Experiment time: 2025-05-09, 18:00 - 2025-05-14, 17:00

Flow Cytometric Analysis of the Cell Cycle (II)

I. Objective of the experiment

This experiment aimed to determine the cell cycle distribution of T24 wild-type and low-, medium-, and high-drug-resistant T24-RC48 cell lines, both untreated and following treatment with Disitamab vedotin (RC48), using a Cell Cycle Staining Kit. The analysis of cell cycle alterations in the resistant variants provides an experimental basis for investigating 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 36 hours respectively)

Number of repetitions: 3 samples per group.

2.2 Measurement principle

During the cell cycle, cells in the G0/G1 phase contain 2N of DNA, those in the S phase have DNA levels between 2N and 4N, while cells in the G2/M phase carry 4N DNA. The Cell Cycle Staining Kit utilizes the DNA-binding dye propidium iodide (PI) to stain cells. Flow cytometry is then employed to detect DNA content and generate a cell cycle distribution histogram. By comparing pre-and post-treatment histograms, it enables the analysis of drug-induced effects on cell cycle progression, such as whether they induce specific phase blockages.

III. Materials and reagents

3.1 Materials

Pipette tips and 15ml centrifuge tubes were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

10-cm cell culture dishes (Corning Company)

Human bladder transitional cell carcinoma cell line (T24)

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

3.2 Reagents

PBS, trypsin and fetal bovine serum were purchased from Gibco, USA

McCoy's 5A medium purchased from Shanghai Yuanpei Biotechnology Co., Ltd., China

Cell cycle detection kit (Lianke Biology, CCS012)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Flow cytometers (BD, not model C6)

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 2×10^5 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 remaining three wells with fresh complete medium containing 200μg/mL Disitamab vedotin (RC48). Treat the cells for 36 hours.

5.3 Discard the culture medium and wash the cells with PBS for 3 times.

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

5.5 Collect the cell suspension from each dish 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 Resuspend the cell pellet in 1 mL of DNA Staining Solution containing 10μL of Permeabilization Solution. Vortex briefly for 5-10 seconds to mix, and incubate at room temperature in the dark for 30 minutes.

5.8 Analyze the stained cells using a flow cytometer.

5.9 Analyze the flow cytometry data using FlowJo software to determine the percentage of cells

in each cell cycle phase and compare the cell cycle distributions between the different drug-resistant groups and the wild-type cell line.

VI. Experimental results

After 36 hours of RC48 treatment, the flow cytometry detection showed that the cell cycle distribution of each group changed significantly: from the histogram of cell cycle distribution of each group (Figure 1), it can be seen that the G2 phase peak of all cells treated with the drug was significantly increased, especially in wild-type T24 cell line.

Under the condition of no drug addition, there was no obvious difference between the histograms of low, medium and high drug-resistant cell strains under naked eye observation; under the condition of drug addition, there was no discernible difference between the histograms of low, medium and high drug-resistant cell strains by visual inspection.

The horizontal coordinates of the G1 and G2 peaks in wild-type cells, both untreated and treated, were different from those observed in the drug-resistant cell lines under corresponding conditions.

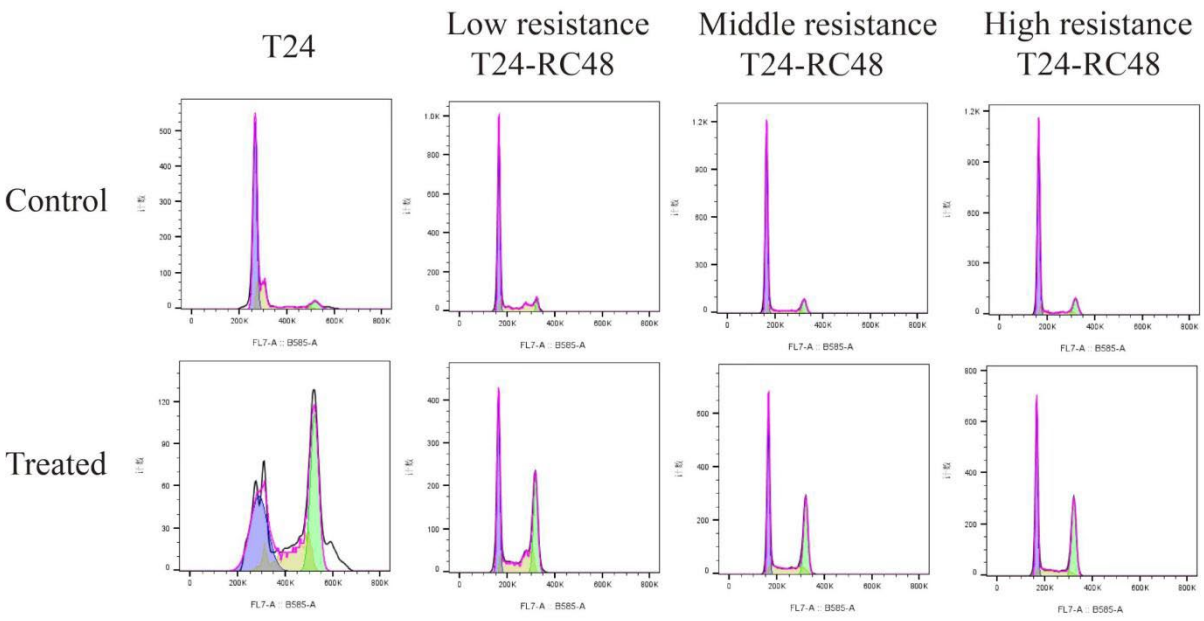

Figure 1 Histogram of cell cycle distribution in each group

Figure 2 demonstrates that the proportion of G1 phase cells in all treatment groups significantly decreased ($p < 0.0001$), while the proportion of G2 phase cells increased markedly ($p < 0.0001$). Regarding S phase: the proportion of wild-type S phase cells decreased significantly after treatment

($p < 0.05$). The S phase proportion showed no significant difference between treated and untreated groups in the low-dose resistance group. However, both the moderate-dose resistance group and high-dose resistance group exhibited a marked increase in S phase proportion following treatment ($p < 0.0001$).

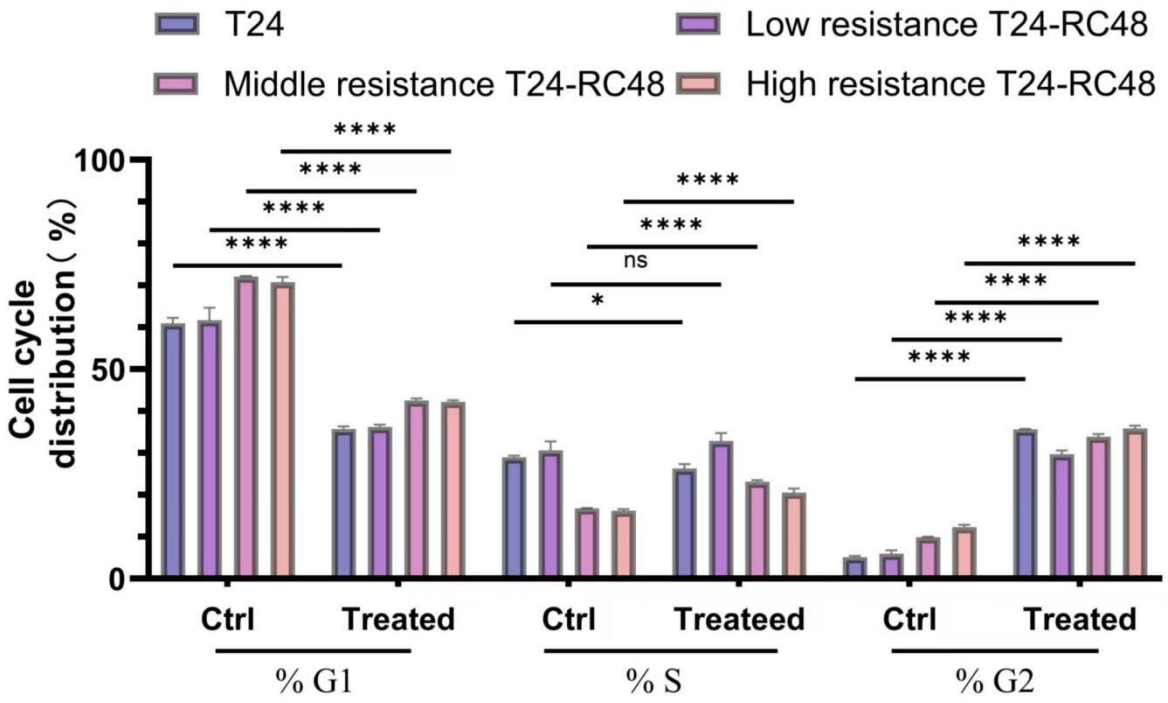

Figure 2 Analysis of the effect of RC48 treatment on the proportion of cell cycle stages in T24 wild-type and drug-resistant cell lines

VII. Results analysis

The difference in the horizontal coordinate (DNA content distribution position) between wild-type and drug-resistant cells at the peak of G1 and G2 periods indicates that drug-resistant cells may have adaptive changes in DNA replication mode or chromosome stability due to long-term drug stress, thus changing the characteristics of DNA content distribution.

After RC48 treatment, the proportion of G1 phase decreased and the proportion of G2 phase increased in all groups, indicating that the drug may inhibit cell proliferation by inducing G2/M phase blockade. Among them, the wild-type T24 cell line showed the largest increase in G2 phase, indicating that it was highly sensitive to RC48.

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Significant differences were observed in the proportion of S-phase cells between drug-treated and untreated groups: the low-drug resistance group showed no change in S-phase, while both medium and high-drug resistance groups experienced increased S-phase progression. This progression may be associated with enhanced DNA damage repair capacity (e.g., activation of ATM/ATR pathways) or improved replication stress tolerance in drug-resistant cells. Additionally, the increase in G2 phase progression was less pronounced in drug-resistant cells compared to wild-type cells, suggesting potential mechanisms like upregulation of anti-apoptotic proteins (e.g., BCL-2) or weakening G2/M checkpoint functions to evade drug effects.

In conclusion, RC48 has a significant effect on the cycle regulation of sensitive cells, while drug-resistant cells may mediate drug resistance through S-phase adaptive enhancement and special regulation of G2/M phase.