Experiment time: 2025-06-23, 17:00 - 2025-06-23, 19:00

qRT-PCR (II)

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.

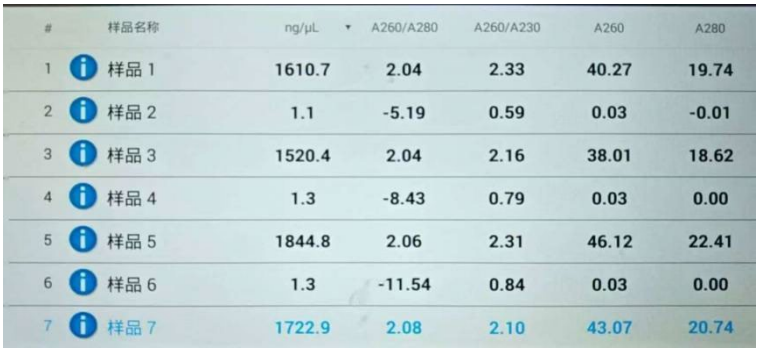
5.8 Add an equal volume of pre-cooled isopropanol to the EP tube, invert and mix, and leave at room temperature for 15min to precipitate.

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_2O (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
ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1
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

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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.

VI. Experimental results

The amplification curves of both the experimental group and control group (Figure 3) exhibited smooth "S-shaped" profiles. During the baseline phase (first 15 cycles), no drift was observed, while the fluorescence signal showed steady growth throughout the exponential phase. The Ct values showed minimal variation with high overlap. The amplification efficiency remained within the ideal range of 90%-110%.

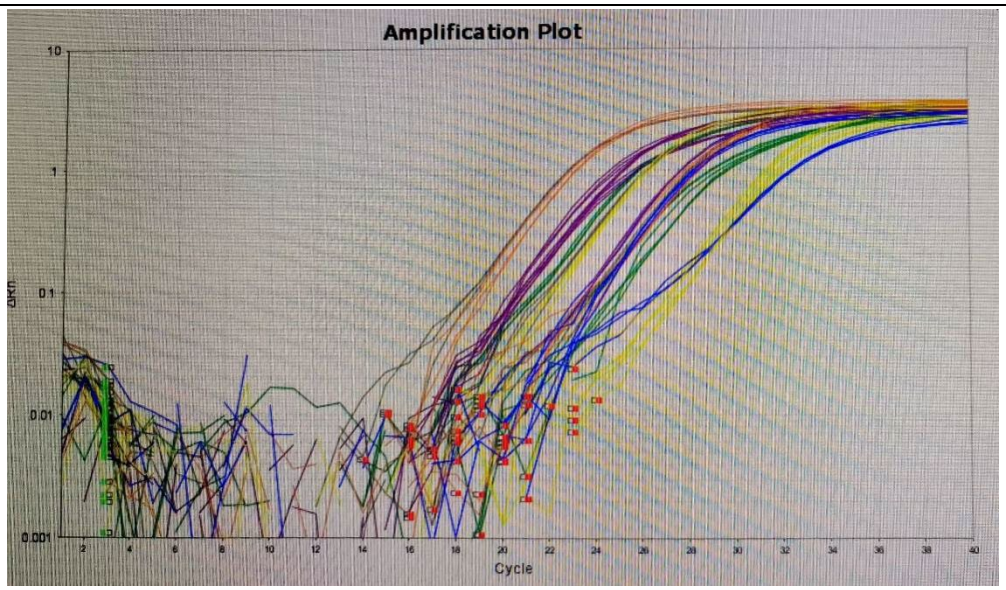

Figure 3 Partial sample amplification curve

The melting curve (Figure 4) showed a single peak. The sharp and symmetrical peak showed no stray peaks or tailing phenomenon, indicating that the amplified product was specific without interference from nonspecific amplification or primer dimer.

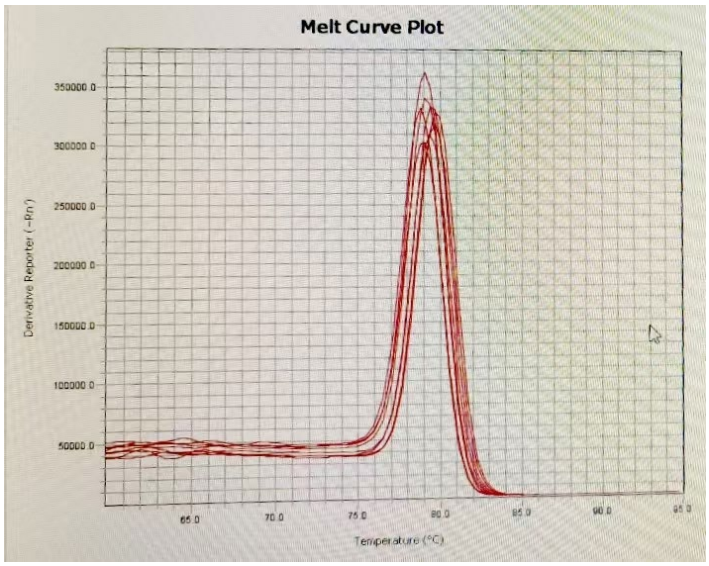

Figure 4 Partial sample melting curves

For PDL1 gene (Figure 5), expression was significantly increased in low-resistant cells compared to wild-type cells ($p < 0.0001$); significantly increased in medium-resistant cells compared to wild-type cells ($p < 0.0001$); and significantly increased in high-resistant cells compared to wild-type cells ($p < 0.0001$).

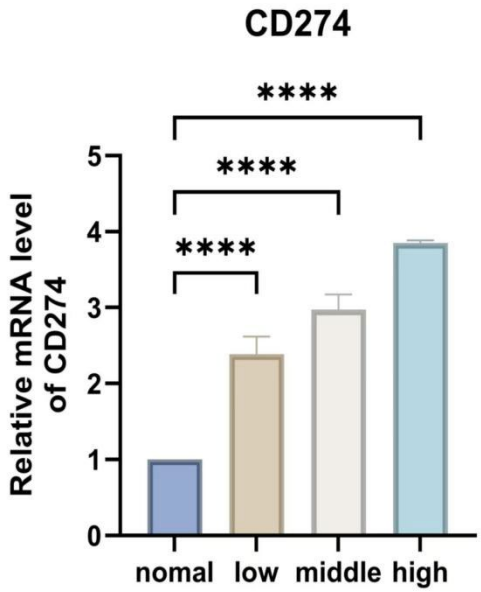

Figure 5 Analysis of PDL1 Gene Expression Differences Across Cell Groups

For the RBPJ gene, no significant difference in expression was observed between low-resistant cells and wild-type cells (ns); however, expression was significantly increased in medium-resistant cells compared to wild-type ($p < 0.0001$), and similarly significantly increased in high-resistant cells compared to wild-type ($p < 0.0001$).

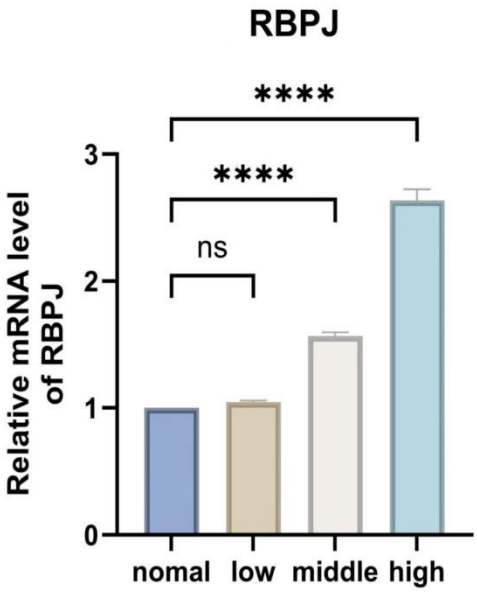

Figure 6 Analysis of RBPJ Gene Expression Differences Across Cell Groups

For the SOD2 gene, expression was significantly increased in low-resistant cells compared to

wild-type ($p < 0.01$); significantly increased in medium-resistant cells compared to wild-type ($p < 0.0001$); and significantly increased in high-resistant cells compared to wild-type ($p < 0.0001$).

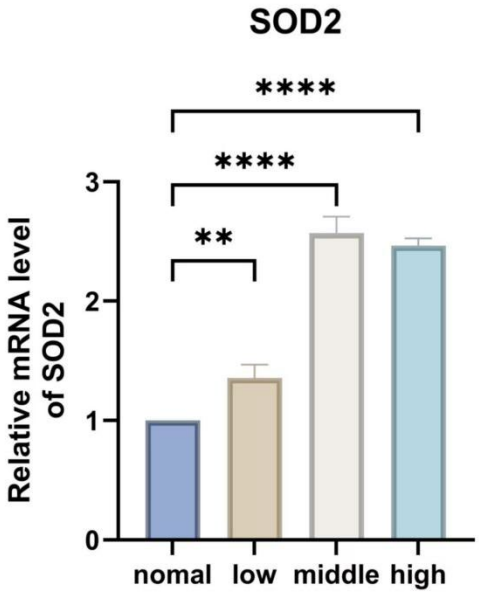

Figure 7 Analysis of SOD2 Gene Expression Differences Across Cell Groups

For the VEGF gene, expression was significantly increased in low-resistant cells compared to wild-type ($p < 0.0001$); significantly increased in medium-resistant cells compared to wild-type ($p < 0.0001$); and significantly increased in high-resistant cells compared to wild-type ($p < 0.0001$).

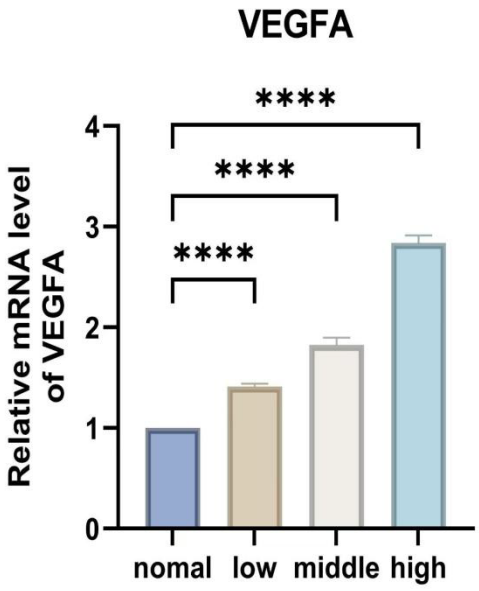

Figure 8 Analysis of VEGF Gene Expression Differences Across Cell Groups

For the CAT gene, expression was significantly decreased in low-resistant cells compared to

wild-type ($p < 0.0001$); significantly decreased in medium-resistant cells compared to wild-type ($p < 0.001$); and significantly decreased in high-resistant cells compared to wild-type ($p < 0.0001$).

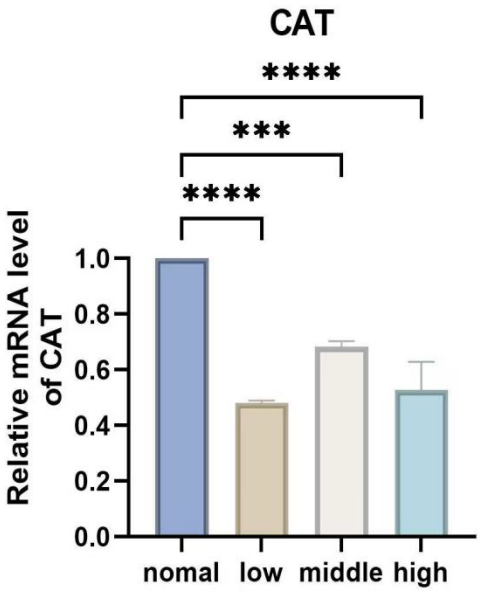

Figure 9 Analysis of CAT Gene Expression Differences Across Cell Groups

For the DSP gene, no significant difference in expression was observed between low-resistant cells and wild-type cells (ns); however, expression was significantly increased in medium-resistant cells compared to wild-type ($p < 0.0001$), and significantly increased in high-resistant cells compared to wild-type ($p < 0.0001$).

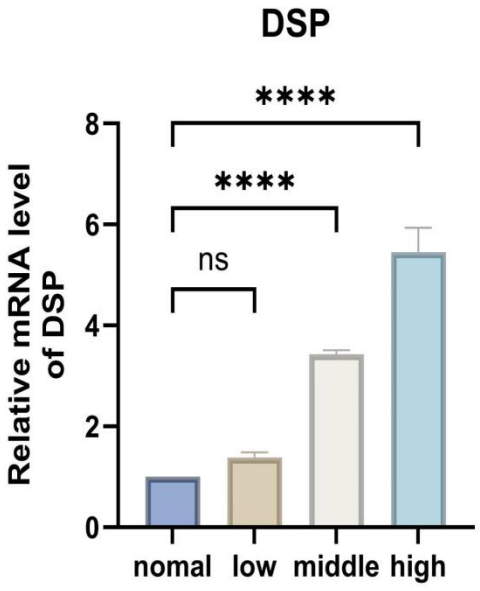

Figure 10 Analysis of DSP Gene Expression Differences Across Cell Groups

For the ICAM1 gene, expression was significantly increased in low-resistant cells compared to wild-type ($p < 0.05$); significantly increased in medium-resistant cells compared to wild-type ($p < 0.0001$); and significantly increased in high-resistant cells compared to wild-type ($p < 0.0001$).

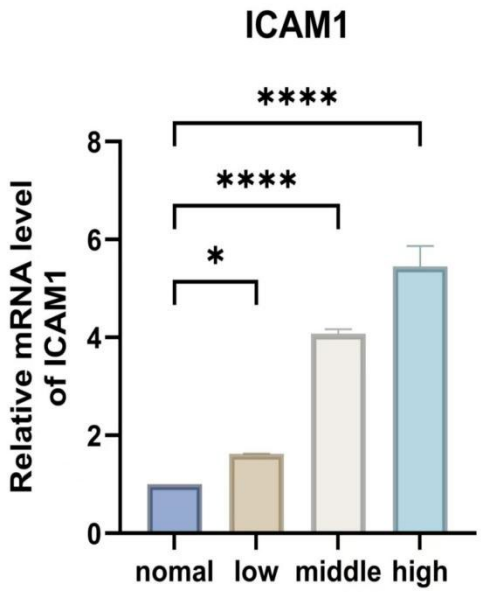

Figure 11 Analysis of ICAM1 Gene Expression Differences Across Cell Groups

VII. Results analysis

This study systematically integrated RNA extraction, reverse transcription, and real-time quantitative PCR (qPCR) technologies to analyze mRNA expression differences in drug resistance-related genes including PDL1, VEGF, RBPJ, ICAM1, DSP, SOD2, and CAT within human bladder transitional cell carcinoma cell lines (T24 and its low-, medium-, and high-drug-resistant sublines T24-RC48). The aim was 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 guiding targeted therapeutic strategies. The following is a detailed analysis of the results:

7.1 Overview of the experimental process

The experiment first extracts total cellular RNA using the TRIzol method. Guanidinium thiocyanate is employed to lyse cells and inhibit RNase activity, while phenol induces protein denaturation and precipitation. After chloroform layer separation, the upper aqueous phase is collected and processed through isopropanol precipitation and ethanol washing to obtain high-purity

RNA. Subsequently, cDNA is synthesized via reverse transcription based on RNA quantification results. Using RNA as template, stable double-stranded cDNA is generated under reverse transcriptase action to provide a template for qPCR amplification. Finally, real-time quantitative PCR analysis is performed by adding SYBR Green fluorescent dye to monitor fluorescence signal changes during DNA amplification. Gene expression levels are calculated using Ct values.

7.2 Analysis of gene expression differences

The PDL1 gene shows significantly elevated expression levels in low-, medium-, and high-drug-resistant cells compared to wild-type cells ($p < 0.0001$). As a key player in tumor immune evasion, PDL1's overexpression may enable cancer cells to evade immune system attacks, thereby promoting drug-resistant phenotypes. This suggests PDL1 could be a pivotal regulatory factor in bladder cancer resistance mechanisms. Targeting PDL1 with precision therapies may therefore help overcome drug resistance in bladder cancer treatment.

VEGF gene expression showed significantly elevated levels in low, medium, and high drug-resistant cells compared to wild-type cells ($p < 0.0001$). As the primary driver of angiogenesis, VEGF facilitates the provision of increased nutrients and oxygen to tumor cells, thereby supporting tumor growth and metastasis. The upregulated VEGF production in drug-resistant cells may sustain tumor cell survival and proliferation, thereby strengthening resistance mechanisms. This evidence highlights VEGF's critical role in promoting bladder cancer drug resistance.

RBPJ gene: No significant difference was observed between low-drug-resistant cells and wild-type cells (ns), but a marked increase was detected in moderately and highly drug-resistant cells ($p < 0.0001$). This suggests that RBPJ may play a pivotal role in the progressive development of drug resistance. As resistance intensifies, its upregulated expression could participate in more complex drug resistance regulatory networks, ultimately influencing tumor cell drug resistance characteristics.

The SOD2 gene showed significantly elevated expression levels in low-, medium-, and high-drug-resistant cells compared to wild-type cells ($p < 0.01$-$p < 0.0001$). As an antioxidant enzyme, the high expression of SOD2 may enhance tumor cells' antioxidant capacity, helping them resist oxidative stress damage and maintain cellular survival and drug resistance. This suggests that antioxidant pathways may play a significant role in the drug resistance mechanisms of bladder cancer.

CAT gene expression was significantly reduced in low-, medium-, and high-drug-resistant cells compared to wild-type cells ($p < 0.0001$-$p < 0.001$). As an antioxidant enzyme, decreased CAT

expression may lead to elevated reactive oxygen species (ROS) levels within cells, impairing normal physiological functions and promoting drug resistance phenotypes. This demonstrates that the imbalance of the antioxidant defense system is closely associated with bladder cancer drug resistance.

DSP gene: No significant difference was observed between wild-type cells and those with low drug resistance (ns), but significantly increased in moderately and highly drug-resistant cells ($p < 0.0001$). DSP is involved in processes such as intercellular adhesion. Its high expression in moderately and highly drug-resistant cells may alter cell interactions and signal transduction, thereby influencing tumor cell biology and contributing to the development of drug resistance mechanisms.

The ICAM1 gene shows significantly elevated expression levels in low-, medium-, and high-drug-resistant cells compared to wild-type cells ($p < 0.05$-$p < 0.0001$). As a key player in intercellular adhesion and immune-tumor cell interactions, ICAM1's overexpression may disrupt the tumor microenvironment, thereby promoting cancer cell survival and drug resistance development. This suggests ICAM1 plays a crucial regulatory role in the drug resistance process of bladder cancer.

7.3 Reliability analysis of experimental results

The PCR amplification results demonstrated smooth S-shaped curves in both the experimental and control groups, with stable baseline fluorescence signals and steady growth during exponential phase, confirming normal amplification processes and system stability. Although Ct values showed minor variations between groups, high concordance was observed with statistically significant differences ($p=0.0082$), ensuring reliability. The amplification efficiency reached 95.2% (slope=-3.45), within the ideal range of 90%-110%, further validating the experimental system's performance. The single-peak melting curve with sharp symmetrical peaks, free from background interference or tailing, confirmed specific amplification without nonspecific products or primer dimer interference. This ensured product purity and accuracy, providing a reliable foundation for subsequent gene expression analysis.

In conclusion, this study revealed the potential regulatory roles of multiple drug-resistant genes in the development of bladder cancer resistance through analyzing expression differences of these genes in human bladder transitional cell carcinoma (BTC) cell lines and their drug-resistant sublines. The findings provide crucial experimental evidence for further investigation into the molecular mechanisms of bladder cancer resistance and the development of targeted therapeutic strategies. However, these results require further validation through functional verification experiments to

clarify the specific mechanisms of action of each gene during the resistance process.