

Research Article

Mechanistic Investigation of Resistance to SHR-A1811 and T-DXd, the Third Generation of HER2-ADC, in Breast Cancer

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Abstract

Background: In recent years, the therapeutic landscape for cancer patients has undergone a profound transformation with the emergence of third-generation HER2-targeted ADCs, including T-DXd and SHR-A1811. Nevertheless, recurrence and metastasis are inevitable for most patients, and the mechanisms driving resistance to these ADCs remain poorly understood. Against this backdrop, the present study seeks to elucidate the resistance mechanisms of breast cancer cells to SHR-A1811 and T-DXd, while identifying potential targets to enhance sensitivity to these agents. **Methods:** Two resistant cell lines-SHR-A1811-resistant and T-DXd-resistant-were established from the parental breast cancer JIMT-1 cell line via successive drug administration. To identify alterations in protein expression profiles, label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) and single-cell RNA sequencing (scRNA-seq) were employed. Validation of the findings was performed using Western blotting, RNA interference, transfection, and the CellTiter-Glo (CTG) Luminescent Cell Viability Assay. **Results:** A total of 5,664 differentially expressed proteins were quantitatively identified via proteomic analysis comparing SHR-A1811/T-DXd-resistant cells with their sensitive counterparts. Integrated proteomic and scRNA-seq analyses revealed significant up-regulation of CD44 and PLOD2 in resistant cells. Subsequent validation studies confirmed that CD44 expression was substantially higher in both resistant cells relative to their sensitive counterparts, and CD44 knockdown enhanced the sensitivity of the resistant cells to the ADCs. **Conclusions:** Our findings demonstrate that CD44 is a critical factor in the development of resistance to SHR-A1811 and T-DXd, with potential utility as both a resistance biomarker for third-generation HER2-ADCs in breast cancer and a therapeutic target to overcome such resistance.

Keywords

SHR-A1811, T-DXd, HER2-ADC, Resistance Mechanism, Proteomic Analysis, sc-RNA-seq Analysis

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1. Introduction

Breast cancer (BC) remains the most common cancer and is one of the leading causes of cancer-related deaths in women worldwide. [1] More than two million new cases of breast cancer are diagnosed each year, resulting in approximately 666,000 deaths globally. [1] Historically, human epidermal growth factor receptor 2 (HER2)-positive BC was acknowledged as having a poor prognosis, with a median overall survival of 15 months in the metastatic setting. [2] The emergence of monoclonal antibodies (mAbs), such as trastuzumab and pertuzumab, and tyrosine kinase inhibitors, namely lapatinib, pyrotinib, tucatinib, and neratinib, significantly improved the clinical benefit and outcomes of this subtype. [3].

The therapeutic landscape was reshaped by antibody-drug conjugates (ADCs). These drugs took advantage of the synergistic effects of HER2-inhibition and chemotherapy through binding a cytotoxic payload to a monoclonal antibody (mAb) via a molecular linker. [4] The third generation of HER2-ADC overcame the drawbacks of previous ADCs with the progressive transition from non-cleavable to cleavable linkers, the membrane permeable payload (a topoisomerase I inhibitor), and a high drug-to-antibody ratio (DAR). This potentially enables a potent cytotoxic effect on neighboring tumor cells irrespective of target expression (called “bystander effect”). [5] T-DXd (fam-trastuzumab deruxtecan-nxki, DS-8201a, ENHERTU[®]) and SHR-A1811 (Trastuzumab Rezetecan, Ai Wei Da[®]), as representatives of the third generation of HER2-ADC, have shown a revolutionary improvement of antitumor efficacy with tolerable toxicity. [6, 7] T-DXd, developed by Daiichi Sankyo and AstraZeneca, is characterized by a DAR of 8, a tetrapeptide-based cleavable linker, and a topoisomerase I inhibitor payload (deruxtecan). [8] It was initially approved by the US Food and Drug Administration (FDA) in 2019 for the management of advanced-stage HER2-positive BC treated with at least two prior lines of HER2-targeted therapy. [9] Then, four more indications were successively approved in terms of unresectable or metastatic HER2-low breast cancer, unresectable or metastatic HER2-mutant non-small cell lung cancer (NSCLC), locally advanced or metastatic HER2-positive gastric or gastroesophageal junction adenocarcinoma, and unresectable or metastatic HER2-positive solid tumors. [10] SHR-A1811, developed by Jiangsu Hengrui Pharmaceuticals Co., Ltd., optimized the structure of payload SHR169265 with an introduction of a chiral cyclopropyl, and a DAR of 6. The chemical modification would augment the linker-payload stability and reduce the amount of toxins into human body, which cannot only sustain outstanding therapeutic effectiveness but also further mitigate toxicity. [11] The agent has been granted 9 Breakthrough Therapy Designations (BTDs) by National Medical Products Administration (NMPA) of China, and the first new drug application for the treatment of HER2-mutant non-small cell lung cancer (NSCLC) was approved in China in May 2025. [12].

However, most patients would eventually experience innate or acquired resistance to HER2-targeted therapies including ADCs, which leads to an early occurrence or progression of advanced disease. The molecular characteristics of resistance mechanisms against these agents display significant heterogeneity and have not been completely depicted yet. [13] Therefore, understanding the mechanisms of T-DXd and SHR-A1811 resistance is critical for developing new treatment strategies and addressing unmet medical needs.

2. Materials and Methods

2.1. Establishment of T-DXd-Resistant or SHR-A1811-Resistant Cell Line-derived Xenograft (CDX) Models

The breast cancer JIMT-1 cell lines were procured from Shanghai Hengrui Pharmaceuticals Co., Ltd. (Shanghai, China). JIMT-1 cells were subcutaneously inoculated into the right flank region of each Balb/c-nude mouse. Once the tumor volume reached approximately 140–170 mm³, the mice were randomly allocated into three experimental groups, which received treatment with vehicle control, SHR-A1811 (10mg/kg, administered every three weeks [Q3W]), or T-DXd (10 mg/kg, Q3W), respectively. Treatment was continued until the xenografts demonstrated progression. When tumor volumes reached approximately 900 mm³, xenografts were serially passaged into naive mice to stabilize the resistant phenotype. In this study, mice were maintained under a 12-h light/dark cycle (dark phase: 18:00-06:00) in a controlled environment with a temperature of 19–21 °C and relative humidity of 40-70%. Ad libitum access to standard chow and water was provided throughout the study. No anesthesia was administered during the experimental period, and mice were euthanized via carbon dioxide (CO₂) inhalation in accordance with ethical guidelines.

2.2. Mass Spec-based Proteomic Analysis

2.2.1. Protein Extraction and Digestion

Three groups of tumor tissues, including T-DXd-resistant, SHR-A1811-resistant, T-DXd and SHR-A1811-sensitive tumor tissues (4 samples each group, around 10 mm³/sample) were harvested and lysed with lysis buffer containing 100 mM PMSF and 8 M urea. Cutting, grinding (freezing grinder, 4 °C, 2 min), and ultrasonication (sonicated on ice 3 times, 15s pulses with 60s intervals) also contributed to the fully isolation of proteins. Then, the cell lysate was centrifuged at 16,000 relative centrifugal force (rcf) for 30 min at 4 °C. After that, the supernatant was transferred into a fresh tube. BCA protein quantification kit (Beyotime, Shanghai, China)

was used for the determination of protein concentration. Upon reduction with dithiothreitol (DTT, Sigma, St. Louis, MO, USA) followed by alkylation using iodoacetamide (IAA, Aladdin, Shanghai, China), the proteins were subjected to an eight-fold dilution with 1 M HEPES buffer (pH 8.0). Subsequently, the diluted proteins underwent enzymatic digestion with trypsin (at an enzyme-to-substrate ratio of 1:50; Promega, Madison, WI, USA) at a temperature of 37 °C for an overnight incubation. Then, trifluoroacetic acid (TFA, sequencing grade, Thermo-Fisher Scientific, Waltham, MA, USA) was added to the digested peptide solution to a final concentration of 1% to stop the digestion reaction.

2.2.2. Label-free Based LC-MS/MS Analysis

The lyophilized peptides were resuspended in 0.1% formic acid (Thermo-Fisher Scientific) in water. Samples were run on Q-Exactive Plus (Thermo-Fisher Scientific) equipped with DNV PepMap Neo 2 μ m C18 75 μ m*150 mm (Thermo-Fisher Scientific). Samples were fractionated with buffer A (0.1% FA) and buffer B (0.1% FA, 98% ACN). The gradient was configured as follows: 5% B, 0 min; 5%~8% B, 15 min; 8%~32% B, 120 min; 99% B, 30 min. Regarding the parameters of the MS method, the spray voltage was set at 1.8 kV. Data-dependent analysis (DDA) was selected as the data acquisition method to obtain a secondary spectrum of superior quality. The full-mass scanning resolution was configured to 70,000, with a scan range spanning from 400 to 1,600 m/z. Meanwhile, the tandem scanning resolution was set at 35,000.

2.3. scRNA-seq Analysis

2.3.1. Sample Preparation and Cell Isolation

The SHR-A1811-resistant and T-DXd-resistant JIMT-1 tissues were washed three times with RPMI 1640 Medium (ATCC modified) (RWL047) and minced into 1~2 mm fragments. The tissue debris was digested with 5 mL Meltenyi enzyme at 37 °C for 35 min. The cells were filtered through 40 μ m sterile strainers (Falcon; Cat. No.352340) and then centrifuged (Eppendorf, 5810R) at 500 $\times$ g for 5 min. Following centrifugation, the supernatant was carefully aspirated and discarded. The cell pellet was then resuspended in 500 μ L of RPMI 1640 Medium. Aliquots of the cell suspension were stained with AO/PI (countstar, RE010212), and cell counting was carried out using an inverted microscope (Olympus, CKX53). Further processing of samples was initiated only when the cell viability exceeded 80%.

2.3.2. Cell Capture and Library Construction

Single-cell suspensions were introduced into microfluidic devices. The construction of scRNA-seq libraries was executed in strict accordance with the 10X GENOMICS protocol using a Chromium Next GEM Single Cell 3' Reagent Kit (10X GENOMICS). The procedure encompassed multiple sequential steps, including cell lysis, mRNA trapping, label-

ing of cells (barcode) and mRNA (unique molecular identifiers, UMI), reverse transcription of mRNA into complementary DNA (cDNA), amplification, and processing of the cDNA fragments. Then, individual libraries were diluted to a concentration of 4 nM and multiplexed to generate pooled sequencing libraries. The resulting pools were sequenced on an Illumina HiSeq X sequencer (Illumina), configured with a 150-base-pair (bp) paired - end read protocol.

2.4. Data Processing and Bioinformatic Analysis

2.4.1. Proteomics Data

PEAKS Studio software (Version 11) was used for label-free quantitation (LFQ) analysis. Variable modifications included oxidation, acetyl (K), and acetyl (protein N-terminal); fixed modifications consisted of carbamidomethyl. The cut-off value for false discovery rate (FDR) was established at 0.01. Unique acetylated peptides were screened out. The gene list was run against the UniProt-Human database.

Further bioinformatic analysis was conducted using R software (version 4.2.0) with prcomp function and ggplot2 (version 3.5.2) for principal component analysis (PCA), enrichplot (version 1.28.2) for Gene Ontology (GO) enrichment analysis, clusterProfiler (version 4.16.0) for Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Pathways with an adjusted *p* value below 0.05 were identified as significantly enriched.

2.4.2. scRNA-seq Data

The computational workflow for single-cell RNA sequencing (scRNA-seq) analysis was systematically implemented as follows:

1) Data Integration & Batch Correction

Seurat framework (v4.0+) was employed for initial data integration and quality control. Harmony algorithm (Korsunsky *et al.*, 2019) was applied to address technical batch effects while preserving biologically relevant variations, with parameters optimized via the RunHarmony (v4.0+) function.

2) Feature Selection

Highly Variable Genes (HVGs) were identified post-covariate correction to minimize confounding technical artifacts. Selection criteria included dispersion-to-mean ratios (FindVariableFeatures in Seurat, mean-variance threshold=0.0125).

2.5. Western Blot Assay

Cells resistant to SHR-A1811 or T-DXd were harvested and lysed using RIPA buffer (Thermo-Fisher Scientific, Cat# 89900) supplemented with a protease inhibitor cocktail (Thermo-Fisher Scientific, Cat# 78442). Protein concentration was quantified by means of a BCA protein quantification kit (Thermo-Fisher Scientific, Cat# 23227). Equivalent

amounts of proteins (15–20 µg) were then loaded onto SurePAGE precast gels featuring a linear gradient ranging from 4% to 12% (GenScript, Nanjing, China; Cat #M00654). Subsequently, the Trans-Blo Turbo protein transfer system (Bio-Rad, Hercules, CA, USA) was utilized to transfer proteins onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with a blocking buffer (5% nonfat-dried milk with 0.1% tween 20 TBST) for 1 h at room temperature and primary antibody [CD44 (E7K2Y) XP[®] rabbit mAb] overnight at 4 °C. After washing, the membranes were incubated with anti-rabbit HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were made visible through a Super-Signal WestDura Substrate (Thermo Scientific, Cat# 34076). Image acquisition was carried out with the ChemiDoc MP Imaging System (Bio-rad). The expression of GAPDH was employed as the loading control.

2.6. RNA Interference and Transfection

Negative control siRNA (NC siRNA) and CD44-specific siRNAs were custom-designed and synthesized by GENEWIZ (Suzhou, China). To mitigate potential off-target effects, a dual-siRNA strategy targeting distinct regions of the CD44 transcript was employed for gene knockdown. The specific nucleotide sequences of the siRNAs utilized in this study are provided in Table 1 and archived in the Figshare database. [14].

Table 1. The Specific Nucleotide Sequences of the siRNAs.

siRNA	Sequence
SiRNA-NC	Sense UUCUCCGAACGUGUCACGUTT
	Antisense ACGUGACACGUUCGGAGAATT
hCD44 si-1	Sense GGACCAAUUACCAUAAC-UAAUUTT
	Antisense AAUAGUUAUGGUAAU-UGGUCCTT
hCD44 si-2	Sense ACCUCCAGUAUGACACAUAUTT
	Antisense AUAUGUG-UCAUACUGGGAGGUTT

JIMT-1 cells were seeded at a density of 3×10^4 cells per well in 24-well plates and cultured overnight to achieve 30–50% confluency. On the subsequent day, 20 nM siRNA was delivered to the cells using the Lipofectamine RNAi Max transfection reagent (GENEWIZ) according to the manufacturer's protocol, followed by a 3-day incubation period.

2.7. Cell-viability Assay

JIMT-1 cells were seeded in 96-well plates at a density of

2×10^3 cells per well. Following the transfection, cells were treated with escalating concentrations of SHR-A1811 or T-DXd for 6–7 days, with medium replacement after 3–4 days. Cell viability was assessed using the CellTiter-Glo (CTG) Luminescent Cell Viability Assay (Promega): plates were equilibrated to room temperature for 30 min, CellTiter-Glo reagent was added to each well, and plates were shaken for 10–20 min to induce cell lysis. Luminescence signals were measured using a microplate reader (PerkinElmer Envision) with an integration time of 0.1 sec per well.

2.8. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ standard deviation (SD). For statistical comparisons, unpaired two-tailed Student's *t*-test was employed, as specified in the figure legends. Statistical significance was defined as a *p*-value < 0.05 , with exact *p*-values reported where applicable.

3. Results

3.1. Proteomic Features Differentiating SHR-A1811/T-DXd-Sensitive and Resistant Cell Lines

Through a comprehensive proteomic analysis, a total of 5,664 proteins were initially identified from the SHR-A1811/T-DXd-resistant cell lines and sensitive cell lines. 789 regulated proteins were selected employing a significance threshold of $p < 0.05$. Further filtering based on a fold change (FC) > 2 in resistant groups yielded 397 candidate proteins for in-depth study (Figure 1A).

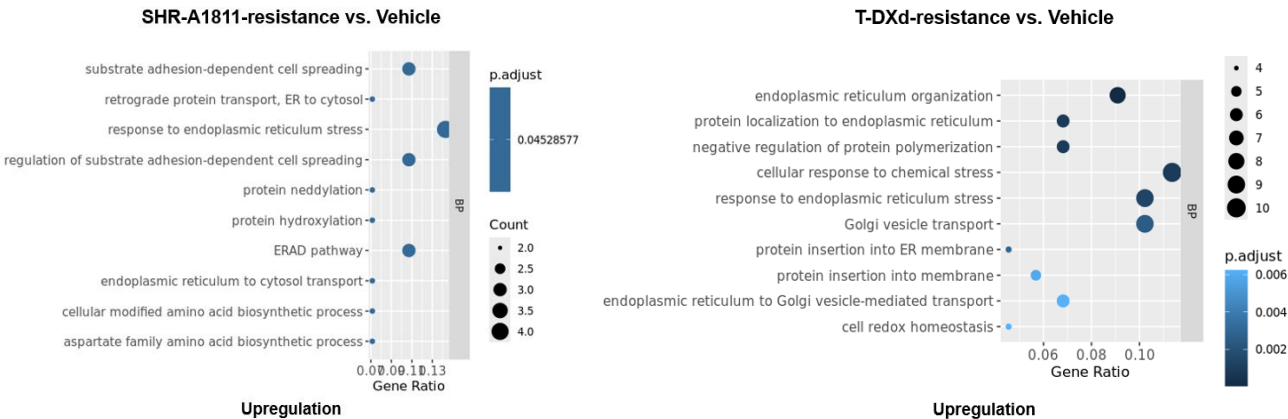
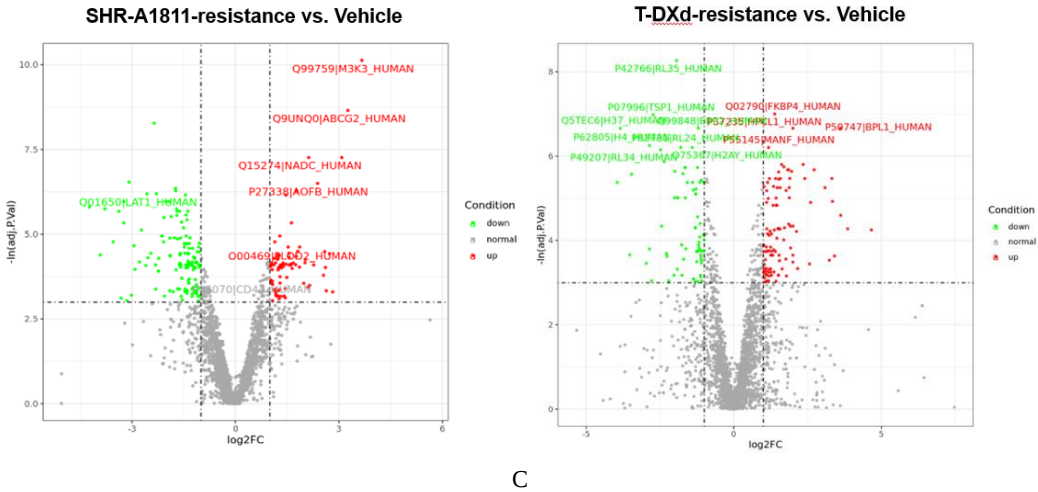
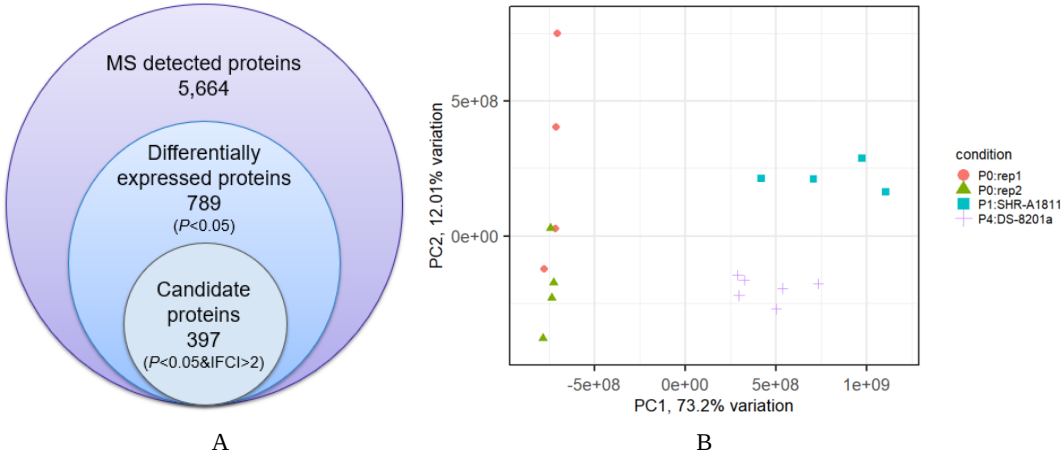
Principle component analysis (PCA) revealed distinct patterns of protein expression profiles between the drug-resistant groups and the control group, suggesting significant differences in proteomic landscapes. Moreover, the PCA results also imply potential divergence in the drug resistance mechanisms underlying the SHR-A1811-resistant and T-DXd-resistant groups, indicating that the two types of resistance may be slightly different (Figure 1B).

Meanwhile, SHR-A1811-resistant group exhibited a predominant pattern of gene down-regulation, while the T-DXd-resistant group was characterized by a significant up-regulation of gene expression (Figure 1C).

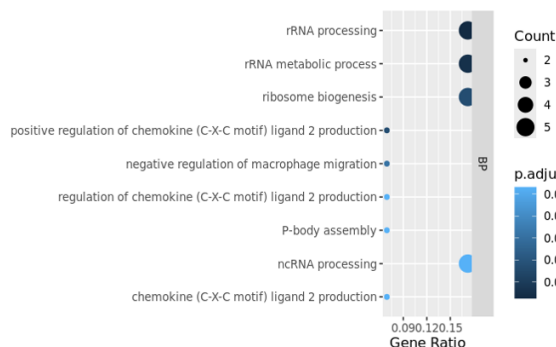
Gene Ontology (GO) analysis showed that the upregulated genes were predominantly enriched in the substrate adhesion-dependent cell spreading pathway (Figure 1D). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis further demonstrated significant enrichment of proteins in the chemical carcinogenesis pathway within SHR-A1811-resistant cell lines. Concurrently, multiple pathways were significantly downregulated in both resistant

cell lines, including the cAMP signaling pathway, Rap1 signaling pathway, and VEGF signaling pathway. This concordant enrichment or decreased pattern in GO and KEGG analyses underscores the critical role of these dysregulated pathways in mediating resistance to HER2-ADCs and provides a mechanistic foundation for developing pathway-targeted therapies (Figure 1E).

Following the identification of activated signaling pathways, our analytical focus transitioned to proteins exhibiting significant regulatory changes for in-depth investigation. Through comparative profiling of protein expression between the resistant and sensitive cohorts, 15 most commonly regulated target proteins were identified, with details summarized in Table 1.

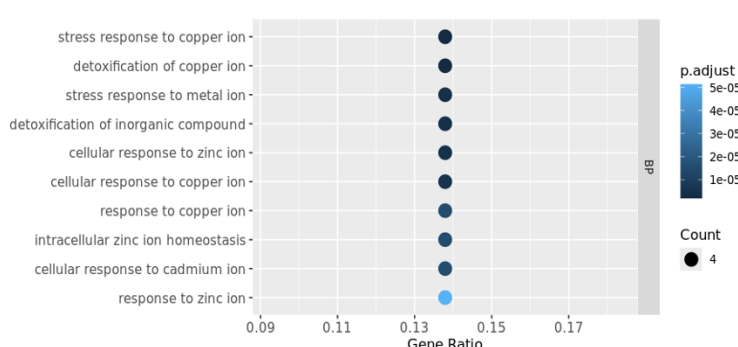


SHR-A1811-resistance vs. Vehicle



Downregulation

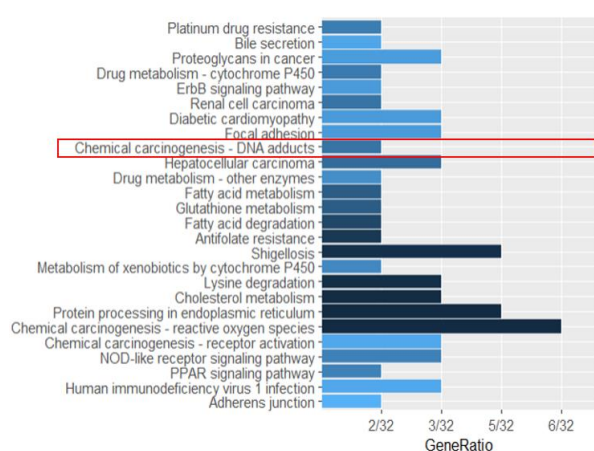
T-DXd-resistance vs. Vehicle



Downregulation

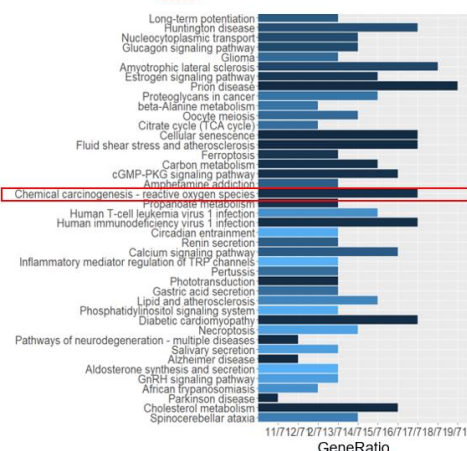
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SHR-A1811-resistance vs. Vehicle



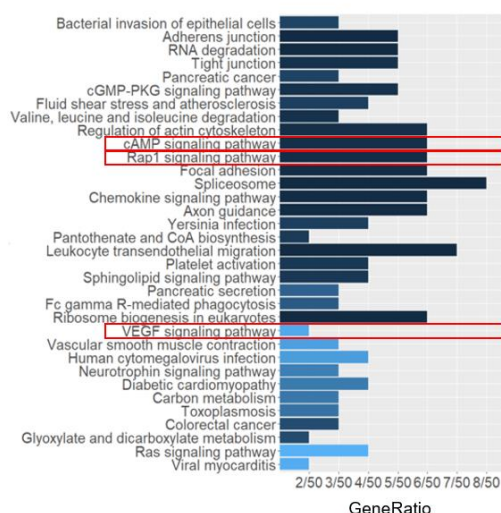
Upregulated pathways

T-DXd-resistance vs. Vehicle



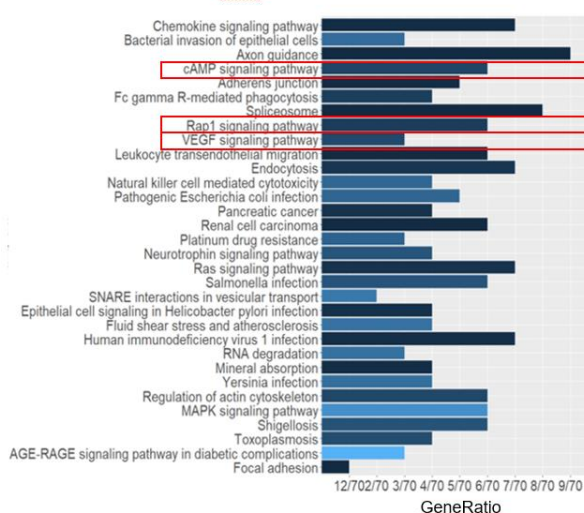
Upregulated pathways

SHR-A1811-resistance vs. Vehicle



Downregulated pathways

T-DXd-resistance vs. Vehicle



Downregulated pathways

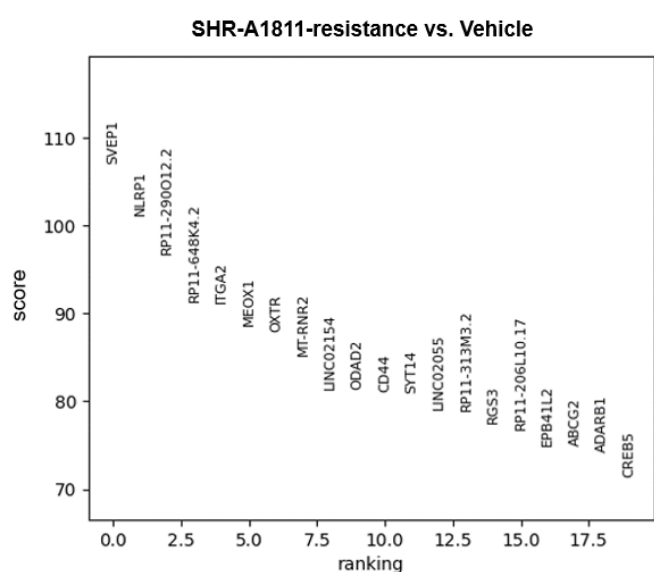
E

Figure 1. Bioinformatic analysis of differentially expressed proteins. (A) The Venn diagram of MS results. Differentially regulated proteins were identified by p -value < 0.05 in SHR-A1811/T-DXd-resistant versus sensitive cell lines, with candidate proteins further filtered by a fold-change threshold > 2 . (B) Principal component analysis revealed distinct differences and shared characteristics between resistant and sensitive groups. (C) Volcano plots of identified proteins. Selected candidate protein names were listed herein. (D) GO enrichment analysis of differentially regulated genes showed significant enrichment in the substrate adhesion-dependent cell spreading pathway. (E) KEGG analysis of regulated genes revealed proteins enrichment in the chemical carcinogenesis pathway in SHR-A1811-resistant cell lines, while multiple pathways were downregulated in both resistant lines.

Table 2. The Most Commonly Differentially Regulated Proteins in SHR-A1811- and T-DXd-Resistant Cell Lines.

Gene Symbol	Description	Biological Process (GO Terms)
EDC3	Enhancer of mRNA decapping 3	mRNA decay; P-body assembly; miRNA-mediated silencing
LSM4	LSM4 homolog, mRNA degradation associated	mRNA splicing; RNA catabolism; stress granule formation
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	Collagen cross-linking; lysine hydroxylation; ECM organization
PLOD3	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	Collagen glycosylation; hydroxylysine biosynthesis; skeletal development
GGH	Gamma-glutamyl hydrolase	Polyglutamate hydrolysis; folate metabolism; collagen maturation
RBX1	RING-box protein 1	Ubiquitin ligase complex assembly; protein neddylation; proteasomal degradation
NEDD8	Neural precursor cell expressed, developmentally downregulated 8	Protein neddylation; cullin-RING ligase activation; cell cycle progression
CRK	CRK proto-oncogene	Signal transduction; EGFR endocytosis; apoptotic process regulation
MIF	Macrophage migration inhibitory factor	Inflammatory response; NF- κ B signaling; angiogenesis regulation
POSTN	Periostin	Collagen fibril organization; extracellular matrix assembly; wound healing
CNN2	Calponin 2	Actin cytoskeleton organization; smooth muscle contraction; cell migration
MANF	Mesencephalic astrocyte-derived neurotrophic factor	ER stress response; unfolded protein response; neuron survival
RCN3	Reticulocalbin 3	Calcium ion homeostasis; protein folding; ER-Golgi transport
NOP56	Nucleolar protein 56	rRNA processing; snoRNA localization; ribosome assembly
NOP2	NOP2 nucleolar protein	rRNA methylation; ribosome biogenesis; cell cycle regulation

3.2. The Up-regulation of CD44 May Contribute to SHR-A1811 or T-DXd Resistance



SHR-A1811_names	SHR-A1811_p value	SHR-A1811_logFC
SVEP1	<0.001	8.150118
NLRP1	<0.001	4.48399
RP11-290O12.2	<0.001	7.406161
RP11-648K4.2	<0.001	3.931928
ITGA2	<0.001	2.555433
MEOX1	<0.001	3.894613
OXTR	<0.001	2.005837
MT-RNR2	<0.001	0.820435
LINC02154	<0.001	3.878088
ODAD2	<0.001	3.069118
CD44	<0.001	1.394527
SYT14	<0.001	2.973338
LINC02055	<0.001	3.614854
RP11-313M3.2	<0.001	7.365934
RGS3	<0.001	5.084136
RP11-206L10.17	<0.001	1.374275
EPB41L2	<0.001	1.414576
ABCG2	<0.001	3.063468
ADARB1	<0.001	2.682421
CREB5	<0.001	2.734725

A

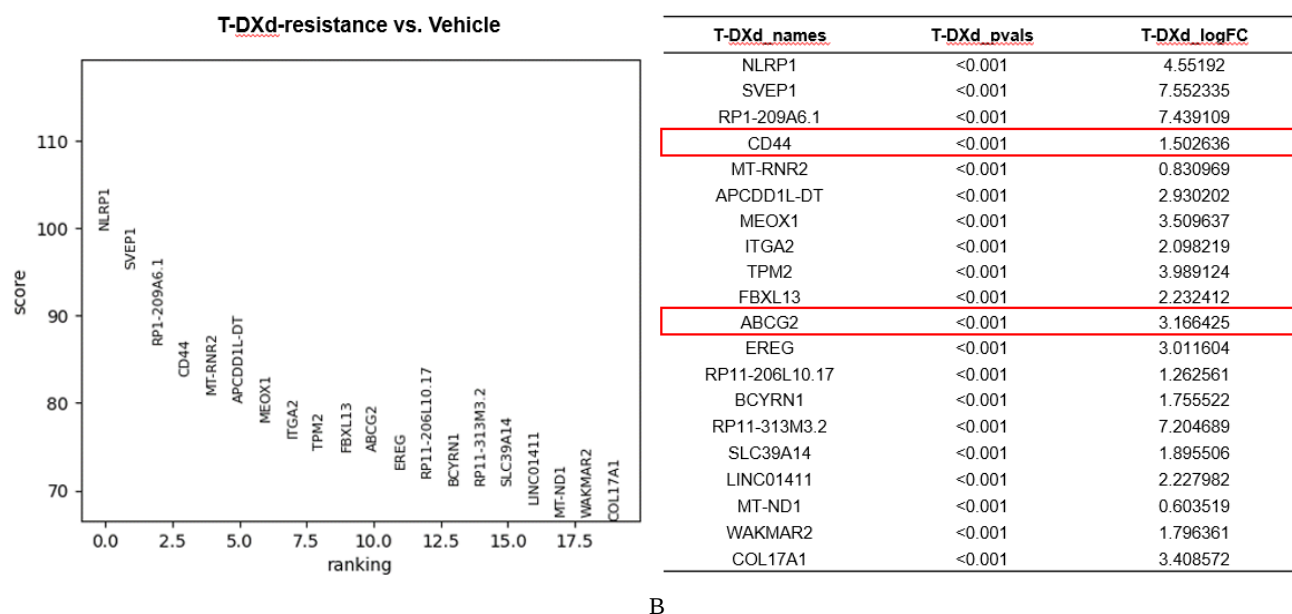


Figure 2. Pseudobulk RNA-seq results revealed the significantly upregulated genes. (A) The top 20 upregulated genes between SHR-A1811-resistant group and vehicle controls. (B) The top 20 upregulated genes between T-DXd-resistant group and vehicle controls.

Figure 2 presented the outcomes of pseudobulk RNA-seq, contrasting the gene expression profiles of the SHR-A1811-resistant group (Figure 2A) and the T-DXd-resistant group (Figure 2B) with their respective vehicle controls. The table enumerated the top 20 upregulated genes. Several shared genes, including SVEPI, NLRP1, CD44, and ABCG2, exhibited upregulated expression in both resistant groups.

Integrative analysis of proteomic and scRNA-seq data revealed a significant up-regulation of CD44 and PLOD2 expression in both SHR-A1811-resistant and T-DXd-resistant cell lines. These findings, as illustrated in [Figure 3](#), strongly implicate CD44 as a potential key determinant contributing to the development of resistance against SHR-A1811 and T-DXd.

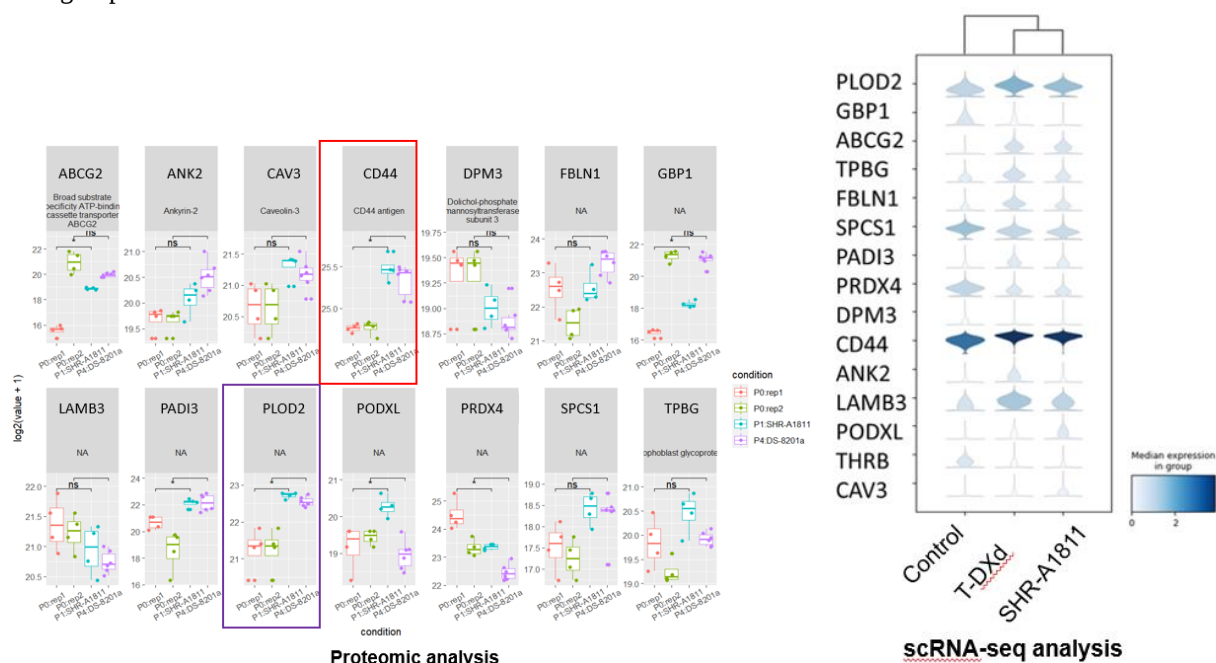


Figure 3. A comparative analysis of proteomic and scRNA-seq results indicated that CD44 and PLOD2 were both significantly upregulated. (A) Box plot of proteomic results. (B) Violin plot of scRNA-seq results.

3.3. Validation of the Role of CD44 in Mediating Resistance to SHR-A1811 and T-DXd

To elucidate the potential association between resistance to SHR-A1811/T-DXd and the upregulation of CD44, Western blot analysis was conducted. The findings demonstrated a substantial increase in CD44 expression in both SHR-A1811-resistant and T-DXd-resistant cell lines relative to their sensitive counterparts, providing direct protein-level evidence supporting the role of CD44 in mediating resistance to this ADC drug (Figure 4A, 4B).

In order to further investigate the functional interplay between CD44 upregulation and resistance to SHR-A1811 and T-DXd, we implemented a siRNA-mediated knockdown strategy to decrease CD44 expression. Firstly, the CTG assay confirmed that the SHR-A1811 and T-DXd-resistant cell lines

exhibited high resistance to the corresponding ADC drugs (Figure 5A). Besides, Western blot results demonstrated a significant reduction in CD44 levels within both SHR-A1811-resistant and T-DXd-resistant cells (Figure 5B). Consistent findings were also observed in the cell viability assay. As depicted in Figure 5C, siRNA-mediated reduction of CD44 significantly attenuated cell viability compared to the control group, suggested the knockdown of CD44 could increase the sensitivity of these resistant cell lines to SHR-A1811 or T-DXd, which providing quantitative evidence that CD44 upregulation is mechanistically linked to resistance against SHR-A1811 and T-DXd. These results establish a functional correlation between CD44 expression levels and therapeutic insensitivity to these ADCs, underscoring CD44’s potential role in mediating resistance pathways.

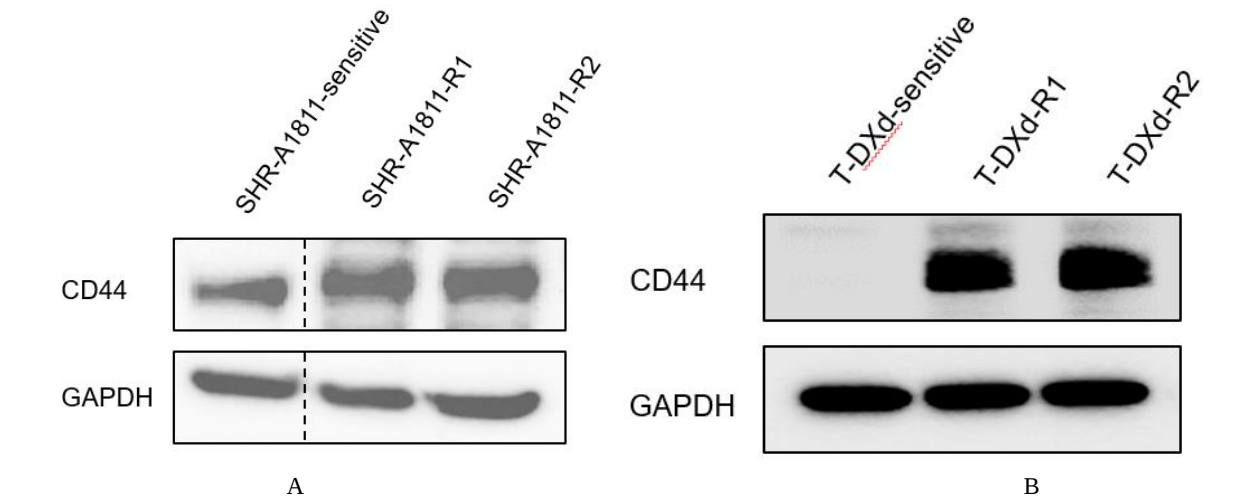
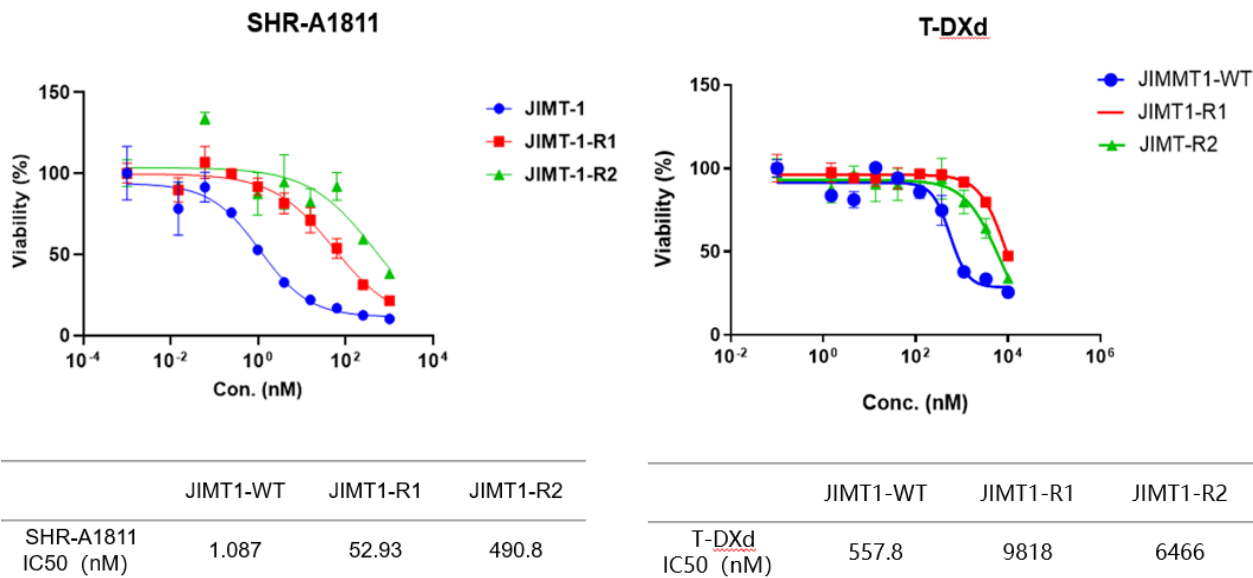


Figure 4. Western Blot Analysis Validated a Substantial Upregulation of CD44 in SHR-A1811-resistant or T-DXd-resistant Cell Lines Compared to Their Sensitive Counterparts.



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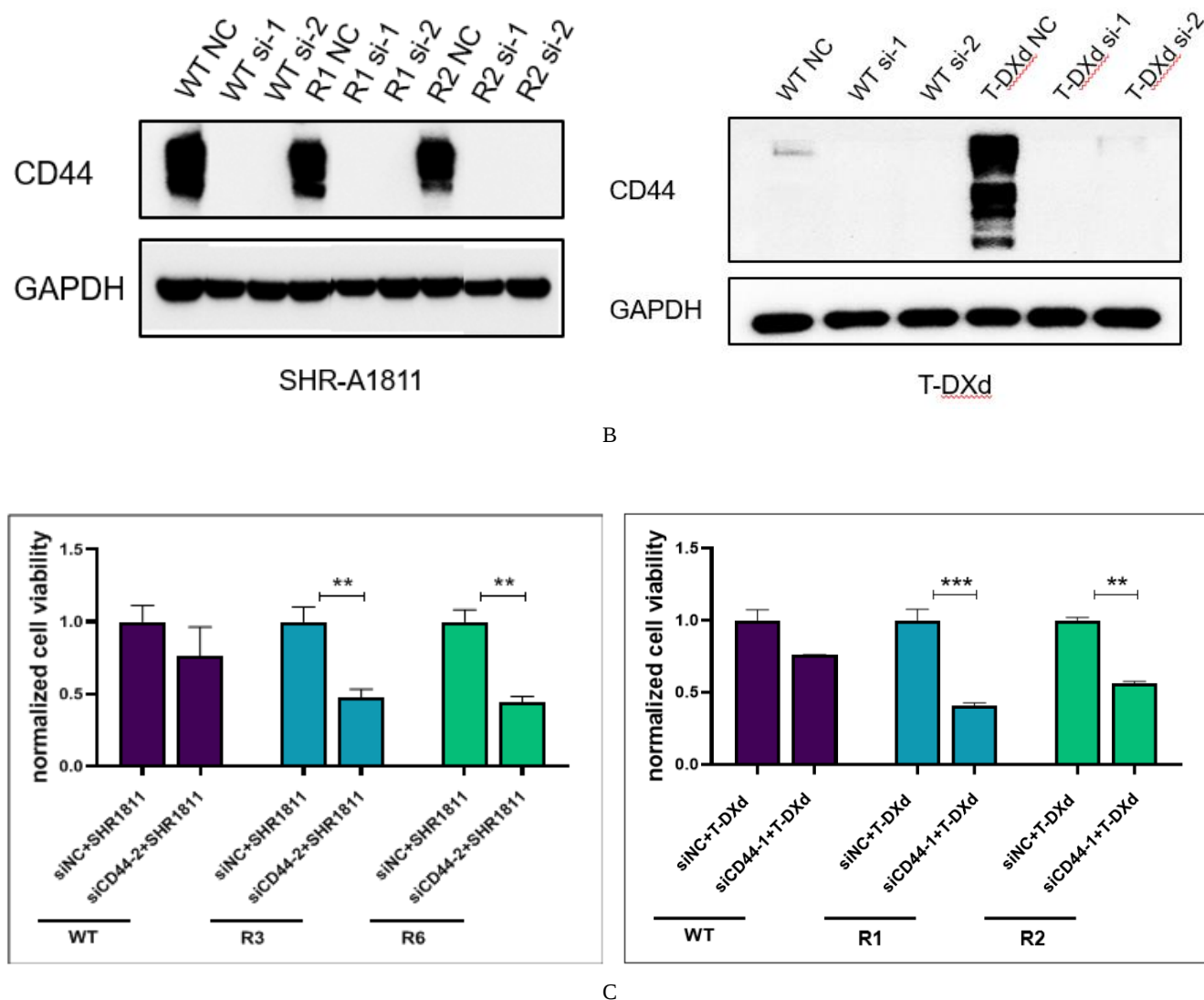


Figure 5. CD44 knockdown sensitized SHR-A1811/T-DXd-resistant JIMT-1 cells. (A) Resistant cell lines were highly resistant to ADCs. (B) Western blot verified reduced CD44 expression via siRNA intervention. (C) Cell viability assays showed CD44 knockdown sensitized resistant cell lines to SHR-A1811/T-DXd.

4. Discussion

Despite the prognosis of breast cancer patients has been significantly improved with the major advancements in ADC technology, therapeutic resistance persists as a critical challenge. It may emerge via diverse mechanisms, including reduced antigen levels [15], impaired drug trafficking [16, 17], disrupted lysosomal function [18], and payload-related resistance [19, 20], etc., some of which remain incompletely characterized. Thus, the exploration of strategies to overcome resistance represents a complex and ongoing research field. In this study, quantitative proteomics and scRNA-seq analysis were performed to identify differently expressed proteins and genes between ADC-resistant and ADC-sensitive breast cancer cell lines. Our results revealed that a subset of proteins in SHR-A1811-resistant or T-DXd-resistant cells displayed

upregulated expression relative to their sensitive counterparts. Subsequent functional experiments elucidated the critical role of CD44 in sustaining resistance to third generation anti-HER2 ADCs in breast cancer cells. Further investigations indicated that CD44 serve as a potential therapeutic target for overcoming resistance to SHR-A1811 or T-DXd in breast cancer cells.

CD44, a cell surface adhesion molecule, is overexpressed in cancer stem cells (CSCs). [21] Cells with CD44 overexpression display key CSC properties, such as self-renewal capacity, epithelial-mesenchymal transition (EMT) potential, and resistance to chemo- and radiotherapy, which may contribute to poor clinical outcomes in affected patients. [22] However, the functional role of CD44 in breast cancer remains elusive. Our study demonstrated that CD44 expression is upregulated in breast cancer cells resistant to SHR-A1811 or T-DXd. Furthermore, Western blot analysis validated increased CD44 expression in resistant cells compared to their

sensitive counterparts. Notably, inhibition of CD44 significantly enhanced the sensitivity of SHR-A1811- or T-DXd-resistant breast cancer cell lines to these agents. These findings suggest that in breast cancer, CD44 expression may act as a prognostic indicator for recurrence and harbor therapeutic implications.

Nevertheless, this study is subject to several limitations. Firstly, the research was confined to in vitro cellular experiments, necessitating extension to in vivo models and expanded clinical validation for deeper investigation. Secondly, functional assays solely verified that CD44 upregulation contributes to resistance against SHR-A1811 or T-DXd, while the roles of other significantly dysregulated genes remain unelucidated. Thirdly, the precise mechanism by which CD44 modulates resistance to SHR-A1811 or T-DXd in resistant cell lines remains obscure and warrants further exploration.

5. Conclusions

In summary, our study preliminarily identified differential protein expression profiles between SHR-A1811/T-DXd-resistant and -sensitive breast cancer cell lines. These findings not only deepen our understanding of the cellular mechanisms underlying responses to third-generation HER2-targeted antibody-drug conjugates (ADCs), but also provide novel insights for clinical intervention. Specifically, this research uncovers potential resistance mechanisms to third-generation HER2-targeted ADCs in breast cancer cells and highlights opportunities to optimize sensitization strategies for clinical practice. By modulating specific targets and their associated signaling pathways, it may be possible to improve the therapeutic efficacy of these ADCs in breast cancer patients.

Abbreviations

ADC	Antibody-Drug conjugate
BC	Breast Cancer
BTD	Breakthrough Therapy Designations
cDNA	Complementary Deoxyribonucleic Acid
CDX	Cell Line-Derived Xenograft
CSCs	Cancer Stem Cells
CTG	CellTiter-Glo
DAR	Drug to Antibody Ratio
DDA	Data-Dependent Analysis
DTT	Dithiothreitol
EMT	Epithelial-Mesenchymal Transition
FC	Fold Change
FDA	Food and Drug Administration
FDR	False Discovery Rate
GO	Gene Ontology
HER2	Human Epidermal Growth Factor Receptor 2
HVGs	Highly Variable Genes
IAA	Iodoacetamide

KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Label-Free Liquid Chromatography-Tandem Mass Spectrometry
LFQ	Label-Free Quantitation
mAbs	Monoclonal Antibodies
MS	Mass Spectrometry
NMPA	National Medical Products Administration
NSCLC	Non-Small Cell Lung Cancer
PCA	Principal Component Analysis
Q3W	Every Three Weeks
scRNA-seq	Single-Cell RNA Sequencing
SD	Standard Deviation
TFA	Trifluoroacetic Acid
UMI	Unique Molecular Identifiers

Supplementary Material

The supplementary material can be accessed at <https://doi.org/10.11648/j.ijcocr.20251004.18>

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Data Availability Statement

The mass spectrometry data and siRNA sequence data have been archived in the Figshare database with the DOI of 10.6084/m9.figshare.30575879. Additional datasets utilized and/or analyzed in the present study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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