1) The cell inoculation density was adjusted to 1×10^5/cell to reduce contact inhibition and improve the interaction efficiency between drugs and cells.

2) The processing time of RC48 was extended to 48 hours to ensure that the apoptosis process was fully completed.

3) Collect the floating cells from the culture medium prior to trypsinization, and combine them with the trypsinized cells before centrifugation to avoid losing any apoptotic cells.

The improvements in this experiment significantly enhanced the reliability and scientific validity of the results. Data indicate that the drug resistance level of bladder cancer cell lines correlates positively with apoptosis resistance: higher drug resistance corresponds to weaker apoptosis induction and survival inhibition effects. Notably, although drug-resistant cells have developed adaptive mechanisms, they still retain some drug sensitivity. This suggests that apoptosis suppression may be one of the key mechanisms behind Vedotin resistance in bladder cancer cells. Such apoptosis suppression likely stems from the gradual adaptation mechanisms formed by T24-RC48 cells during long-term culture and screening processes, which reduce the drug's cytotoxicity. These findings hold significant implications for further investigation into T24 cell resistance mechanisms against RC48 drugs. The results validate T24-RC48 cell lines as suitable models for studying RC48 resistance mechanisms, laying the foundation for deeper exploration of resistance mechanisms. This indicates that subsequent research could focus on investigating specific resistance mechanisms in T24-RC48 cells, providing theoretical support for developing more effective anticancer drugs and therapeutic strategies.