Protocol

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Contents

1. T-24 Cell Culture and Determination of Drug Half-Maximal Inhibitory Concentration (IC_{50})

I. Objectives of the experiment

1. To revive and establish a stable in vitro culture of the human bladder transitional cell carcinoma cell line T-24.

2. To determine the half-maximal inhibitory concentration (IC_{50}) of the antibody-drug conjugate Disitamab Vedotin (RC48) against T-24 cells following 72 hours of treatment using the CCK-8 assay, thereby establishing the critical initial drug concentration for subsequent experiments.

II. Materials and reagents

2.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

96-well cell culture plates and T-25 cell culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

2.2 Reagents

Phosphate-Buffered Saline (PBS), trypsin and Dimethyl Sulfoxide (DMSO) were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

Disitamab vedotin (RC48) from Rongchang Biopharmaceutical Co., Ltd., China

Cell Counting Kit-8 (CCK-8)

III. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

37°C, 5% CO_2 cell culture incubator: Thermo Fisher Scientific

Inverted phase-contrast microscope

Microplate reader

IV. Experimental steps

4.1 T-24 Cell Revival and Culture

4.1.1 The biological safety cabinet was sterilized by 30-minute UV irradiation. Complete culture medium and PBS were pre-warmed to 37°C in a water bath.

4.1.2 A cryovial containing T-24 cells was rapidly retrieved from liquid nitrogen storage and immediately placed in a 37°C water bath. The vial was gently agitated until only a small ice crystal remained (approximately 1-1.5 minutes).

4.1.3 The exterior surface of the cryovial was wiped thoroughly with 75% ethanol before being transferred into the biological safety cabinet. The cell suspension was aseptically transferred to a 15 mL centrifuge tube containing 5 mL of pre-warmed complete medium. The tube was gently mixed. Centrifugation was performed at 800 rpm for 5 minutes at room temperature.

4.1.4 After centrifugation, the supernatant was carefully decanted. The cell pellet was resuspended in 5 mL of fresh complete medium by gentle pipetting to generate a single-cell suspension. The entire suspension was transferred into a new T-25 culture flask. The flask was labeled with the cell line name (T-24) and the date of revival.

4.1.5 The culture flask was placed horizontally in a humidified incubator maintained at 37°C with 5% CO_2. Cell attachment and morphology were examined under an inverted phase-contrast microscope after 24 hours to confirm cell viability and normal growth status.

4.1.6 Upon reaching 80-90% confluence, cells were subcultured. The spent medium was aspirated, and the cell monolayer was rinsed once with PBS. An appropriate volume of trypsin-EDTA solution was added to cover the cell layer. Following incubation at 37°C, when cells appeared rounded and began to detach under microscopic observation, the digestion was terminated by adding complete medium. The cells were gently pipetted to achieve a homogeneous suspension and subcultured into new flasks at an appropriate split ratio for continued expansion.

4.2 Determination of RC48 IC_{50} Using CCK-8 Assay

4.2.1 T-24 cells in the logarithmic growth phase were harvested by trypsinization, centrifuged, and resuspended in complete medium. Cell density was adjusted to 4×10^4 cells/mL following counting.

4.2.2 The cell suspension was dispensed into a 96-well plate at 100 μL per well (resulting in 4×10^3 cells/well). The peripheral wells were filled with PBS to minimize evaporation-related artifacts.

The plate was incubated overnight at 37°C with 5% CO_2 to allow for cell attachment.

4.2.3 A serial dilution of RC48 was prepared in complete medium to achieve the desired concentration range (0, 1, 5, 10, 50, 100, 200, 300, 400, 500 μg/mL). After overnight incubation, the culture medium in the 96-well plate was carefully aspirated. Experimental wells then received 100 μL of fresh complete medium containing the respective concentrations of RC48. Each concentration was tested in six replicate wells. Wells containing only complete medium without cells served as the blank control. The plate was returned to the incubator for a continuous 72-hour treatment period.

4.2.4 After 72 hours of drug exposure, 110 μL of fresh medium containing 10% (v/v) CCK-8 reagent was added to each well. The plate was gently shaken to ensure thorough mixing and incubated in the incubator for 1 hour, protected from light.

4.2.5 Following the incubation period, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader.

4.2.6 The average absorbance (OD_{450}) was calculated for each drug concentration group and the control group. Cell viability was calculated as a percentage using the formula:

$$\text{Cell Viability (\%)} = [(OD_s - OD_6) / (OD_c - OD_6)] \times 100\%$$

Where OD_s is the absorbance of the test well, OD_c is the absorbance of the control well (untreated cells), and OD_6 is the absorbance of the blank well (medium only).

A dose-response curve was generated by plotting the drug concentration on the logarithmic X-axis against the cell viability (%) on the Y-axis.

The IC_{50} value, defined as the drug concentration that results in a 50% reduction in cell viability, was determined by non-linear regression analysis of the dose-response curve.

2. Construction of RC48-Resistant Cell Line T24-RC48

I. Objective of the experiment

To establish a stable RC48-resistant human bladder transitional cell carcinoma cell line (T24-RC48) through continuous induction with incrementally increasing concentrations of Disitamab vedotin (RC48).

II. Materials and reagents

2.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

6-well plates and T-25 cell culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

2.2 Reagents

Phosphate-Buffered Saline (PBS) and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

Disitamab vedotin (RC48) from Rongchang Biopharmaceutical Co., Ltd., China

III. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

37°C, 5% CO_2 cell culture incubator: Thermo Fisher Scientific

Inverted phase-contrast microscope

IV. Experimental steps

4.1 Culture T24 cells in complete medium until reaching appropriate confluence. Then seed the cells into 6-well plates and T25 flasks. At 24 hours post-seeding, replace the medium with fresh complete medium containing RC48 at the IC_{50} concentration (40 μg/ml).

4.2 Following 24 hours of RC48 treatment, replace the medium with drug-free complete medium. Continue culturing until the cells reach 80% confluence. Passage the cells once. Repeat this

procedure while incrementally elevating RC48 concentrations.

4.3 Implement the following concentration sequence during the induction process: 40 μg/ml → 50 μg/ml → 70 μg/ml → 100 μg/ml → 150 μg/ml → 250 μg/ml → 300 μg/ml → 400 μg/ml → 500 μg/ml.

4.4 Continue the cyclic induction until the cells demonstrate normal survival and proliferation capability after 24-hour exposure to 100 μg/ml RC48. Subsequently, extend the treatment duration to 48 hours while maintaining the established induction protocol. Through these iterative adaptation cycles, the cells develop tolerance to progressively higher RC48 concentrations. The resulting stable cell population is designated as the T24-RC48 drug-resistant cell line.

4.5 Monitor cell viability and proliferation capacity throughout the entire induction process. Maintain consistent culture conditions during all adaptation phases.

3. Cell Thawing

I. Objective of the experiment

To revive T24 cell lines and T24-RC48 cell lines with varying degrees of drug resistance, and transfer them into cell culture flasks.

II. Materials and reagents

2.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

T-25 cell culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

T24-RC48 cell lines with low, medium, and high drug resistance

2.2 Reagents

Dimethyl sulfoxide (DMSO) from WAK CHEMIE, Germany

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

Fetal bovine serum (FBS, Gibco)

III. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

HX-20 constant temperature metal bath (room temperature): Shanghai Huxi

IV. Experimental steps

4.1 The surface of the biosafety cabinet was wiped with alcohol wipes. All required reagents and consumables were placed inside the cabinet, which was then irradiated with UV light for 30 minutes for disinfection.

4.2 The frozen cell vial was rapidly thawed in a 37°C constant-temperature metal bath (pre-warmed) by gently agitating until just ice crystals remained (approximately 1 minute).

4.3 A 15 mL centrifuge tube was prepared with 5 mL of pre-warmed complete culture medium.

4.4 The cell suspension was aseptically transferred to the prepared centrifuge tube. Centrifugation was performed at 800 rpm for 5 minutes at room temperature.

4.5 After centrifugation, discard the supernatant and add fresh culture medium to resuspend the cells.

4.6 The resuspended cells were seeded into a new, sterile T-25 culture flask. Cell morphology and initial density were observed using an inverted microscope.

4.7 The culture flask was placed horizontally in a 37°C incubator with a 5% CO_2 humidified atmosphere.

4.8 Cell attachment and morphology were examined under an inverted phase-contrast microscope after 24 hours of incubation.

4. Cell Subculture and Medium Change

I. Objectives of the experiment

1.1 The T24 cell line and the moderately resistant and highly resistant T24-RC48 cell lines were passaged to maintain continuous cell proliferation and ensure an adequate supply of nutrients.

1.2 The low-dose resistant T24-RC48 cell line was was performed to remove metabolic waste products and replenish fresh culture medium.

II. Materials and reagents

2.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

T-25 cell culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

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

2.2 Reagents

Phosphate-Buffered Saline (PBS) and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

III. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

IV. Experimental steps

4.1 Wipe the surface of the ultra-clean workbench with alcohol cotton. Sort out the experimental tools. Put the required reagents and consumables into the ultra-clean workbench. Turn on the ultraviolet lamp, and disinfect for 30 minutes.

4.2 Disinfection: Hands were disinfected with alcohol. An alcohol lamp was ignited. Using forceps, a graduated serological pipette was taken. The caps and openings of all reagent bottles were briefly passed through the outer flame of the alcohol lamp. PBS and McCoy's 5A medium bottles were opened. The complete culture medium, supplemented with FBS and antibiotics, was

placed in an accessible position.

4.3 Take the cell culture bottle containing T24 and T24-RC48 cell lines out from the 37℃ constant temperature box, and observe the cell culture under microscope: a large number of cells grow on the wall.

4.4 The neck of the culture flask was briefly passed through the flame of the alcohol lamp to sterilize it before the medium was decanted into a waste container.

4.5 Transfer about 4ml of PBS with a graduated pipette into the culture bottle. Place the culture bottle horizontally. Gently shake the culture bottle front and back. Rinse off cell fragments and residual culture medium, and pour the cleaning liquid into the waste tank. Repeat this step twice.

4.6 Transfer 5ml of complete medium with a graduated pipette into the culture bottle containing low drug resistance T24-RC48 cell line.

4.7 1 mL of trypsin solution was added to each flask using a new sterile pipette. The flasks were incubated at 37°C for 2 minutes. Upon microscopic examination, cells appeared shrunk and rounded but not fully detached. Incubation was continued for an additional 30 seconds. Subsequent observation revealed that the cells detached and assumed a 'sandy' appearance under the microscope. Intercellular connections were lost, and rounded cells were suspended in the medium without significant clumping.

4.8 Add 2 mL of complete culture medium to the culture flask using a calibrated pipette to terminate the trypsin digestion. After gently pipetting the medium up and down several times to dissociate the cells, transfer the remaining liquid to a 15 mL centrifuge tube. Mark the tube and place it in the centrifuge. After equilibration, centrifuge at 800rpm for 5 minutes.

4.9 During centrifugation, add two tubes of complete culture medium (3ml/bottle) to each of the three cell culture bottles with a new pipette.

4.10 Take out the centrifuge tube and pour out the supernatant. Add about 6ml of full culture liquid into the centrifuge tube with a graduated pipette, and the cell pellet was resuspended by gently pipetting up and down. Then divide the culture liquid into three cell culture bottles with a graduated pipette. The flasks were gently rocked back and forth and side-to-side to ensure even cell distribution.

4.11 Label the date and cell type on the cell culture bottle.

4.12 Set up the ultra-clean table and wipe it with alcohol cotton balls.

5. Growth Curve Measurement (I)

I. Objective of the experiment

The proliferative capacity of the T24 cell line and the highly drug-resistant T24-RC48 cell line under 200 μg/ml RC48 treatment was assessed using the CCK-8 assay over a 72-hour period. Growth curves were plotted to evaluate the drug's inhibitory effects on the growth of the different cell lines. The conditions for formal growth curve measurement were explored.

II. Experimental content

2.1 Experimental design

Cell types: T24 wild-type cell line and highly drug-resistant T24-RC48 cell line.

Drug treatment: Vedotin (RC48) was administered at a concentration of 200μg/ml.

Time: 0h,24h,48h,72h.

Number of replicates: 6 wells per group.

2.2 Measurement principle

WST-8 in CCK-8 reagent was reduced to an orange-colored formazan product by mitochondrial dehydrogenase of cells, and its absorbance (OD=450nm) was positively correlated with the number of living cells. The absorbance is directly proportional to the number of viable cells, allowing for the assessment of cell viability.

III. Materials and reagents

3.1 Materials

The 1000μl and 100μl pipette tips and 15ml centrifuge tubes were purchased from Axygen

96-well plates (Corning Inc.) x 4

Micropipettes: Eppendorf

T-25 cell culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

Highly drug-resistant cell line T24-RC48

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

Disitamab vedotin (RC48, Rongchang Biopharmaceutical Co., LTD.)

CCK-8 kit

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Microplate reader

V. Experimental steps

5.1 T24 and highly drug-resistant T24-RC48 cell lines in the logarithmic growth phase were harvested, digested with trypsin, centrifuged, and resuspended. The cell density was adjusted to 4 $\times 10^4$ cells/mL.

5.2 The cell suspension was inoculated into four 96-well plates according to the experimental design, with each plate receiving 12 wells per cell type. Each well contained 100 μl of culture medium (containing 4×10^3 cells), and PBS was added to the edge wells to prevent evaporation. The plates were then cultured in an incubator maintained at 37°C with 5% CO_2 concentration.

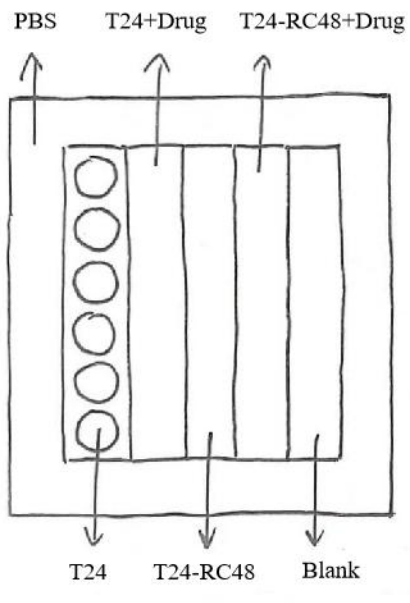

Figure 1 Distribution of cell suspensions in each group in the 96-well plate

5.3 After overnight cell seeding, aspirate the old medium and replace with fresh medium in three 96-well plates (add 6 wells per plate with fresh medium containing 200μg/ml RC48). Set the time to 0 and incubate at 37°C with 5% CO_2. For the remaining plate (Plate 1), add the chromogenic solution to the culture wells according to the CCK-8 kit instructions. Incubate for 1 hour and measure the absorbance at 450nm using a microplate reader.

5.4 At each time point (24, 48, and 72 hours), the corresponding 96-well plate was taken out from the incubator. Treat it according to the instructions of CCK-8 kit and measure the absorbance value at 450nm.

5.5 The time of treatment with RC48 was taken as the horizontal coordinate, and the OD value of the experimental group at 450nm was taken as the vertical coordinate with the OD value of the blank control hole. The time-OD value curve, namely the cell proliferation curve, was plotted.

6. Growth Curve Measurement (II)

I. Objective of the experiment

The proliferative ability of T24 cell lines and low, medium and high drug-resistant T24-RC48 cell lines under the treatment of 200ug/ml RC48 was dynamically monitored using the CCK-8 assay. The absorbance values were continuously measured for 5 days (0h,24h,48h,72h,96h,120h), and the growth curve was plotted to evaluate the long-term inhibitory effects of the drug on cell proliferation.

II. Experimental content

2.1 Experimental design

Cell types: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Drug treatment: Vedotin (RC48) concentration gradient (0,50,100,200,500μg/ml).

Time: 0h (D0),24h (D1),48h (D2),72h (D3),96h (D4),120h (D5).

Number of replicates: 6 wells per group.

2.2 Measurement principle

WST-8 in CCK-8 reagent was reduced to an orange-colored, water-soluble formazan dye by mitochondrial dehydrogenase of living cells, and the absorbance (OD450nm) was positively correlated with the number of living cells.

III. Materials and reagents

3.1 Materials

The 1000μl and 100μl pipette tips and 15ml centrifuge tubes were purchased from Axygen

96-well plates (Corning Inc.) × 6

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

Low-, medium-, and high-resistance T24-RC48 cell lines

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

Disitamab vedotin (RC48, Rongchang Biopharmaceutical Co., Ltd., China)

CCK-8 kit

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Microplate reader (Thermo Fisher Scientific)

37℃,5% CO_2 cell culture chamber (Thermo Fisher Scientific)

V. Experimental steps

5.1 T24 and low/medium/high drug-resistant T24-RC48 cell lines in the logarithmic growth phase were harvested, trypsinized, centrifuged, and resuspended. The cell density was adjusted to 3×10^4 cells/mL.

5.2 The cell suspension was inoculated into six 96-well plates according to the experimental design, with each plate containing 12 wells per cell type. Each well received 100μL of culture medium (containing 3×10^3 cells), and edge wells were covered with PBS to prevent evaporation. The plates were then cultured in a 37°C incubator maintained at 5% CO_2 concentration.

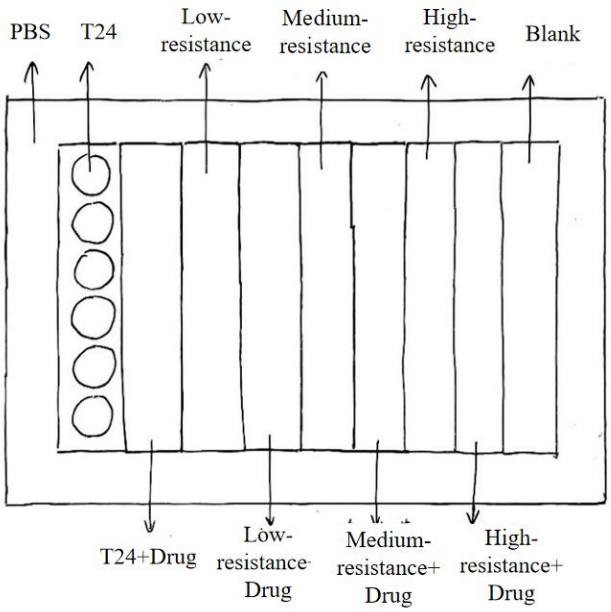

Figure 1 Distribution of cell suspensions in each group in the 96-well plate

5.3 After overnight cell seeding, the culture medium was carefully aspirated from the wells and replace with fresh medium in five 96-well plates (each plate contains six wells of fresh medium supplemented with 200μg/ml RC48). Set time 0 as the reference point and incubate at 37°C with 5% CO_2. For the remaining plate (Plate 1), add the chromogenic solution to the culture wells according to the CCK-8 kit instructions. Incubate for 1 hour and measure the absorbance at 450nm using a microplate reader.

5.4 At each time point (24, 48, 72, 96, and 120 hours), the corresponding 96-well plate was removed from the incubator and processed according to the CCK-8 kit protocol. The absorbance at 450 nm was measured.

5.5 The time of treatment with RC48 was taken as the horizontal coordinate, and the OD value of the experimental group at 450nm was taken as the vertical coordinate with the OD value of the blank control hole as the vertical coordinate to draw the time-OD value curve, namely the cell proliferation curve.

7. Sample Preparation for Proteome Sequencing (I)

I. Objective of the experiment

The cell precipitation samples of T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines were prepared and sent for proteome sequencing. The resulting data will be used for subsequent analysis of differential protein expression and investigation of drug resistance mechanisms.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Number of repetitions: 3 samples per group.

2.2 Measurement principle

Label-free proteomics sequencing achieves protein relative quantification by directly comparing MS1 (first-level) and MS2 (second-level) signal intensities or spectral counts in different samples through mass spectrometry. The core mechanism involves peptide separation via liquid chromatography, followed by detection of peptides based on four dimensions: mass-to-charge ratio (m/z), retention time, ion intensity, and ion mobility (a newly added dimension). Ion mobility enhances selective detection of low-abundance peptides by leveraging differences in ion migration within the electric field, thereby reducing background interference and improving detection sensitivity and quantification accuracy. Quantification is performed by establishing a positive correlation between signal intensity and protein abundance through either integrating the chromatographic peak area (Label-free quantification, LFQ intensity) or by counting the number of fragmentation spectra (Spectral Count) matched to a peptide. This technique eliminates the need for isotope labeling, simplifies experimental procedures, and is particularly suitable for high-throughput analysis of complex samples.

III. Materials and reagents

3.1 Materials

Pipette tips and 15ml centrifuge tube were purchased from Axygen

Serological pipettes was purchased from a domestic company

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

10-cm cell culture dishes (Corning)

Liquid nitrogen, dry ice

Human bladder transitional cell carcinoma cell line (T24)

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

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

V. Experimental steps

5.1 Low, medium, high drug-resistant and wild-type T24 cell lines were seeded in 10-cm cell culture dishes until the cells grew to more than 80%.

5.2 Discard the culture medium and add 4℃ pre-cooled PBS. Shake gently for 1 minute on a flat surface and discard the PBS. Repeat the above operation three times to fully wash off the culture medium.

5.3 Place the culture dish on ice and add 1ml of 4℃ pre-colded PBS into the culture dish. Quickly scrape the cells with a clean cell scraper onto one side of the culture dish. Place the culture dish at an angle on ice so that the buffer flows to one side.

5.4 Use a pipette to aspirate the cell suspension into a pre-cooled 1.5ml EP tube and centrifuge to remove the supernatant.

5.5 Label the sample number. Rapidly frozen in liquid nitrogen, and then stored at -80°C.

5.6 Dry ice packaging and send it for proteome sequencing.

8. Sample Preparation for Proteome Sequencing (II)

I. Objective of the experiment

The TRIzol lysis samples of T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines were prepared. These samples were then shipped on dry ice to the sequencing company for subsequent transcriptome sequencing analysis.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Number of repetitions: 3 samples per group.

2.2 Measurement principle

RNA-seq (RNA sequencing) converts RNA into double-stranded cDNA libraries through reverse transcription, followed by sequencing to quantify gene expression. The workflow involves: extracting total RNA from samples, enriching mRNA using oligo (dT) magnetic beads (which bind to the poly-A tails of eukaryotic mRNA), fragmenting RNA, synthesizing cDNA with random primers, constructing libraries via end repair and ligation, and performing high-throughput sequencing on platforms like Illumina. This technology captures transcript sequences at single nucleotide resolution, where sequencing depth (the number of reads mapped to a gene) correlates directly with the abundance of its transcripts, enabling full transcriptome coverage and precise identification of structural variations such as alternative splicing and fusion genes. RNA-seq eliminates the need for probe design, offering high sensitivity and cross-species applicability. It is widely used in disease mechanism research, developmental regulation analysis, and novel transcript discovery.

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

T25 culture flasks were purchased from Corning

10-cm cell culture dishes (Corning)

Liquid nitrogen, dry ice

Human bladder transitional cell carcinoma cell line (T24)

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

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

V. Experimental steps

5.1 Low, medium, high drug-resistant and wild-type cell lines were seeded in 10-cm cell culture dishes until the cells grew to more than 80%.

5.2 Discard the culture medium and add 4℃ pre-cooled PBS. Shake gently for 1 minute on a flat surface and discard the PBS. Repeat the above operation three times to fully wash off the culture medium.

5.3 Aspirate the PBS completely. Then, add 1 mL of TRIzol reagent per 10 million cells ($1x10^7$) to lyse the cells directly in the culture dish. Ensure the reagent covers the cell monolayer completely.

5.4 Using a pipette, repeatedly aspirate and dispense the TRIzol lysate to homogenize it until no cell clumps are visible. The lysate should appear clear and non-viscous, indicating complete dissociation. Transfer the homogeneous lysate to a pre-chilled, RNase-free 1.5 mL microcentrifuge tube.

5.5 Label the tube, immediately freeze it in liquid nitrogen, and then store it at -80°C.

5.6 Dry ice packing and send it for proteome sequencing.

9. Sample Preparation for Whole Genome Sequencing

I. Objective of the experiment

To prepare whole-genome sequencing (WGS) samples from the T24 wild-type cell line and low-, medium-, and high-drug-resistant T24-RC48 cell lines. The samples will be shipped on dry ice for subsequent genomic variation analysis to investigate drug resistance-related genomic alterations.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Number of repetitions: 3 samples per group.

2.2 Measurement principle

Whole Genome Sequencing (WGS) involves randomly fragmenting genomic DNA, constructing a library from these fragments, and then performing massive parallel sequencing using next-generation sequencing (NGS) technology. Short reads are subsequently assembled into a complete genome sequence using bioinformatic tools. The specific workflow includes: extracting high-quality DNA; randomly shearing long DNA strands into fragments of 200–500 bp via physical or chemical methods; constructing a sequencing library through steps including end repair, adapter ligation, and PCR amplification; sequencing the library using technologies such as sequencing by synthesis (SBS) or single-molecule real-time sequencing, which determines the sequence by detecting fluorescent signals during base extension; and finally, performing sequence alignment, variant detection (e.g., SNPs, CNVs), and functional annotation to reveal genomic structural variations and functional characteristics. WGS provides single-base resolution, high coverage, and unbiased whole-genome detection, making it widely applicable in fields such as cancer genomics and evolutionary studies.

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

T25 culture flasks were purchased from Corning

10-cm cell culture dishes (Corning)

Liquid nitrogen, dry ice

Human bladder transitional cell carcinoma cell line (T24)

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

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

V. Experimental steps

5.1 Low, medium, high drug-resistant and wild-type T24 cell lines were seeded in 10-cm cell culture dishes until the cells grew to more than 80%.

5.2 The culture medium was discarded, and the cells were washed with 4°C pre-chilled PBS. The dish was gently rocked for 1 minute, and the PBS was aspirated. This washing step was repeated twice more.

5.3 The culture dish was placed on ice. Then, 1 mL of ice-cold PBS was added. Cells were quickly scraped off using a sterile cell scraper and pooled to one side of the dish by tilting it on ice.

5.4 Use a pipette to aspirate the cell suspension into a pre-cooled 1.5ml centrifuge tube and and centrifuged. The supernatant was then carefully discarded.

5.5 The tubes were labeled, rapidly frozen in liquid nitrogen, and subsequently stored at -80°C.

5.6 Samples were packaged on dry ice and sent for subsequent genomic variation analysis.

10. Flow Cytometric Analysis of Apoptosis

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.

11. Flow Cytometric Analysis of the Cell Cycle

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.

12. Transwell Migration Assay

I. Objective of the experiment

In this experiment, Transwell was used to detect the migration ability of T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines, and the migration characteristics of drug-resistant cell lines were analyzed to provide an experimental basis for elucidating the mechanism of ADC resistance in bladder cancer.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Number of repetitions: 3 per group.

2.2 Measurement principle

The Transwell cell migration assay operates on the fundamental principle of cellular chemotaxis. In this setup, cells are placed in the upper chamber while the lower chamber contains a culture medium with chemokine gradients. When cells detect these concentration differences, they actively migrate through the permeable membrane toward the higher chemokine concentration. The migration capacity is then assessed by visualizing and counting the number of cells that pass through the membrane during the experiment.

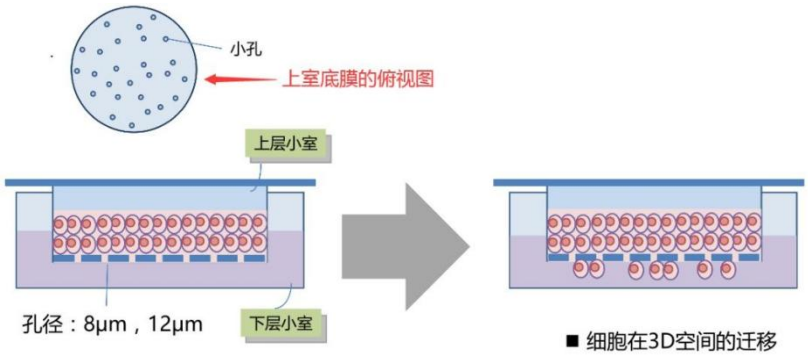

Figure 1 Transwell chamber principle

III. Materials and reagents

3.1 Materials

T25 cell culture bottle

Pipette tips and 15ml centrifuge tubes were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

Transwell chamber (8μm aperture)

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

4% polyformaldehyde

Crystal violet

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Inverted phase contrast microscope

V. Experimental steps

5.1 T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines were inoculated into T25 cell culture bottles respectively.

5.2 After 24 hours, the complete medium in each flask was replaced with McCoy's 5A medium containing 1% FBS for serum starvation for 24 hours.

5.3 Place the Transwell chamber into a 24-well plate and add 500μl McCoy's 5A medium containing 10% FBS to the lower chamber.

5.4 Cells digested with trypsin were prepared into a single cell suspension and the cell concentration was adjusted to 1×10^5 cells/ml.

5.5 Take 100μl cell suspension and add it to the upper chamber of Transwell chamber, with 3 replicates in each group.

5.6 Place the 24-well plate in a 37℃,5% CO_2 incubator for 24 hours.

5.7 The Transwell chamber was removed and gently rinsed with PBS to remove non-migrated

cells.

5.8 Gently wipe away the unmigrated cells in the upper inner layer with a cotton swab.

5.9 Cells were fixed with 4% paraformaldehyde for 15 minutes.

5.10 Rinse twice with PBS for 3 minutes each time.

5.11 Stain with crystal violet for 10 minutes.

5.12 Rinse off excess stain and air-dry.

5.13 Place the stained chamber on a moist glass slide and take pictures along the cross line to record the cell migration.

5.14 Use the image analysis software Image J to measure and calculate the number of migrating cells.

13. Transwell Invasion Assay

I. Objective of the experiment

In this experiment, transwell invasion was used to compare the invasive capabilities of the T24 wild-type cell line with its low-, medium-, and high-drug-resistant T24-RC48 sublines, and to explore the relationship between drug resistance and cell invasion behavior.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line and its low-, medium-, and high-drug-resistant T24-RC48 sublines.

Cell density: 50,000 cells per well.

Repeat times: 3 duplicate holes per group.

Matrix gel: Transwell chambers were pre-coated with Matrigel matrix gel (diluted 1:8)

2.2 Measurement principle

The Transwell invasion assay enhances the migration experiment by incorporating a Matrigel barrier. In this setup, the upper chamber of the Transwell microplate is pre-coated with Matrigel to simulate the extracellular matrix environment. Cells must secrete proteases to degrade the gel before migrating through the permeable membrane into the lower chamber. The invasion capability of cells is assessed by quantifying the number that successfully penetrate the gel barrier.

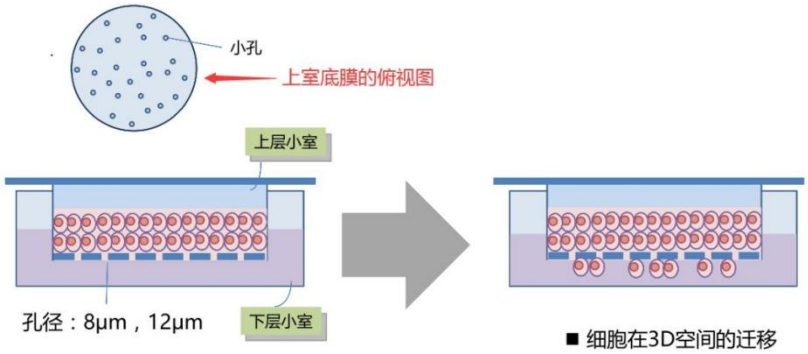

Figure 1 Transwell chamber principle

III. Materials and reagents

3.1 Materials

T25 cell culture bottle

Pipette tips and 15ml centrifuge tubes were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

Transwell chamber (8μm aperture)

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

3.2 Reagents

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

Matrigel matrix gel was purchased from Corning, part number 354234

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

4% polyformaldehyde

Crystal violet

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Inverted phase contrast microscope

V. Experimental steps

5.1 The T24 wild-type and the low-, medium-, and high-resistant T24-RC48 cell lines were seeded into T25 cell culture flasks, respectively.

5.2 After 24 hours, the complete medium of each flask was replaced with McCoy's 5A medium containing 1% FBS for starvation treatment for 24 hours.

5.3 Under condition 4℃ (on ice), Matrigel was diluted with serum-free McCoy's 5A cell culture medium at 1:8.

5.4 Take 60μl and add it uniformly to the upper surface of the Transwell chamber. Incubate in the 37℃ incubator for 3h to allow the Matrigel to polymerize and form a gel layer.

5.5 Following polymerization, the excess liquid was carefully aspirated from the upper chamber, and 100μl of low serum medium was added to each chamber. The plate was then returned to the incubator for 30 minutes to carry out substrate membrane hydration.

Tips:

(1) The gun head is gently pushed along the wall of the chamber to push out Matrigel without bubbles, and avoid poking the filter membrane of the chamber.

(2) The volume of the added Matrigel glue should not be too large, and the polycarbonate film can be soaked.

(3) Matrigel is easy to solidify at too high or too low temperature, so the gun head and other equipment required for operation should be pre-cooled at 4℃.

(4) Ensure that the liquid surface is level when laying the glue, and the thickness of the glue is uniform. Do not produce bubbles.

5.6 Add 20% serum McCoy's 5A medium 600μl/cell, and then place the Transwell chamber into the 24-well plate with forceps.

5.7 Cells digested with trypsin were prepared into a single cell suspension and the cell concentration was adjusted to 5×10^5 cells/ml.

5.8 Take 100μl cell suspension and add it to the upper chamber of Transwell chamber, with three replicates per group, and continue to culture for 48h.

5.9 Add 800 μl PBS to the blank culture well, gently place the chamber into which the medium has been sucked out, and gently washed (care was taken throughout all washing steps to avoid scraping or dislodging the migrated cells on the lower surface of the membrane).

5.10 Gently wipe off the inner layer of cells in the chamber: use a cotton swab with a slightly loosened tip to ensure thorough cleaning, including the edges. After wiping, rinse the inner wall with PBS.

5.11 Transfer the chamber to a new well containing 800μL of 4% paraformaldehyde (PFA) per well and fix at room temperature for 15 min; then wash twice with PBS, 2 min each time (all washes were performed by gentle immersion without shaking or swishing).

5.12 Transfer the chamber to a blank culture hole with 600ul/ hole of crystal violet dye, and stain for 10min; after staining, wash off the floating color with clean water twice, each time for 2min.

5.13 Place the stained chamber on a clean glass slide, and pay attention to not completely dry it. Slightly moistening will make the cell morphology better; capture images following a predefined pattern: first along the crosshairs, and then at each of the four corners.

5.14 Use the image analysis software Image J to measure and calculate the number of migrating

cells.

14. Transcriptomics Data Analysis

I. Objective of the experiment

By analyzing the gene expression profile differences between human bladder transitional cell carcinoma T-24 cells and low, medium and high concentration RC48 drug-resistant strains (T24-RC48) through transcriptome sequencing technology (RNA-seq), this study screened the key genes and signal pathways related to RC48 acquired drug resistance, providing molecular data support for elucidating the drug resistance mechanism.

II. Experimental procedure

2.1 Sample processing and library sequencing

Sample groups: T24 cells (control group), low drug resistance strain, moderate drug resistance strain, and high drug resistance strain, with 3 biological replicates in each group.

RNA extraction and quality control: Agilent 2100 Bioanalyzer was used to detect RNA integrity (RIN value>7).

Library construction: Use chain-specific library construction (retaining the transcription direction information), enrich mRNA by Oligo (dT) magnetic beads, synthesize double-stranded cDNA after random fragmentation, and then connect sequencing adapters for PCR amplification.

Sequencing: Illumina platform was used for double-end sequencing (PE150), and the single sample data volume was greater than or equal to 6 gigabases (Gb).

2.2 Bioinformatics analysis

Data quality control: Filter low quality reads with high proportion of connectors and N to obtain clean reads (Q20≥97%, Q30≥93%).

Reference genome alignment: Clean reads were aligned to the human reference genome using HISAT2 software (alignment rate> 89%).

Gene quantification: Gene expression was calculated by featureCounts (standardized FPKM).

Differential expression analysis: DESeq2 (accounting for biological replicates) was used to identify differentially expressed genes (DEGs) ($|\log2(\text{FoldChange})| \geq 1$ and $\text{p.adj.} \leq 0.05$).

Functional enrichment analysis: Based on GO, KEGG, Reactome, DO and DisGeNET databases, pathway enrichment was performed by clusterProfiler (adjusted p-value (p.adj.) <0.05).

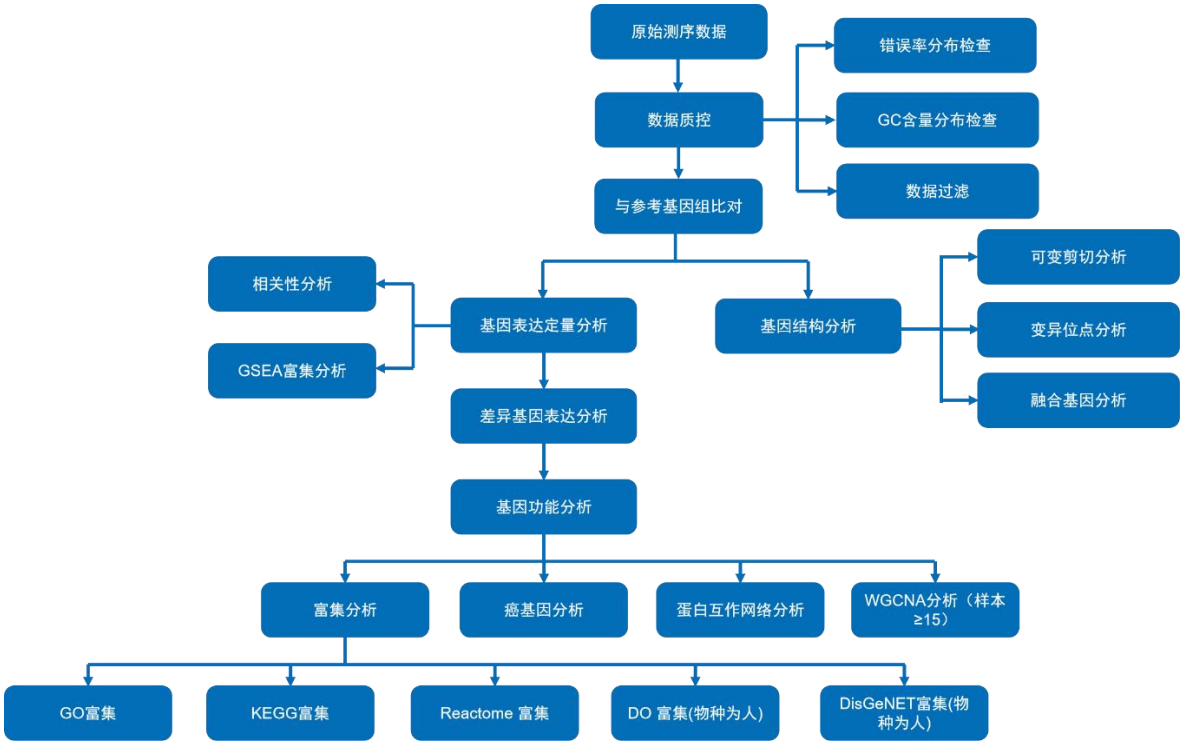

Figure 1 RNA-seq information analysis technology flow

15. Proteomics Data Analysis

I. Objective of the experiment

Through proteomic technology, we conducted comprehensive protein expression profiling analysis on the RC48 drug-resistant human bladder transitional cell carcinoma T24-RC48 cell line (low/medium/high drug resistance group) and the T24 cell line (control group). This approach identified significantly differentially expressed proteins, revealed drug resistance-related functional pathways and key protein biomarkers, thereby providing molecular-level data support for elucidating the mechanisms of acquired drug resistance in RC48.

II. Experimental procedures

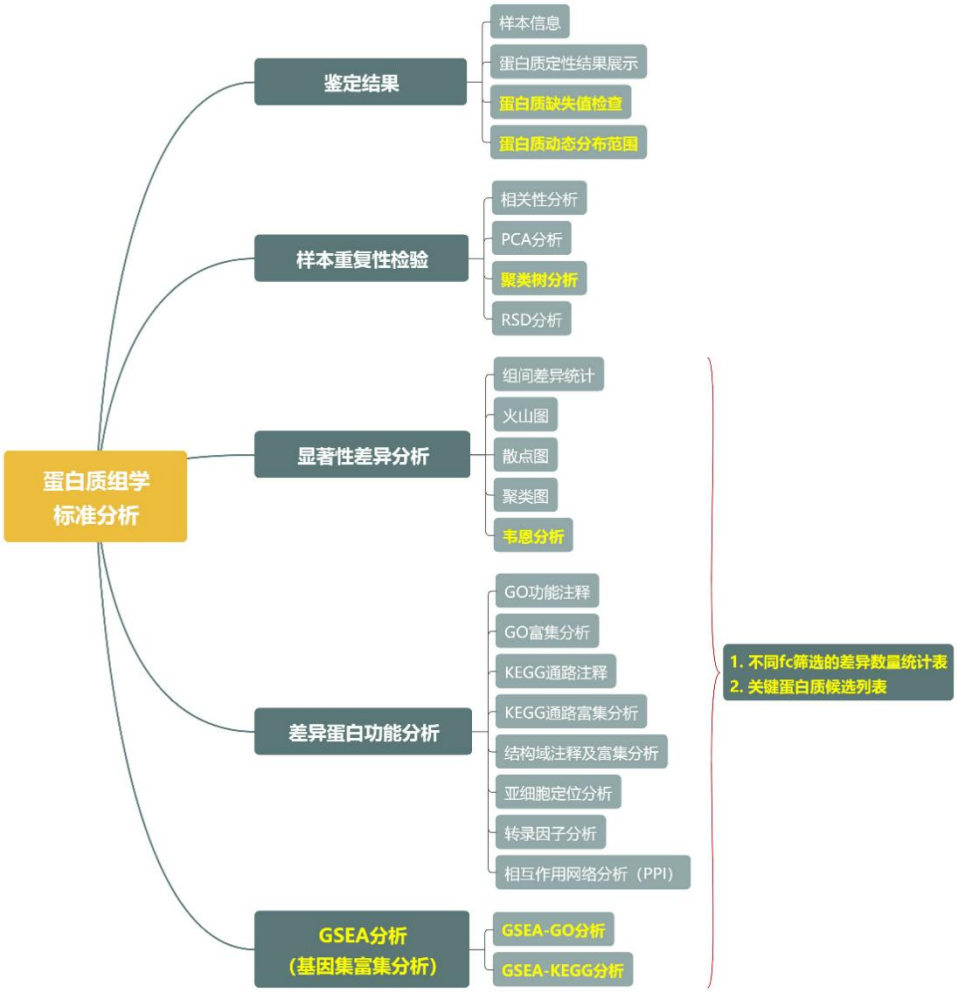

Figure 1 Proteomics information analysis technology flow

2.1 Sample preparation

1) Extract total protein by cell lysis, quantify by BCA method, and verify protein integrity by

SDS-PAGE.

2) Quality control after enzymatic digestion (peptide amount ≥200ng), and data were acquired using a TimsTOF Pro mass spectrometer in Data-Dependent Acquisition (DDA) mode.

2.2 Mass spectrometry data acquisition and analysis

1) The original mass spectrometry data were searched in the database (Homo sapiens UniProt database) by MaxQuant software.

2) Label-free quantification, and screening more than 50% of non-empty data in the group for differential analysis.

3) Significance difference criteria: fold change>1.2 (up or down) and P value <0.05.

2.3 Bioinformatics analysis

1) Data Visualization: Differential proteins are visualized using volcano plots, scatter plots, and hierarchical clustering heatmaps.

2) Functional Annotation:

- GO Annotation: The target protein set is annotated via Blast2GO (v1.4.4) through a sequential process of BLAST alignment (E-value ≤ $1e^{-3}$), GO mapping, annotation, and supplementary annotation using InterProScan and ANNEX.
- KEGG Pathway Annotation: KOALA (v3.0) is used to assign KEGG Orthology (KO) identifiers and associated pathway information by aligning target proteins against the KEGG GENES database.
- Domain Annotation: Functional domains are annotated using the InterPro database.
- Subcellular Localization: Predictions are performed using WoLF PSORT based on sorting signals and amino acid composition.

3) Enrichment Analysis: Fisher's exact test is employed to identify significantly enriched GO terms, KEGG pathways, and protein domains within the target protein set compared to the background proteome.

4) GSEA: Gene Set Enrichment Analysis (GSEA) software is utilized for GSEA-GO and GSEA-KEGG analysis against species-specific molecular signature databases (Msigdb package for Human).

5) Transcript Factor Prediction: Potential transcription factors are predicted using the species-appropriate database (AnimalTFDB 3.0 for animals).

6) Protein-Protein Interaction (PPI) Network Analysis: A PPI network for the target proteins is constructed using the STRING database and visualized with AnyChart (v8.11.0.1934).

16. Integrative Analysis of Transcriptomics and Proteomics

I. Objective of the experiment

1.1 Through the combined analysis of transcriptome and proteome, the correlation and differences between gene expression and protein levels were systematically investigated in a drug-resistant cell model of human bladder transitional cell carcinoma (T24-RC48).

1.2 Select genes/proteins that are significantly changed at both transcription and protein levels, explore their biological functions and signaling pathways, and reveal potential regulatory mechanisms related to drug resistance.

1.3 Verify whether the changes in transcript level directly determine the changes in protein abundance, and explore the coordinated multi-omics regulatory network underlying the drug-resistant phenotype.

II. Experimental procedures

2.1 Sample information and grouping

Transcriptome samples: T24_R_high (highly resistant), T24_R_mode (moderately resistant), T24_R_low (low-resistant), and T24_R_control (wild-type)

Protein group samples: T24_R_high (highly resistant), T24_R_mode (moderately resistant), T24_R_low (low-resistant), and T24_R_control (wild-type)

2.2 Criteria for Differential Analysis

Transcriptome: $|\log2FC| \geq 1.0$, $pAdj < 0.05$

Protein group: $|\log2FC| \geq 0.263$, $p\_value < 0.05$

2.3 Joint analysis process

Overall results comparison: Venn diagram analysis, gene expression distribution, correlation analysis (linear fit R^2 value to assess transcription and protein level consistency).

Comparison of differential results: statistical analysis of differential genes/proteins, Venn analysis, and clustered heatmaps.

2.4 Data mining analysis

GO functional enrichment: Enriched GO terms (Biological Process, Molecular Function, Cellular Component) were identified and visualized using Venn diagrams, heatmaps, bubble plots, and bar charts.

KEGG pathway enrichment: a total of pathway screening combined with pathway and network maps to analyze key signaling pathways.

Screening of key genes/proteins: Identify core drug-resistant related molecules based on co-expression trends (such as UP-UP, DOWN-DOWN, etc.) and PPI network analysis.

2.5 Statistical and Bioinformatics Methods

- Enrichment Analysis: The statistical significance of GO term and KEGG pathway enrichment was evaluated using Fisher's Exact Test, comparing the distribution of terms/pathways in the target gene/protein set against the background set.
- Correlation Analysis: Integrative correlation analysis among differentially expressed genes, proteins, and metabolites was performed using Spearman's rank correlation method, implemented via the cor function in R (v4.2.2).
- PPI Network Analysis: PPI networks for target proteins were constructed by querying the STRING database to retrieve both direct and indirect interactions. The resulting networks were visualized and analyzed using AnyChart software (v8.11.0.1934).

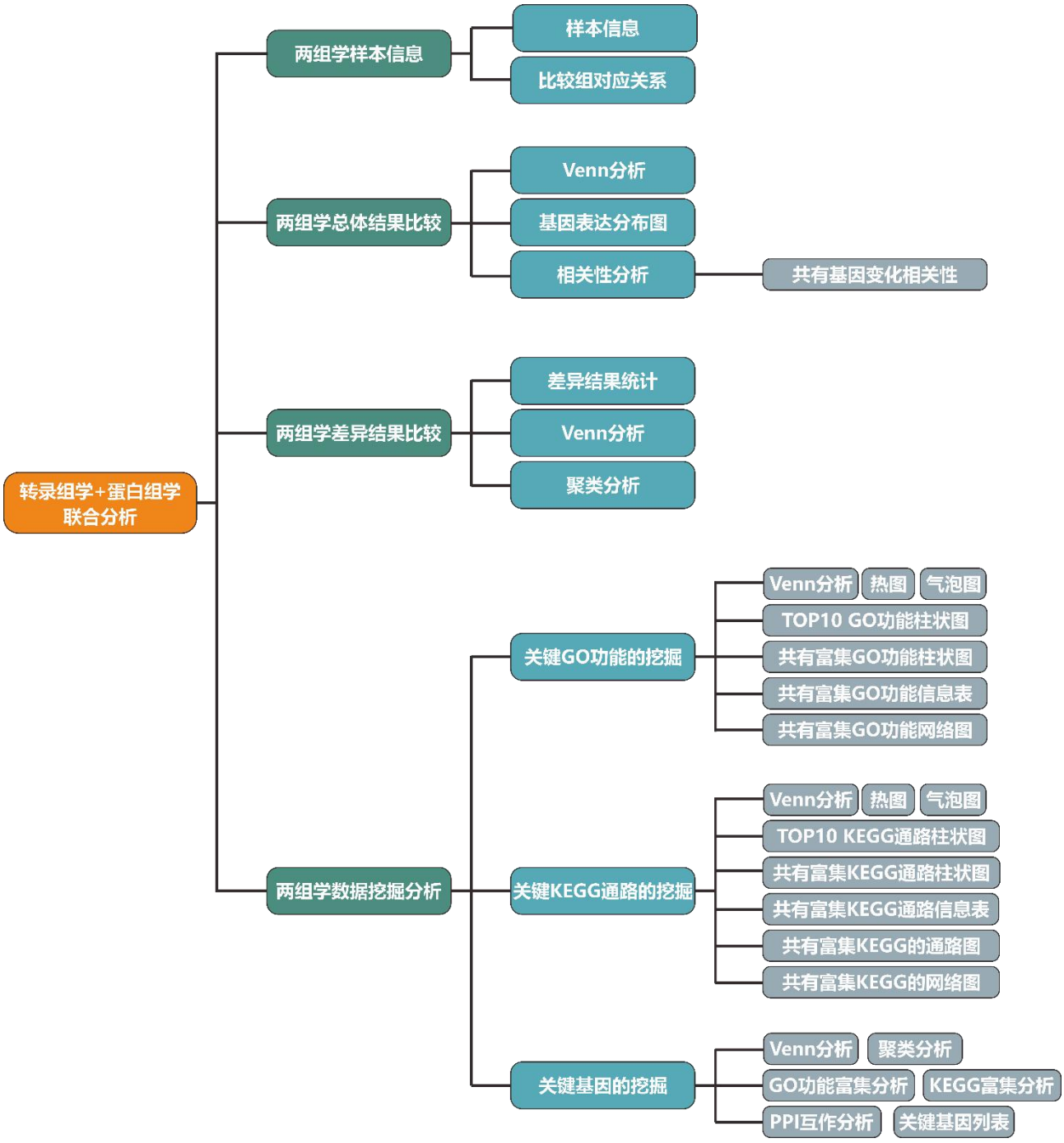

Figure 1 Joint analysis process and method

17. RNA Extraction

I. Objective of the experiment

The T24 cell lines and low, medium and high drug-resistant T24-RC48 cell lines iin two six-well plates were treated with TRIzol reagent to extract RNA, thus providing the material foundation for subsequent qPCR experiments.

II. Experimental content

2.1 Experimental design

Total RNA was extracted by lysing cells in TRIzol reagent firstly. After centrifugation using chloroform for phase separation, collect the supernatant and add isopropanol to precipitate RNA. Wash with 75% ethanol, dry the sample, and dissolve it in RNase-free water. Adherence to standard protocols ensures the yield of high-quality RNA for subsequent molecular biology studies. Improper handling, such as RNA degradation, may compromise experimental accuracy and reliability.

2.2 Sample types

T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

2.3 Measurement principle

The TRIzol method for RNA extraction operates through the synergistic action of phenol and guanidine thiocyanate in the reagent system. Guanidine thiocyanate acts as a strong denaturant to rapidly lyse cells, releasing RNA while inhibiting endogenous RNase activity to prevent degradation. Phenol facilitates protein denaturation and precipitation. After adding chloroform, centrifugation creates a three-phase system: an upper aqueous phase (containing RNA), a middle phase (denatured proteins), and a lower organic phase (containing DNA and lipids). Under acidic conditions, RNA selectively dissolves in the aqueous phase. Subsequent isopropanol precipitation separates RNA, followed by ethanol washing to remove salts, yielding high-purity RNA. This method achieves efficient RNA isolation and purification through chemical denaturation, phase separation, and selective precipitation.

III. Materials and reagents

3.1 Materials

Pipette tips and 15ml centrifuge tubes were purchased from Axygen

Micropipettes: Purchased from Eppendorf

Ice bucket

2ml EP tubes

Human bladder transitional cell carcinoma cell line (T24)

Low/medium/high resistance T24-RC48 cell lines

3.2 Reagents

PBS: Purchased from Gibco, USA

TRIzol reagent: purchased from Thermo Fisher Scientific

Isopropanol and 75% ethanol were purchased from Sensi Chemical

Chloroform

IV. Experimental instruments

Fume hood

Vortex mixer

Benchtop low-speed centrifuge at room temperature: Eppendorf

Spectrophotometer: purchased from Thermo Fisher Scientific

V. Experimental steps

5.1 The T24 cell line and the low, medium and high drug-resistant T24-RC48 cell lines were inoculated into two six-well plates, with two wells per cell line.

5.2 Take two six-well plates out of the 37℃ constant temperature box and place them in a fume hood and on ice.

5.3 Transfer 1ml PBS with a pipette into the eight holes of the experimental group, gently shake and clean, and repeat twice.

5.4 Add 500μl TRIzol reagent to each well to lyse the cells, and pipette repeatedly until the lysate is homogeneous and no visible cell clumps remain. Then suck the positive, low, medium and high drug-resistant cells into four 2ml EP tubes labeled as "positive", "low", "medium" and "high".

5.5 Add chloroform (tri-chloromethane) to each of the four EP tubes at 1/5 of the volume of TRIzol reagent for extraction. Vortex vigorously for 15 seconds to ensure complete emulsification.

5.6 After shaking, the solution was left at room temperature for 5min and then centrifuged at

12,000×g for 15 minutes at 4℃. At this time, the solution was obviously divided into three layers: the upper layer was water phase, the middle layer was protein and the lower layer was organic phase.

5.7 Carefully aspirate the upper aqueous phase, 200μl *2/ per tube (aspirate part, do not touch the middle protein layer) and place it in a new EP tube.

5.8 Add an equal volume of pre-cooled isopropanol to the EP tube, invert and mix, and leave at room temperature for 15min to precipitate.

5.9 12000g 4℃ Centrifuge for 10min, discard the supernatant, add 1ml of pre-cooled 75% ethanol to wash the precipitate twice.

5.10 12000g 4℃ Centrifuge for 5min, discard the supernatant, and dry the centrifuge tube at room temperature to allow the residual ethanol to volatilize.

5.11 The dried pellet was resuspended in 20μL of RNase-free water and left on ice.

5.12 RNA concentration and purity were quantified using a spectrophotometer.

18. Reverse Transcription

I. Objective of the experiment

The RNA of human bladder transitional cell carcinoma cell lines (T24) extracted in the early stage, namely the wild-type (control), low-, medium-, and high-drug-resistant T24-RC48 cell lines, were reverse transcribed to obtain cDNA, which laid the foundation for qPCR in the later stage.

II. Experimental content

2.1 Experimental design

In this experiment, the total RNA of human bladder transitional cell carcinoma cell line (T24) and its drug-resistant subtype (T24-RC48) were reverse transcribed into cDNA to prepare for subsequent qPCR experiments.

First, based on RNA quantification results, calculate the required volume of each reverse transcription reagent for every sample according to the manufacturer's instructions to prepare a 20 μL reaction system. Next, retrieve the RNA samples and reverse transcription reagents from the -80°C freezer and thaw them on ice. Distribute the reagents into corresponding RNA extraction tubes (EP tubes) by group and gently mix. Place the EP tubes in a metal bath: first incubate at 50°C for 15 minutes, then cool to 85°C for 5 seconds. Finally, after allowing the temperature to slightly decrease, seal the EP tubes with sealing film and store them in the -80°C freezer. The entire experiment strictly followed RNase-free protocols conducted under ice bath conditions to ensure RNA quality and obtain high-quality cDNA.

2.2 Sample types

T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

2.3 Measurement principle

Reverse transcriptase synthesizes complementary cDNA strands using RNA as a template and guided by primers. This critical step converts unstable RNA into stable double-stranded cDNA, providing a DNA template for subsequent qPCR amplification and quantification. As the essential pretreatment process in RT-qPCR analysis of RNA (e.g., gene expression studies), it ensures reliable data acquisition through precise DNA preparation.

III. Materials and reagents

3.1 Materials

Micropipettes: All purchased from Eppendorf

RNase-free pipette tips: Purchased from Axygen

EP tubes: Purchased from Axygen

Parafilm

Lens cleaning paper

3.2 Reagents

RNA previously extracted from drug-resistant cell lines

Reverse transcription reagent: purchased from Vazyme

IV. Experimental instruments

Constant temperature metal bath heater: purchased from Biosafer

Ultra-micro spectrophotometer: purchased from Thermo Fisher Scientific

-80 degree refrigerator

V. Experimental steps

This experiment strictly followed the RNase-free operation protocol, and was carried out under ice bath to prevent RNA degradation and nonspecific amplification. The experimental steps were as follows:

5.1 Based on the results of the last RNA extraction and the ratio shown in the instruction manual, calculate the amount of each reverse transcription reagent added.

#	样品名称	ng/μL	A260/A280
1	样品 1	719.7	1.91
2	样品 2	1302.8	1.93
3	样品 3	1015.1	1.91
4	样品 4	1115.4	1.92
5	样品 5	967.1	1.93

Figure 1 RNA quantification results

Table 1. The amount of each reagent in the reverse transcription system of each group

	RNA amount (ng/μl)	**Enzyme Mix (μl)**	**5×All-in-one qRT SuperMix (μl)**	**RNase-free ddH_2O (μl)**
wild type	1302.8	23.4504	117.252	333.756
Low levels of resistance	1015.1	18.2718	91.359	256.077
Intermediate drug resistance	1115.4	20.0772	100.386	283.158
High levels of resistance	967.1	17.4078	87.039	243.117

5.2 Sample pretreatment: Retrieve the cryopreserved RNA samples (four tubes total) from the -80°C freezer and thaw them gradually on ice. Meanwhile, the reverse transcription reagent is slowly thawed on ice.

5.3 Carefully aspirate the calculated volumes of reagents and add them to the respective RNA-containing tubes for the wild-type, low-, medium-, and high-resistance groups. Take care to avoid introducing air bubbles during pipetting.

5.4 After adding all components, mix the reaction gently by pipetting up and down, and briefly centrifuge to collect the contents at the bottom of the tube. Wipe any condensation from the outside of the tube. Place the tubes in a pre-heated metal bath set at 50°C for 15 minutes for the reverse transcription reaction, followed by incubation at 85°C for 5 seconds to inactivate the reverse transcriptase. This process converts the RNA template into complementary DNA (cDNA).

5.5 Once the tubes have cooled slightly, seal them with Parafilm and store at -80°C for subsequent qPCR experiments.

19. qRT-PCR

I. Objective of the experiment

This study aims to systematically analyze the expression profiles of drug resistance-related genes including PDL1, VEGF, RBPJ, ICAM1, DSP, SOD2, and CAT in human bladder transitional cell carcinoma (T24) and its low-, medium-, and high-drug-resistant sublines (T24-RC48) through integrated analysis of RNA extraction, reverse transcription, and real-time quantitative PCR. The research aimed to explore the potential regulatory roles of these genes in the development of drug-resistant phenotypes in bladder cancer, thereby providing experimental evidence for elucidating molecular mechanisms of drug resistance and informing targeted therapeutic strategies.

II. Experimental content

2.1 Experimental design

In this experiment, the mRNA expression differences of PDL1, VEGF and other genes in human bladder transitional cell carcinoma cell lines (T24) and its low/medium/high drug-resistant subtypes (T24-RC48) were systematically investigated to reveal the drug resistance mechanism. The experimental process was divided into three stages:

Total RNA extraction: First, extract total RNA by adding TRIzol lysis to the cells. Centrifuge with chloroform for phase separation, collect the supernatant, and add isopropanol for precipitation. Wash with 75% ethanol, dry, and dissolve in RNase-free water. Quantify RNA concentration and purity using a spectrophotometer (for RNA quantification). Properly standardized procedures yield high-quality RNA suitable for subsequent molecular biology studies. Improper handling (e.g., RNA degradation) may compromise experimental accuracy and reliability.

Reverse Transcription and cDNA Synthesis: Based on RNA quantification results, prepare 20μl reaction mixtures for each sample using calculated reverse transcription reagent ratios. Transfer RNA samples and reagents from the -80℃ freezer to ice. Add reagents to corresponding RNA extraction tubes in groups and gently mix. Place tubes in a metal bath: first incubate at 50℃ for 15 minutes, then cool at 85℃ for 5 seconds. After temperature stabilization, seal with capping film and freeze at -80℃. Note: This procedure strictly follows nucleases-free protocols under ice bath conditions to ensure RNA quality and obtain high-quality cDNA.

qPCR Quantitative Analysis: Finally, the cryopreserved cDNA samples of four drug-resistant cell lines (T24-RC48) at positive, low, medium, and high concentrations were retrieved from the -80°C freezer. After thawing at room temperature, they underwent instantaneous centrifugation and were placed on ice for later use. Subsequently, ROX Reference Dye, RNase-free ddH_2O, and specific gene primers (PDL1, VEGF, etc.) were thawed slowly on ice. The qPCR reaction mix was prepared according to the preset protocol (each group containing 15μl cDNA, 75μl ROX, 3μl each of forward/counterpart primers, and 54μl ddH_2O), then aliquoted into labeled EP tubes labeled "control, low, medium, high". Following the predefined 96-well plate layout (red, orange, yellow, green corresponding to the four concentration groups), the reaction mix was added to the appropriate wells ensuring no bubbles and without touching the well walls with the pipette tip. Finally, the 96-well plate was loaded into a real-time fluorescence quantitative PCR instrument, programmed with denaturation (95℃ 5 seconds) and annealing/extension (60℃ 30 seconds) cycles (40 cycles), and run in SYBR Green detection mode to collect fluorescence signals and export data. Note: This experimental step also requires strict adherence to the entire procedure and reagents. Preparation of the PCR reaction mixture must be conducted on ice while ensuring enzyme-free conditions.

2.2 Sample types

T24 wild-type cell line and low/medium/high drug resistant T24-RC48 cell line.

2.3 Measurement principle

Total RNA Extraction: The TRIzol method for RNA extraction operates through the synergistic action of phenol and guanidine thiocyanate. Guanidine thiocyanate acts as a strong denaturant to rapidly lyse cells, releasing RNA while inhibiting endogenous RNase activity to prevent degradation. Phenol facilitates protein denaturation and precipitation. After adding chloroform, centrifugation creates a three-phase system: an upper aqueous phase (containing RNA), a middle phase (denatured proteins), and a lower organic phase (containing DNA and lipids). Under acidic conditions, RNA selectively dissolves in the aqueous phase. Subsequent isopropanol precipitation and ethanol washing to remove salts yield high-purity RNA. This method achieves efficient RNA isolation and purification through chemical denaturation, phase separation, and selective precipitation.

Reverse Transcription for cDNA Synthesis: Using RNA as a template, reverse transcriptase synthesizes complementary cDNA strands through primer guidance. This critical pretreatment converts unstable RNA into stable double-stranded cDNA, providing essential DNA templates for subsequent qPCR amplification and quantitative analysis. It serves as the pivotal preparatory step in RT-qPCR for RNA studies such as gene expression analysis.

Quantitative PCR (qPCR), also known as real-time PCR, is a technique that monitors DNA amplification through fluorescence signal changes. The principle involves adding fluorescent dyes (e.g., SYBR Green) or specific probes (e.g., TaqMan probes) to the reaction system. Each DNA amplification cycle enhances the fluorescence signal. The instrument generates an amplification curve by continuously monitoring fluorescence levels. When the signal reaches a preset threshold, the corresponding cycle number (Ct value) inversely correlates with the initial template quantity. Using standard curves or relative quantification methods, the original DNA template concentration can be precisely calculated. This technique is widely applied in gene expression analysis, pathogen detection, and gene copy number determination.

III. Materials and reagents

3.1 Materials

RNase-free pipette tips, 15ml centrifuge tubes: purchased from Axygen

Micropipettes: all purchased from Eppendorf

EP tubes: Purchased from Axygen

96-well optical reaction plate, optical sealing film (Pantel): both purchased from domestic companies

RNAase Free EP tubes, octet tubes and pipette tips: purchased from AxyGen

Seal-off film

Lens cleaning paper

Ice box

2ml EP tubes

Human bladder transitional cell carcinoma cell line (T24)

Low/medium/high resistance T24-RC48 cell lines

3.2 Reagents

PBS: Purchased from Gibco, USA

TRIzol reagent: purchased from Thermo Fisher Scientific

Isopropanol and 75% ethanol were purchased from Sensi Chemical

Reverse transcription reagent: purchased from Vazyme

ROX Reference Dye

Primers for target genes and the reference gene (GAPDH)

RNase-free ddH_2O

Chloroform

IV. Experimental Instruments

Draught cupboard

Vibration instrument

-80 degree refrigerator

Benchtop normal temperature low speed centrifuge: purchased from Eppendorf

Spectrophotometer: purchased from Thermo Fisher Scientific

Constant temperature metal bath heater: purchased from Biosafer

Ultra-micro spectrophotometer: purchased from Thermo Fisher Scientific

Micropipettes, benchtop low temperature and low speed centrifuge: purchased from Eppendorf

Real-time quantitative PCR instrument: AB IQ Quant Studio 5 1.5ml

Centrifuge tubes: Purchased from Corning

Cubee centrifuge: Purchased from GeneReach

V. Experimental steps

This experiment strictly followed the RNase-free operation protocol, and was carried out under ice bath to prevent RNA degradation and nonspecific amplification. The experimental steps were as follows:

5.1 The T24 cell line and the low, medium and high drug-resistant T24-RC48 cell lines were inoculated into two six-well plates, with 2 wells per cell line.

5.2 Take two six-well plates out of the 37℃ constant temperature box and place them in a fume hood and on ice.

5.3 Transfer 1ml PBS with a pipette into the eight holes of the experimental group, gently swirl

the plate to wash the cells, and aspirate the PBS. Repeat this washing step twice.

5.4 Add 500μl TRIzol reagent to each well to lyse the cells. Blow until there is no obvious clumping, and then suck the positive, low, medium and high drug-resistant cells into four 2ml EP tubes labeled as "control", "low", "medium" and "high".

5.5 Add chloroform (tri-chloromethane) to each of the four EP tubes at 1/5 of the volume of TRIzol reagent for extraction. Vibration for 15s on the shaker to fully emulsify.

5.6 After shaking, the solution was left at room temperature for 5 minutes and centrifuged at 12000g for 15 minutes. At this time, the solution was obviously divided into three layers: the upper layer was water phase, the middle layer was protein and the lower layer was organic phase.

5.7 Carefully aspirate the upper aqueous phase, 200μl *2/ per tube (aspirate part, do not touch the middle protein layer) and place it in a new EP tube.

5.8 Add an equal volume of pre-cooled isopropanol to the EP tube, invert and mix, and leave at room temperature for 15min to precipitate.

5.9 12000g 4℃ Centrifuge for 10min, discard the supernatant, add 1ml of pre-cooled 75% ethanol to wash the precipitate twice.

5.10 12000g 4℃ Centrifuge for 5min, discard the supernatant, and dry the centrifuge tube at room temperature to allow the residual ethanol to volatilize.

5.11 The precipitate after drying is dissolved in 20μl RNase free water and left on ice.

5.12 Quantitative RNA Concentration and Purity Analysis (Figure 1). Samples 1-7 represent: 1: wild-type cells, 2: RNase-free water (blank), 3: low-resistant cells, 4: RNase-free water (blank), 5: medium-resistant cells, 6: RNase-free water (blank), 7: high-resistant cells.

#	样品名称	ng/μL	A260/A280	A260/A230	A260	A280
1	样品 1	1610.7	2.04	2.33	40.27	19.74
2	样品 2	1.1	-5.19	0.59	0.03	-0.01
3	样品 3	1520.4	2.04	2.16	38.01	18.62
4	样品 4	1.3	-8.43	0.79	0.03	0.00
5	样品 5	1844.8	2.06	2.31	46.12	22.41
6	样品 6	1.3	-11.54	0.84	0.03	0.00
7	样品 7	1722.9	2.08	2.10	43.07	20.74

Figure 1 RNA quantification results

5.13 According to the results of RNA extraction and the ratio shown in the reference manual, the

amount of each reverse transcription reagent added was calculated.

Table 1. The amount of each reagent in the reverse transcription system of each group

	RNA amount (ng/μl)	**Enzyme Mix (μl)**	**5×All-in-one qRT SuperMix (μl)**	**RNase-free ddH_2O (μl)**
Wild type (Control)	1610.7	28.9926	115.9704	416.889
Low Resistance	1520.4	27.3672	109.4688	392.508
Medium Resistance	1844.8	33.2064	132.8256	480.096
High Resistance	1722.9	31.0122	124.0488	447.183

5.14 Sample pretreatment: Take the cryopreserved RNA samples (4 tubes in total) from the -80℃ ultra-low temperature refrigerator and place them on ice for slow thawing. Meanwhile, the reverse transcription reagent is slowly thawed on ice.

5.15 According to the previously calculated amount of reverse transcription reagent, slowly aspirate the corresponding volume and add it to the RNA EP tubes with four concentrations: positive, low, medium and high respectively. Pay attention not to produce bubbles when aspirating and injecting.

5.16 After joining, mix appropriately and gently wipe away the small water droplets on the outside of the EP tube. Put it on the metal bath heater and set the temperature to 50℃ 15min. After completion, set the temperature to 85℃ 5sec immediately. Upon completion, the RNA is reverse transcribed into cDNA.

5.17 When the temperature is slightly reduced, a proper amount of sealing film is torn to seal the EP tube, and then it is frozen in the-80℃ refrigerator to provide the material basis for the next qPCR.

5.18 Take the EP tubes of four kinds of cDNA (positive, low, medium and high) frozen in the last time from the-80 degree refrigerator, tear off the sealing film, let it melt at room temperature for 5 ~ 10 minutes, invert the tubes up and down 5 ~ 10 times to mix thoroughly, then use centrifuge to snap the tubes to the bottom, and put them on ice for standby.

5.19 At the same time, ROX reagent, RNase-free ddH2O and positive and negative primers of specific genes (PDL1, VEGF, RBPJ, ICAM1, DSP, SOD2, CAT and GAPDH) were taken out from the -20 degree refrigerator and slowly thawed on ice.

5.20 Set up a group control with three small holes of the primer gene, and calculate the amount of each reagent in the system according to the qPCR system configuration instructions:

Table 2. The amount of each reagent added to the system

	cDNA (μl)	**ROX Reference Dye (μl)**	**Forward primer (μl)**	**Reverse primer (μl)**	**RNase-free ddH$_2$O (μl)**
Wild type (Control)	15	75	3	3	54
Low Resistance	15	75	3	3	54
Medium Resistance	15	75	3	3	54
High Resistance	15	75	3	3	54

5.21 Use a pipette with a range of 10 μl and 200 μl to draw the above amount and add it to four new EP tubes labeled "control", "low", "medium" and "high". Mix gently without producing bubbles during the process.

5.22 Edit the position relationship between the corresponding primer gene and the "control", "low", "medium" and "high" concentrations on the 96-well plate in Excel (red, orange, yellow and green represent control, low, medium and high concentrations respectively);

PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1
VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF
RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ
ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1	ICAM 1
DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP

IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B
CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT
GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH

Figure 2 Schematic diagram of 96-hole plate layout

5.23 Prepare 12-row eight-tube tubes and carefully add 40μl of qPCR system solution to each well according to the positional diagram. Note: Do not touch the well wall with the pipette tip during the addition process; change the pipette tip when switching samples.

5.24 After the sampling is completed, cover the lid and put the 12-row octuple tube into the high-speed mini centrifuge for 1000rpm centrifugation for 1 minute, and centrifuge the liquid to the bottom of the qPCR plate to ensure the accuracy of the experiment.

5.25 Remove the 96-well plate and its supporting tray, and put the 12-row eight-tube into the tray in order. Pay attention not to touch the middle and bottom of the 96-well plate with your hands, so as to avoid contamination affecting the detection accuracy.

5.26 Place the 96-well plate into the qPCR machine and set the reaction conditions as follows:

Table 3 Standard reaction conditions on the qPCR amplifier

Reaction step	**Temperature**	**Time**	**Recurring number**	**Remarks**
Pre-transformation	95℃	30 seconds	1	Activate hot start DNA polymerase and eliminate cold start effect
Chains of amplification			40	Fluorescence signal acquisition point: at the end of each annealing/elongation cycle
denaturation	95℃	5 seconds		Double strand DNA unwinding
-Annealing/Extending	60℃	30 seconds		Primer binding and chain extension (annealing temperature adjusted according to primer Tm value)
Melting curve analysis	65℃→95℃	Continuous heating	1	Each temperature increase of 0.5℃ for 5 seconds, and the fluorescence signal was monitored (used to verify product specificity)

5.27 Start the device, select SYBRGreen program, mark the sample and primer, and wait for results.

5.28 Take out the 96-well plate in the instrument and copy the data.

5.29 Analyze and process the data to calculate ΔCT, ΔΔCT and $2^{-\Delta\Delta CT}$ values.

20. Whole Genome Data Analysis

I. Objective of the experiment

Through whole genome sequencing (WGS) technology, we systematically screened genomic variations (including single nucleotide variants, insertions/deletions, copy number variations, and structural variations) in human bladder transitional cell carcinoma T-24 cells and their low-, medium-, and high-grade RC48-resistant sublines (T24-RC48). This study identified potential driver genes and molecular mechanisms associated with RC48-induced drug resistance, providing genomic evidence for subsequent functional validation and drug resistance pathway research.

II. Experimental procedures

2.1 Sample preparation

Cell lines: Parental cell T24 cell line and low, medium and high drug resistant T24-RC48 cell line (3 biological repeats per group).

DNA extraction: Use a high-purity genomic DNA extraction kit (e.g., QIAGEN DNeasy Kit), and detect the DNA quality by agarose gel electrophoresis and Nanodrop (OD260/280=1.8-2.0, concentration ≥50 ng/μL).

2.2 Library construction and sequencing

Library preparation: Illumina TruSeq DNA PCR-Free Library Prep Kit was used to construct the whole genome library, which was fragmented to 350 bp, and then end repair, linker connection and purification were performed.

Sequencing platform: Illumina NovaSeq 6000 platform, dual-end sequencing (PE150), target sequencing depth ≥30×.

2.3 Data Analysis

- Quality control of raw data: FastQC was used to evaluate the quality of sequencing data, and Trimmomatic was used to filter out low-quality reads (Q score < 20, length < 50 bp).
- Genome alignment: Clean reads were aligned to the human reference genome (GRCh38/hg38) using BWA-MEM.
- Mutation testing:

SNV/InDel: GATK HaplotypeCaller is used to detect variations, and ANNOVAR was used for functional annotation of the identified variants.

Copy number variation (CNV): CNVkit analyzes copy number variations.

Structural variation (SV): Manta and Delly detect chromosomal structural variation.

Drug resistance-related gene screening: The mutation frequencies in resistant cell lines were compared with the parental line to screen for candidate genes that were significantly enriched ($p < 0.05$) and associated with the drug-resistant phenotype.

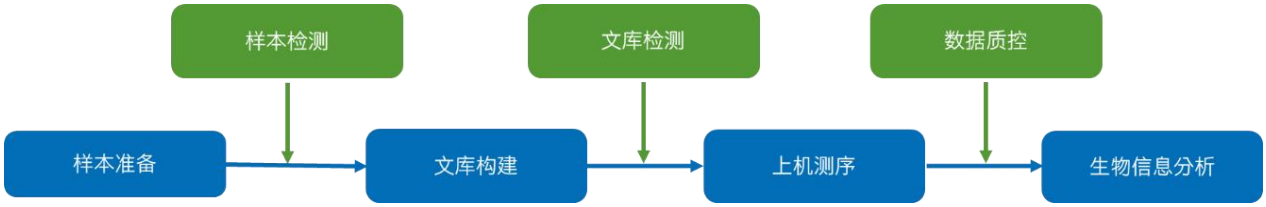

Figure 1 Project flow chart

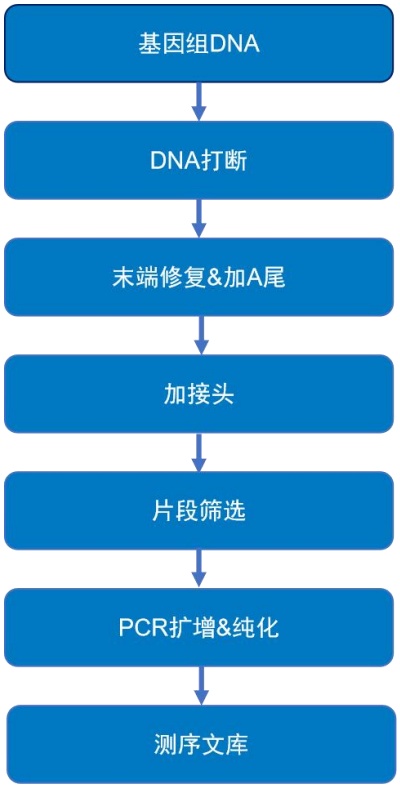

Figure 2 Library building process

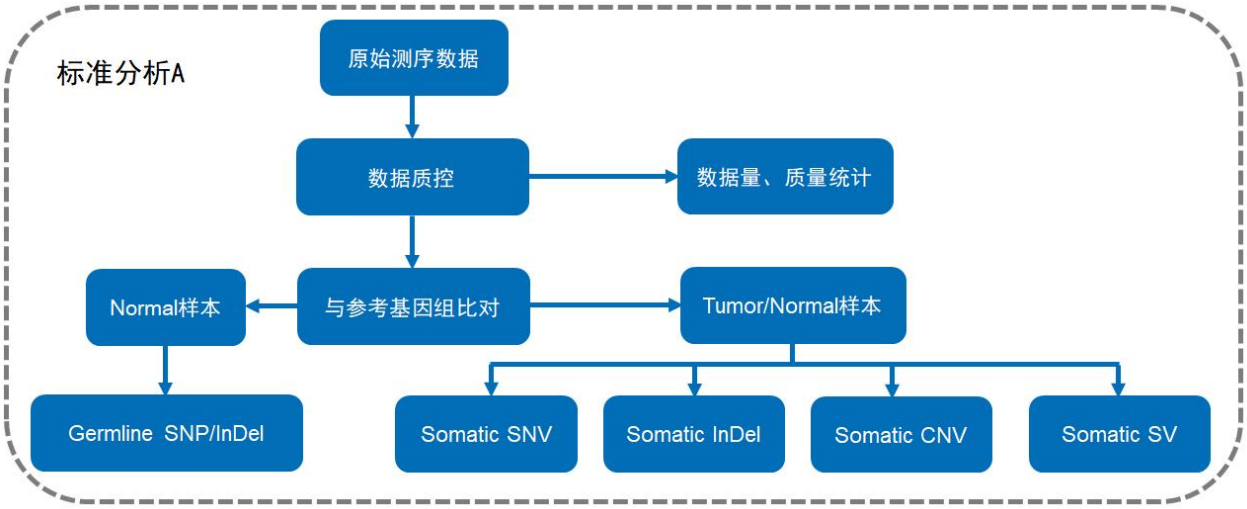

Figure 3 Information analysis flow chart

21. Western Blot

I. Objective of the experiment

Through WB experiments, we identified relevant proteins extracted from the drug-resistant RC48 cell line T24-RC48 and the wild-type T24 cell line. Using the previously incubated primary antibodies combined with corresponding secondary antibodies for incubation and development, we determined the expression changes of drug-resistant proteins RBPJ, PD-1, and VEGF2.

II. Materials and reagents

2.1 Materials

Micropipettes: Purchased from Eppendorf

EP tubes: Purchased from Axygen

RNase-free pipette tips: Purchased from AxyGen

Seal-off film

Ice box

Cell scraper

Human bladder transitional cell carcinoma cell line (T24)

Highly drug-resistant T24-RC48 cell line

Proteins extracted from the highly RC48-resistant T24-RC48 cell line and the wild-type T24 cell line

Gel preparation kit was purchased from Yase Company

Antibody 1 (Tau antibody, p-Tau antibody): Purchased from Proteintech

The primary antibodies (BAD antibody, phospho-GSK3 beta antibody, GSK3 beta antibody, phospho-BAD antibody) and the endogenous reference (GAPDH antibody) were purchased from Affinity Biosciences LTD.

The membrane with incubated primary antibody from the previous step

2.2 Reagents

PBS: Purchased from Gibco, USA

Cell lysate: Purchased from Thermo Fisher Scientific

Proteinase inhibitor mixture and phosphatase inhibitor mixture: purchased from Yase Company

Ethanol (absolute), deionized water

Transfer buffer, stripping solution, primary antibody dilution solution, secondary antibody dilution buffer, electrophoresis buffer, blocking buffer, and TBST were all purchased from Yase Company

Developing A and Developing B solutions: Purchased from Millipore

III. Experimental instruments and equipment

Antibody incubation box, shaker, Chemiluminescence imager

Draught cupboard

-80 degree refrigerator

Benchtop low temperature high speed centrifuge: Purchased from Eppendorf

BioRAD gel kit, electrophoresis kit, transfer kit, centrifuge

IV. Experimental steps

4.1 Protein Extraction

4.1.1 Observe the cell morphology, aspirate the culture medium, and wash twice with PBS, paying attention to avoid washing away the cells.

Figure 1 Aspiration of culture medium

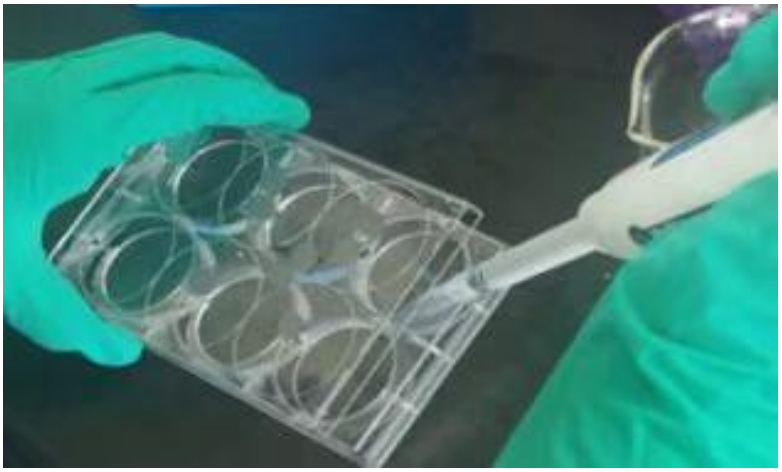

Figure 2 Washing cells with PBS

4.1.2 Lysis mixture preparation: According to the instructions of phosphatase inhibitor mixture and protease inhibitor mixture, the two were mixed with cell lysate in a ratio of 1:100 (1.5ml cell lysate, 15ul phosphatase inhibitor mixture, 15ul protease inhibitor mixture).

4.1.3 Lysis of cells: To four wells of the six-well plate, add 100ul mixture of lysis solution to each hole, gently shake and mix, and place on ice for 5min.

4.1.4 Collection of cells: With a cell scraper, the cells treated with the lysis mixture were scraped off, and the four holes of the mixture were respectively loaded into the corresponding four EP tubes and marked.

Figure 3 Cells are scraped by a cell scraper

4.1.5 Centrifugation: Put the above 4 EP tubes into the centrifuge for centrifugation, at 14,000×g,

4℃ for 10 minutes.

4.1.6 Protein collection: The supernatant from the four EP tubes was transferred to four new centrifuge tubes, marked and stored in -80℃ refrigerator.

4.2 Gel preparation

4.2.1 Clean the glass plate, clamp it and check for leakage.

4.2.2 Preparation of lower layer gel (separation gel) 5ml: 2.5ml of lower layer gel solution and 2.5ml of lower layer gel buffer solution. Add 60ul of catalyst. Add the lower layer gel, press it flat with deionized water, and wait for the lower layer gel to solidify.

4.2.3 Preparation of upper layer gel (concentrated gel) 1.5ml: 0.75ml of lower layer gel solution and 0.75ml of lower layer gel buffer. Add 20ul of catalyst. Pour out deionized water, add upper layer gel, insert the gel comb, and wait for upper layer gel to solidify.

4.2.4 After the gel is solidified, clamp it with a clamp and put it into the electrophoresis tank. Check for leakage with electrophoresis liquid. If there is no leakage, continue to pour electrophoresis liquid until the tooth comb part is covered.

4.3 Protein quantification

The protein samples previously extracted were taken out, thawed and tested on the machine. The results are shown in the figure below.

Figure 4 Protein quantification results

4.3.1 Preparation of samples: According to the standard configuration of 15ug protein sample on each protein sample, the volume of protein sample: buffer=1:4, the sample was loaded. Each protein sample corresponded to one tube, and the specific data were shown in Table 4-1. In addition, two tubes were flattened. After configuration, the sample was centrifuged.

Table 1 Calculation of Western Blot Loading Volumes

Number	Protein sample concentration (μg/μl)	Volume of protein sample (μL)	Buffer volume (μl)	Total sample volume (μl)
Comparison 1	3.272	4.28	1.07	5.35
Comparison 2	3.066	4.89	1.22	6.11
Drug resistance 1	4.008	3.74	0.94	4.68
Drug resistance 2	3.631	4.13	1.03	5.16

4.3.2 Sample loading and start electrophoresis:

① Remove the comb and load the samples in the order of marker, sample 1-4 from left to right. At the same time, in order to avoid edge effect, equal amount of sample buffer can be added to the unsampled hole.

② Start electrophoresis and set the parameters: constant V, 200V,45min.

4.4 Protein Transfer

① Prepare two black filters, two filter papers and a piece of plywood. Put them all into a white porcelain plate and soak them with transfer buffer.

② Take a PVDF film of appropriate size and immerse it in the transfer solution of a white porcelain plate.

③ Remove the electrophoresis completed gel and clamp it in a sandwich structure: black side of the plate + black filter screen + filter paper + gel + PVDF film + filter paper + black filter screen + transparent side of the plate.

④ Place the sandwich into the transfer membrane tank, clamp the black side of the plate to the black side of the tank and the transparent side to the red side of the tank, pour in the transfer buffer, and perform the transfer with the entire apparatus placed in an ice bath.

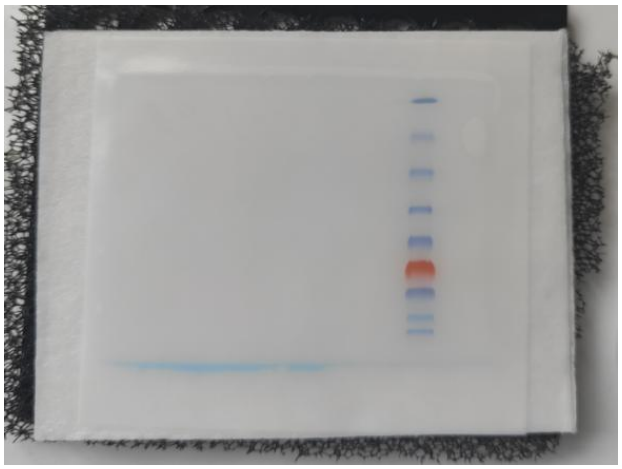

Figure 5 Transmembrane

4.5 Blocking

① With TBST as the solvent, prepare a 5% skimmed milk powder solution.

② Soak the membrane in a 5% solution of skim milk powder and shake it at 40r/min for 60min.

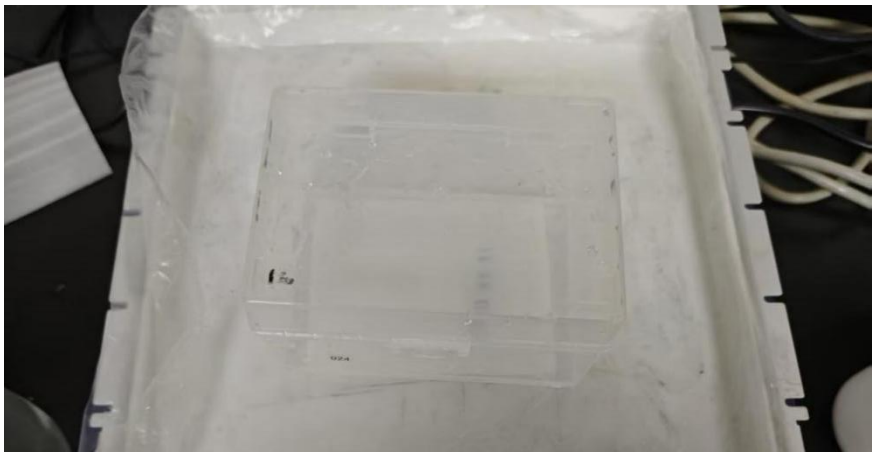

Figure 6 Closure

4.6 Primary antibody incubation

① Wash the closed membrane with TBST for 3 times, each time for 3min, and shake the bed at 60r/min.

② Add the corresponding primary antibody. For targets with substantially different molecular weights, the membrane can be cut prior to incubation; for targets with similar molecular weights, the membrane may be stripped with stripping buffer and re-probed with another antibody.

③ Overnight incubation in a 4℃ refrigerator.

Table 2 Target Proteins, Expected Band Sizes, and Corresponding Secondary Antibodies

Number	Protein	Corresponding molecular weight of developing band (estimated range)	Corresponding to the type of secondary antibody
1	phospho-GSK3 beta	46KD	anti-rabbit
2	phospho-BAD	24KD	anti-rabbit

4.7 Secondary antibody incubation

① Collect the primary antibody solution for potential reuse and wash with TBST for 3 times, shaking the bed at 60rpm/min for 3 minutes each time.

② Add the corresponding secondary antibody as shown in the following table. The corresponding secondary antibody for each protein is shown in Table 3.

③ Incubate the membrane on a shaker, 40rpm/min, 60min.

Table 3 Target Proteins, Expected Band Sizes, and Corresponding Secondary Antibodies

Number	Protein	Corresponding molecular weight of developing band (estimated range)	Corresponding to the type of secondary antibody
1	phospho-GSK3 beta	48KD	anti-rabbit
2	phospho-BAD	24KD	anti-rabbit

22. Angiogenesis Assay

I. Objective of the experiment

To evaluate the impact of VEGFA changes in drug-resistant cell lines on the microenvironment by performing a tube formation assay using Human Umbilical Vein Endothelial Cells (HUVEC) cultured with conditioned media from untreated T24 wild-type cells, low/medium/high drug-resistant T24-RC48 cells, and corresponding drug-treated T24 wild-type and T24-RC48 cells.

II. Experimental content

2.1 Experimental design

Conditioned media from untreated T24 wild-type cells, low/medium/high drug-resistant T24-RC48 cells, and corresponding drug-treated T24 wild-type and T24-RC48 cells were used to culture HUVECs for the tube formation assay. By observing and recording the growth status and interactions of the various cell lines in the co-culture system for both untreated and drug-treated groups, we analyzed whether the drug inhibited angiogenesis in the drug-resistant T24-RC48 cells in the treated group, and the differential impact of changes in VEGFs expression in the drug-resistant T24-RC48 cells compared to the ordinary T24 cells on the surrounding microenvironment between the two groups. This investigation aimed to determine whether VEGF changes in drug-resistant cells significantly affect angiogenesis-related processes and the composition and function of the entire cellular microenvironment, thereby providing insights into the mechanisms of microenvironment regulation by drug-resistant cells.

2.2 Sample types

T24 wild-type cell lines

Highly drug-resistant T24-RC48 cell line.

2.3 Experimental principle

Under the stimulation of angiogenic factors, endothelial cells can self-assemble into tubular networks. When tumor cells (T24 and drug-resistant strain T24-RC48) are co-cultured with human vascular endothelial cells (HUVEC), VEGFAs (vascular endothelial growth factor A) secreted by the tumor cells exert paracrine effects on adjacent HUVEC cells. As a key angiogenic signal, VEGFA activates endothelial cell proliferation, migration, and lumen-forming capabilities, driving their connection to form three-dimensional tubular structures. By comparing the differences in tube formation induced by drug-resistant strains versus wild-type strains, this experiment directly reflects

the regulatory efficacy of VEGFA expression changes in the angiogenic microenvironment, revealing how drug resistance reshapes the tumor microenvironment through altered secretion of pro-angiogenic factors.

III. Materials and reagents

3.1 Materials

The 1000μl and 100μl pipette tips and 15ml centrifuge tubes were purchased from Axygen

24-well plates: Corning

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

Human Umbilical Vein Endothelial Cells (HUVECs), Passage 3

Low/medium/high drug-resistant T24-RC48 cell lines

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Matrigel (Corning Matrigel Matrix, Growth Factor Reduced, GFR)

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Benchtop room temperature low speed centrifuge (Eppendorf)

Ultra-clean workbench (Suzhou purification equipment factory)

37℃,5% CO_2 cell culture chamber (Thermo Fisher Scientific)

Cell counter

V. Experimental steps

5.1 One day in advance, transfer Matrigel from -20°C to a 4°C refrigerator for overnight slow thawing. All subsequent operations are performed in the ultra-clean workbench using pre-cooled pipette tips and centrifuge tubes.

5.2 Twenty-four hours in advance, take two flasks each of T24 wild-type cells and low/medium/high drug-resistant T24-RC48 cells at 70% confluence. For each cell type, replace the

medium in one flask with serum-free medium, and in the other flask with serum-free medium containing 300μg/ml Ivonescimab. After 24 hours of culture, collect the supernatants to obtain a total of 8 conditioned media samples.

5.3 Twelve hours in advance, starve HUVEC cells for 12 hours using DMEM medium with 1% FBS.

5.4 Mix 4.8ml Matrigel with 2.4ml DMEM medium at a 2:1 ratio. Using pre-cooled pipette tips, slowly add 300μl of the mixture to each of the 24 wells along the wall, avoiding bubbles. Transfer the 24-well plate to a 37°C incubator and incubate for 30 minutes to allow the Matrigel to polymerize and solidify completely.

5.5 Take HUVECs in the logarithmic growth phase, discard the old medium, and wash once with PBS. Add trypsin to digest the cells. Once the cells become rounded, add complete medium to stop digestion. Gently pipette to form a single-cell suspension and distribute equally into 8 new centrifuge tubes. Centrifuge at 1000 rpm for 5 minutes and discard the supernatant.

5.6 Resuspend the cell pellets from the 8 tubes in the 8 different conditioned media respectively, pipetting to mix thoroughly. Count the cells and adjust the density to 3×10^5 cells/ml.

5.7 After 30 minutes, the Matrigel should be fully polymerized and solidified. According to the planned layout (see Table 1 below), add 0.5 ml of cell suspension to each well of the 24-well plate sequentially. Each conditioned media group has 3 replicate wells.

Table 1 Layout of the 24-well Plate

WT Treated	WT Treated	WT Treated	WT Untreated	WT Untreated	WT Untreated
LowR Treated	LowR Treated	LowR Treated	LowR Untreated	LowR Untreated	LowR Untreated
MedR Treated	MedR Treated	MedR Treated	MedR Untreated	MedR Untreated	MedR Untreated
HighR Treated	HighR Treated	HighR Treated	HighR Untreated	HighR Untreated	HighR Untreated

*WT: Wild Type T24; LowR: Low-resistant T24-RC48; MedR: Medium-resistant T24-RC48; HighR:

High-resistant T24-RC48

5.8 Observe tube formation under an inverted phase-contrast microscope at 2h, 4h, and 12h after plating. Analyze the tube formation images using ImageJ software.