Experiment time: 2025-07-03, 18:00 - 2025-09-14, 21:30

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

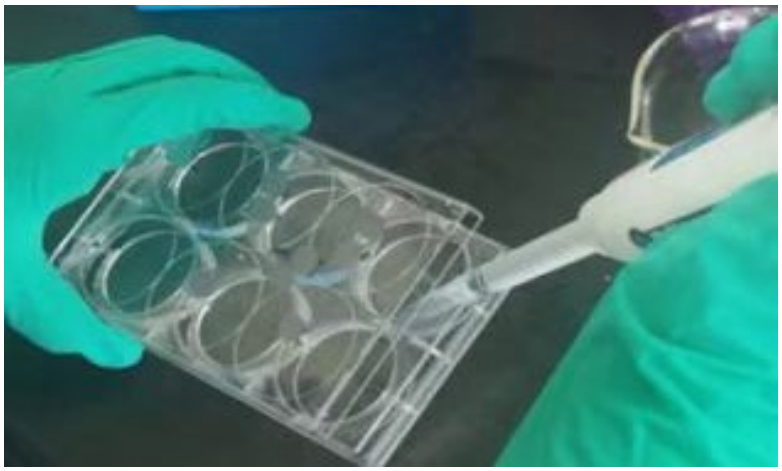


Figure 2 Washing cells with PBS

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.

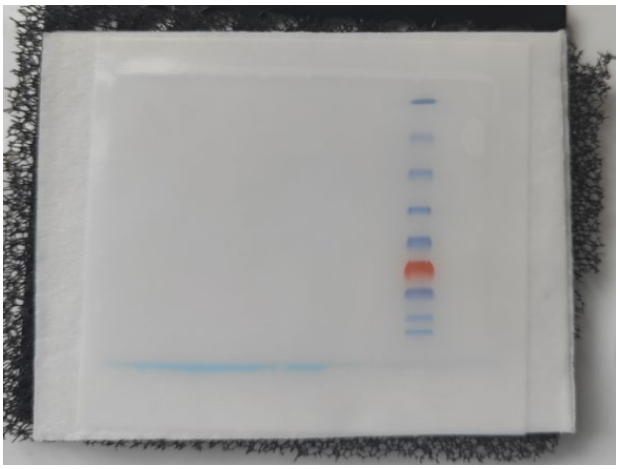


Figure 5 Transmembrane

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.

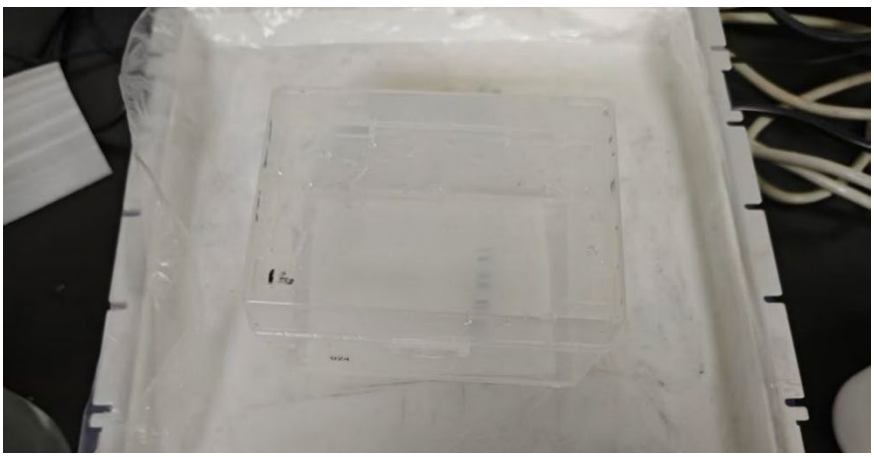


Figure 6 Closure

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

V. Experimental results

Analysis of protein expression by Western Blot showed a significant increase in RBPJ levels in both moderate ($P < 0.05$) and high ($P < 0.0001$) drug-resistance groups relative to the control. Conversely, VEGF levels showed no significant alteration across all resistant groups. Furthermore, PD-L1 protein expression was also significantly higher in the moderate resistance group compared to controls ($P < 0.01$).

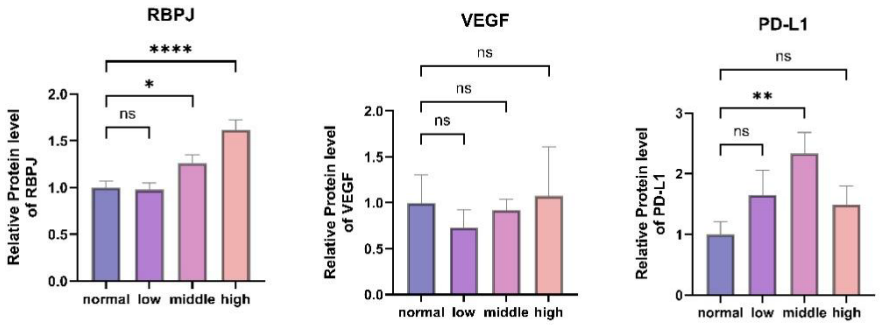
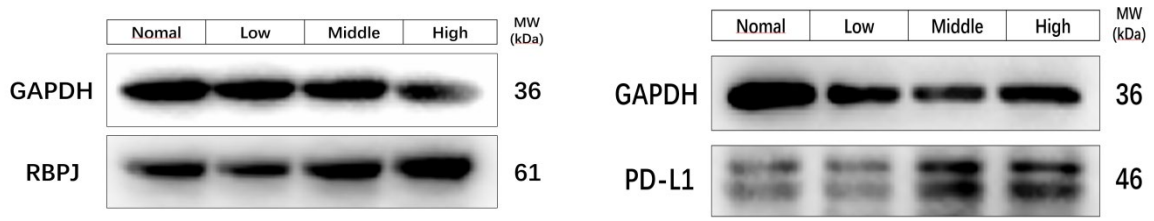
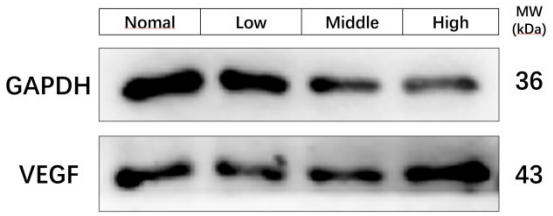


Figure 7 Relative expression levels of the target proteins across different groups (ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001)



（a）RBPJ　　（b）PD-L1



（c）VEGF

Figure 8 Representative Western Blot images of the target proteins from each group

VI. Results analysis

The expression of RBPJ was significantly up-regulated in both the moderate ($P < 0.05$) and high ($P < 0.0001$) drug-resistance groups. This result clearly confirms the successful activation of

RBPJ in the drug-resistance model, and its expression level is positively correlated with the degree of resistance, laying a solid foundation for subsequent mechanistic studies.

Contrary to expectations, the intracellular relative protein expression level of VEGF showed no significant increase in any drug-resistance groups compared to the control. This phenomenon might be attributed to the characteristic of VEGF as a predominantly secreted protein. Since Western Blot detects intracellular proteins, it may not accurately reflect the dynamics of VEGF secreted into the extracellular environment. This finding is indirectly supported by omics data: while VEGF mRNA showed an up-regulated trend in transcriptome sequencing, the protein itself was not reliably detected in the proteomics data. This further suggests that the intracellular steady-state level of VEGF might remain constant due to its continuous secretion. Consequently, to more comprehensively evaluate the role of VEGF in the drug-resistance process, follow-up studies should include analysis of the conditioned medium (e.g., via Western Blot or the more sensitive ELISA) combined with functional assays like angiogenesis formation experiments to draw more scientific conclusions.

PD-L1 expression was significantly up-regulated in the moderate drug-resistance group ($P < 0.01$) but did not reach statistical significance in the high drug-resistance group. To ensure the reliability of this result and rule out the possibility of random chance, further independent experimental replicates are necessary for PD-L1, particularly for the high drug-resistance group.