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Original Article

Exploring the role of YES1 kinase in regulating cisplatin resistance through iTRAQ-based quantitative proteomic analysis in urothelial carcinoma

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Abstract: Urothelial carcinoma (UC) is a highly metastatic cancer that frequently develops resistance to platinum-based chemotherapy, although the underlying mechanisms remain unclear. While certain genes have been implicated in UC drug resistance, their specific roles require further validation. In this study, we established a cisplatin-resistant UC cell line (BFTC909 Cis-R) and used iTRAQ analysis to compare differences in protein expression between BFTC909 Cis-R cells and their parental BFTC909 counterparts. iTRAQ mass analysis revealed decreased expression of the tyrosine kinase YES1 in BFTC909 Cis-R cells, along with reduced levels of YES1 and YAP in both BFTC909 Cis-R and T24 Cis-R cells. Moreover, we found that bladder cancer patients with higher YES1 expression had significantly better survival outcomes in our in-house cohort and two public datasets (GSE13507 and GSE169455). Treatment with dasatinib, a YES1 inhibitor, reduced cisplatin-induced cytotoxicity in UMUC-14 cells, suggesting that YES1 influences cisplatin efficacy in UC cells. Our findings indicate that YES1 plays a critical role in cisplatin resistance and may represent a promising therapeutic target in bladder cancer.

Keywords: Urothelial carcinoma, cisplatin resistance, chemotherapy, YES1, iTRAQ

Introduction

Over 95% of urothelial carcinomas (UC) arise in the bladder, with some originate in the upper urinary tract [1]. Unlike non-muscle invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC) is a lethal disease featured with a high rate of distant metastasis [2]. For patients with metastatic urothelial carcinoma (mUC), platinum-based chemotherapy has been the cornerstone of systemic treatment and the gold standard for the past 30 years [3, 4]. Although the treatment landscape for mUC has gradually shifted towards using immune checkpoint inhibitors (ICIs), and the pivotal study has shown better overall survival (OS) with the combination of ICIs and antibody-drug conjugates (ADCs) compared to platinum-based chemotherapy, in real-world practice, platinum-based chemotherapy remains the most commonly used treatment regimen [5, 6].

Cisplatin (CDDP) is a platinum-based compound that interacts with DNA to form adducts, leading to cell death and has demonstrated anti-neoplastic effects on various types of cancer [7]. For patients with MIBC, cisplatin-based chemotherapy is the preferred treatment. The most commonly used chemotherapy regimens are gemcitabine combined with cisplatin (GC) and methotrexate, doxorubicin, vinblastine, and cisplatin (MVAC) [8, 9]. Urothelial carcinoma is typically initially sensitive to platinum-based chemotherapy; however, tumor progression and the development of chemoresistance usually occur after approximately six months with-

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out therapeutic advancement [8, 9]. Consequently, overcoming chemoresistance in mUC is a significant challenge that requires further scientific research to find a solution.

Tyrosine kinase YES1, a member of the Src family of nonreceptor tyrosine kinases (SFKs), plays a role in various cellular functions and intracellular signaling pathways, including cellular growth, adhesion, proliferation, migration, and survival [10, 11]. Several studies have indicated that YES1 activation is associated with drug resistance in cancers [12]. In breast and lung cancers, YES1 has been shown to protect tumors against the effects of EGFR and HRE2 inhibitors, respectively [13, 14]. Additionally, overexpression of YES1 in breast cancer cells activates the EGFR, PI3K, and MAPK signaling pathways to counteract the effects of trastuzumab emtansine (T-DM1) [15]. Furthermore, 5-fluorouracil (5-FU)-resistant colorectal cancer cells exhibit high levels of YES1, which suppresses the nuclear localization of Yes-associated protein (YAP) and induces a quiescent state in tumor cells, enabling them to evade 5-FU-induced cell death [16]. However, the relationship between YES1 and cisplatin-resistant bladder cancer remains unclear.

In this study, we present the results of an iTRAQ (isobaric tagging for relative and absolute quantification) assay conducted to elucidate the differences in protein expression between cisplatin (CDDP)-resistant and non-resistant UC cells, specifically BFTC909. The data revealed a decreased expression of the protein YES1 in CDDP-resistant BFTC909 cells. Furthermore, inhibition of YES1 activity using Dasatinib treatment was observed to enhance the resistance of UMUC14 cells to the cytotoxic effects of CDDP. These findings indicate that YES1 is integral to the mechanism of CDDP resistance in bladder cancer cells, suggesting a potential novel therapeutic strategy for the treatment of bladder cancer.

Material and methods

Cell culture and drug treatment

Human urothelial carcinoma (UC) cell lines BFTC-909 (renal pelvis) and T24 (bladder) from the Bioresource Collection and Research Center (BCRC), Taiwan, were used to induce cisplatin resistance. BFTC909 cells, derived from patients with renal pelvis urothelial carcinoma, were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. To establish the CDDP-resistant cell line (BFTC909 Cis-R), BFTC909 cells were treated with increasing doses of CDDP (0.01 to 0.8 μM) (Kemoplat®, Fresenius Kabi Oncology Limited, Solan, India) over six months. The cells were treated with CDDP until they reached 20-30% confluence, at which point a higher concentration of CDDP was added when the cells grew to 80-90% confluence (approximately 4-8 days of cultivation). This procedure was repeated until the cells were able to grow in DMEM with 0.8 μM CDDP. Similarly, human bladder cancer cells T24 were maintained in McCoy's 5A medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. T24 Cis-R cells were selected using the same regimen, with CDDP doses ranging from 0.1 to 3.5 μM.

J82 (bladder) and RT4 (bladder) cell lines were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured in DMEM and McCoy's 5A medium, respectively. The UMUC14 cell line was procured from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in Eagle's Minimum Essential Medium, supplemented with 2 mM glutamine (Thermo Fisher Scientific), 1% nonessential amino acids (Thermo Fisher Scientific), and 10% FBS. The SV-HUC-1 (uroepithelium) cell line (CRL-9520, ATCC) was purchased from the American Type Culture Collection (ATCC) and cultured in F-12K medium (Thermo Fisher Scientific). All cell lines were incubated at 37°C with 5% CO_2.

Cell viability assay (MTT assay)

A total of 2.5×10^3 CDDP-resistant or non-resistant human UC cells were seeded into 96-well plates and cultured overnight. Subsequently, 50 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 hours following various treatments. After removing the supernatant, 200 μl of DMSO (Sigma-Aldrich) was added to dissolve the formazan. Cell viability was then determined using an ELISA reader at an optical density of 595 nm (OD_{595}).

Colony formation assay

A total of 100 human UC cells, both CDDP-resistant and non-resistant, were seeded into 6-well plates and treated individually with 0, 1, 3, 5, and 7 μM CDDP. After 14 days of culture, the colonies were fixed with methanol and stained with 0.1% crystal violet for 15 minutes at room temperature. The number of colony formations was then recorded by direct counting.

RNA extraction and Real-Time PCR (RT-PCR)

Total RNA of human urothelial cancer cells was purified using QIAGEN RNA purification kit (Qiagen, Hilden, Germany) according to the instruction manual. One microgram of RNA from each sample was reverse transcribed using RevertAid™ H Minus Reverse Transcriptase (Fermentas, Waltham, MA, USA). Real-time PCR was performed using a mixture of SYBR Green PCR master mix (Life Technologies, Carlsbad, CA, USA) and the following specific primers: MDR1 (NM_001348945.2; forward 5'-GCTGTCAAGGAAGCCAATGCCT-3'; reverse: 5'-TGCAATGGCGATCCTCTGCTTC-3'). ERCC1 (NM_; forward 5'-GAGTGGCCAAGCCCTTATT-3'; reverse: 5'-GAGGCTGTGAGATGGCATATT-3'). YES1 (NM_005433.4; forward 5'-GAGAATCTTTGCGACTAGAGG-3'; reverse: 5'-CTGGCATCATTGTACCTGG-3'). YAP (NM_001130145.3; forward 5'-TGTCCCAGATGAACGTCACAGC-3'; reverse: 5'-TGGTGGCTGTTTCACTGGAGCA-3'). GAPDH (NM_002046.7; forward 5'-GGGGAAGGTGAAGGTCGGAGTC-3'; reverse: 5'-CAAGCTTCCCGTTCTCAGCCTT-3') (GENOMICS, New Taipei City, Taiwan). The Ct values of the samples were determined using the ABI 7500 sequence detection system (Life Technologies), and the relative mRNA expression levels of the genes of interest were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting assay

Cell lysates from CDDP-resistant or nonresistant human UC cells, subjected to various experimental treatments, were prepared using RIPA buffer with proteinase inhibitor (Roche, Basel, Switzerland). Twenty micrograms of protein from each lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Merck Millipore, Burlington, MA, USA). Following blocking with 5% skim milk, the membranes were probed with primary antibodies, including MDR1 (cat# ab170904, Abcam, Cambridge, UK, 1:400), ERCC1 (cat# GTX110562, GeneTex, Irvine, CA, USA, 1:1000), α-actin (cat# ZRB1312, Sigma-Aldrich, 1:5000), p-YES1 (cat# ab188319, Abcam, 1:1000), YES1 (cat# PA5-80243, Invitrogen, Waltham, MA, USA, 1:1000) and YAP (cat# 14074S, Cell Signaling, Danvers, MA, USA, 1:1000) at 4°C overnight. After three washes with PBST (10 mM NaH_2PO_4, 130 mM NaCl, 0.05% Tween 20), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. The signal on the membranes, treated with enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA), was then captured using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

Cell cycle assay

Human UC cells were seeded into 6-well plates and cultured overnight. Following treatment with CDDP, the tyrosine kinase inhibitor Dasatinib, or a combination of the two, the cells were washed with PBS and fixed in 70% cold ethanol at 4°C for 30 minutes. Subsequently, the cells were treated with ribonuclease A for 30 minutes to extract RNA. The cell cycle distribution was then analyzed by staining the cells with propidium iodide (PI) and using the BD FACSAria (BD Bioscience, Singapore). The results were determined using FlowJo software (BD Bioscience).

Protein extraction, digestion and iTRAQ proteomics analysis

The protein expression profiles of BFTC909 and BFTC Cis-R cells were comprehensively analyzed using iTRAQ gel-free proteomics. Briefly, BFTC909 and BFTC909 Cis-R cells were lysed using a lysis buffer containing 8M urea, 50 mM Tris-HCl (pH 8.0), and protease inhibitors to prevent protein degradation. Cells were disrupted by sonication on ice and centrifuged at 12,000 g for 30 minutes at 4°C to remove debris. The supernatant, containing soluble proteins, was collected, and protein concentration was determined using the BCA assay. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested overnight with trypsin at a 1:50 ratio. The resulting peptides were then purified and labeled with the iTRAQ reagents Multiplex Kit

(Cat# 4352135, Sigma-Aldrich) and analyzed using an LC/Q-Exactive Orbitrap MS (Thermo Fisher Scientific) for 24 hours.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

The iTRAQ-labeled samples were pooled and desalted using Sep-Pak C18 cartridges (Waters, Milford, MA, USA). After desalting, the peptide mixtures were dried with a SpeedVac and re-suspended in 0.5% trifluoroacetic acid. They were then loaded onto an EASY-Spray™ C18 column (Thermo Fisher Scientific) for separation using a gradient of acetonitrile (5-80%) in 0.1% formic acid. The peptides were analyzed on a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific) coupled with an UltiMate™ 3000 RSLCnano HPLC system. The raw mass spectrometry data were processed using the Mascot search algorithm (version 2.5, Matrix Science) against the Swiss-Prot human protein database in Proteome Discoverer (version 2.1, Thermo Fisher Scientific).

Gene ontology, pathway and interaction analyses

The identified proteins and their associated signaling pathways were analyzed according to molecular functions, cellular components, and biological processes using the Gene Ontology (GO) database and the KOBAS 3.0 software (http://kobas.cbi.pku.edu.cn), which provides orthology-based annotations linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO and KEGG enrichment analysis was performed using Fisher's exact test, and functional categories and pathways were considered significant when *p*-values fell below the 0.05 threshold. This approach highlights key biological processes and pathways impacted by the proteins of interest. The highly expressed candidate proteins were mapped using the STRING database (https://string-db.org) to construct functional networks of protein-protein interactions.

Immunohistochemical (IHC) assay

The human UC tissue samples used in the study were obtained from the tissue bank at Kaohsiung Chang Gung Memorial Hospital, Taiwan. In this cohort, cancer tissue samples were collected from 71 patients with mUC who were undergoing systemic platinum-based chemotherapy for analysis. The tumor samples were fixed in 10% formalin, dehydrated, embedded in paraffin, and then sectioned 4 μm slices. The sections were deparaffinized in xylene and rehydrated using a graded series of ethanol solutions. Antigen retrieval was performed using a repair solution. The sections were then stained overnight at 4°C with primary antibodies against YES1. Finally, the sections were incubated with an HRP-conjugated secondary antibody at 37°C, and HRP activity was detected using 3,3'-diaminobenzidine (DAB). YES1 expression was assessed using H-score calculations, with tumors defined as having low YES1 expression if the H-score was below 100.

Public database analysis

Two publicly available datasets, GSE13507 and GSE169455, were retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The GSE13507 dataset comprises 165 bladder cancer samples and explores disease invasiveness and progression through microarray panel analysis (Illumina Human-6 BeadChip). The GSE169455 dataset included 149 bladder cancer patients receiving neoadjuvant cisplatin-based chemotherapy, with gene expression profiles analyzed using the Affymetrix Human Gene 1.0 ST Array.

Statistical analysis

All experiments in this study were conducted independently at least three times, and the data were presented as the mean ± standard error (SE). Differences between experimental groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. An independent t-test was employed to compare differences between two groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Induction of CDDP-resistant cell lines in urothelial carcinoma cell lines

To elucidate the molecular mechanism underlying cisplatin resistance in UC cells, BFTC909 and T24 cells were treated with increasing concentrations of CDDP every 4-7 days to develop CDDP-resistant cell clones, named BFTC909-R

and T24-R (**Figure 1A**). The IC_{50} values of BFTC909-R and T24-R were found to be 3-fold and 5-fold higher, respectively, compared to those of the parent cells (**Figure 1B**, **1C**). After 2 days of CDDP treatment, the morphology of BFTC909-R and T24-R appeared more intact than that of their parental cells (**Figure 1D**). Furthermore, as the CDDP doses increased, both BFTC909-R and T24-R exhibited higher survival rates compared to their parental cells (**Figure 1E**).

Validation of CDDP-resistant ability in BFTC909-R and T24-R cells

To further validate the CDDP-resistant capability of BFTC909-R cells, a colony formation assay was performed to assess the survival impact of CDDP treatment. The results showed that 3 μM CDDP fully inhibited colony formation in BFTC909 cells, while BFTC909-R cells formed colonies even at 7 μM CDDP (**Figure 2A**). Similarly, T24-R cells survived at 4 μM CDDP, whereas 2 μM was sufficient to eliminate all T24 cells (**Figure 2B**). We further confirmed the expression of common drug-resistance genes, including multidrug resistance 1 (MDR1) and DNA excision repair protein ERCC1, in BFTC909-R and T24-R cells. We observed that the mRNA expression levels of MDR1 and ERCC1 were significantly higher in BFTC909-R cells (**Figure 2C**, left) and T24-R cells (**Figure 2C**, right) compared to their parental BFTC909 and T24 cells. Similarly, the protein levels of MDR1 and ERCC1 were also elevated in BFTC909-R (**Figure 2D**, left) and T24-R cells (**Figure 2D**, right) relative to their parent cells. These findings demonstrated that BFTC909-R and T24-R cells exhibit cisplatin resistance characteristics.

Analysis of differentially expressed proteins identified by iTRAQ assay and GO/KEGG functional characterization

To identify proteins involved in the mechanism of CDDP resistance, differences in protein expression between BFTC909 and BFTC909-R cells were compared after labeling with stable isotope reagents 114, 115, 116, and 117, respectively (**Figure 3A**). The iTRAQ analysis identified a total of 95,717 peptides, including 5,261 unique peptides and 4,655 proteins. Differentially expressed proteins (DEPs) between drug-resistant and parental UC cell lines were determined using the following criteria: (1) proteins detected in all four datasets; (2) proteins with an average $\log_2$ ratio change less than -1 or greater than 1. Compared to parental cells, 154 DEPs were up-regulated and 124 were down-regulated in CDDP-resistant cells. The top-ranked proteins based on fold-change are listed in **Table 1**.

To begin our analysis, we examined the dominantly expressed proteins based on Gene Ontology (GO) database categorizations into biological process (BP), cellular component (CC), and molecular function (MF). In the BP category, the most differentially expressed proteins were involved in nucleosome assembly, response to virus, defense response to virus, type I interferon signaling pathway, and negative regulation of viral genome replication. For the CC category, these proteins were primarily associated with extracellular exosomes, cytoplasm, nucleosomes, cytosol, and nuclear heterochromatin. Additionally, in the MF category, most proteins were identified with functions such as protein binding, poly(A) RNA binding, protein homodimerization activity, histone binding, and protein heterodimerization activity (**Figure 3B-D**). Furthermore, KEGG pathway analysis indicated that the top three pathways associated with the DEPs were systemic lupus erythematosus, glutathione metabolism, and alcoholism (**Figure 3E**). We further investigated protein-protein interactions (PPIs) using STRING software. The PPI network included the most highly DEPs as nodes. These proteins might participate in resistance mechanisms by interacting with each other (**Figure 3F**).

Identification of YES1 protein associated with CDDP resistance

Among the DEPs associated with CDDP resistance, we observed that the expression of the tyrosine kinase YES1 protein was lower in BFTC909-R cells compared to BFTC909 cells. This was confirmed by the detection of the amino acid sequence LLLNPGNQR through LC-MS/MS analysis (**Figure 4A**). We further confirmed that the expression levels of YES1 and its downstream protein YAP and its phosphorated form (p-YAP) were decreased in BFTC909-R and T24-R cells compared to their parental cisplatin-sensitive counterparts (**Figure 4B**). Additionally, we assessed the expression of YES1 and YAP at both mRNA and

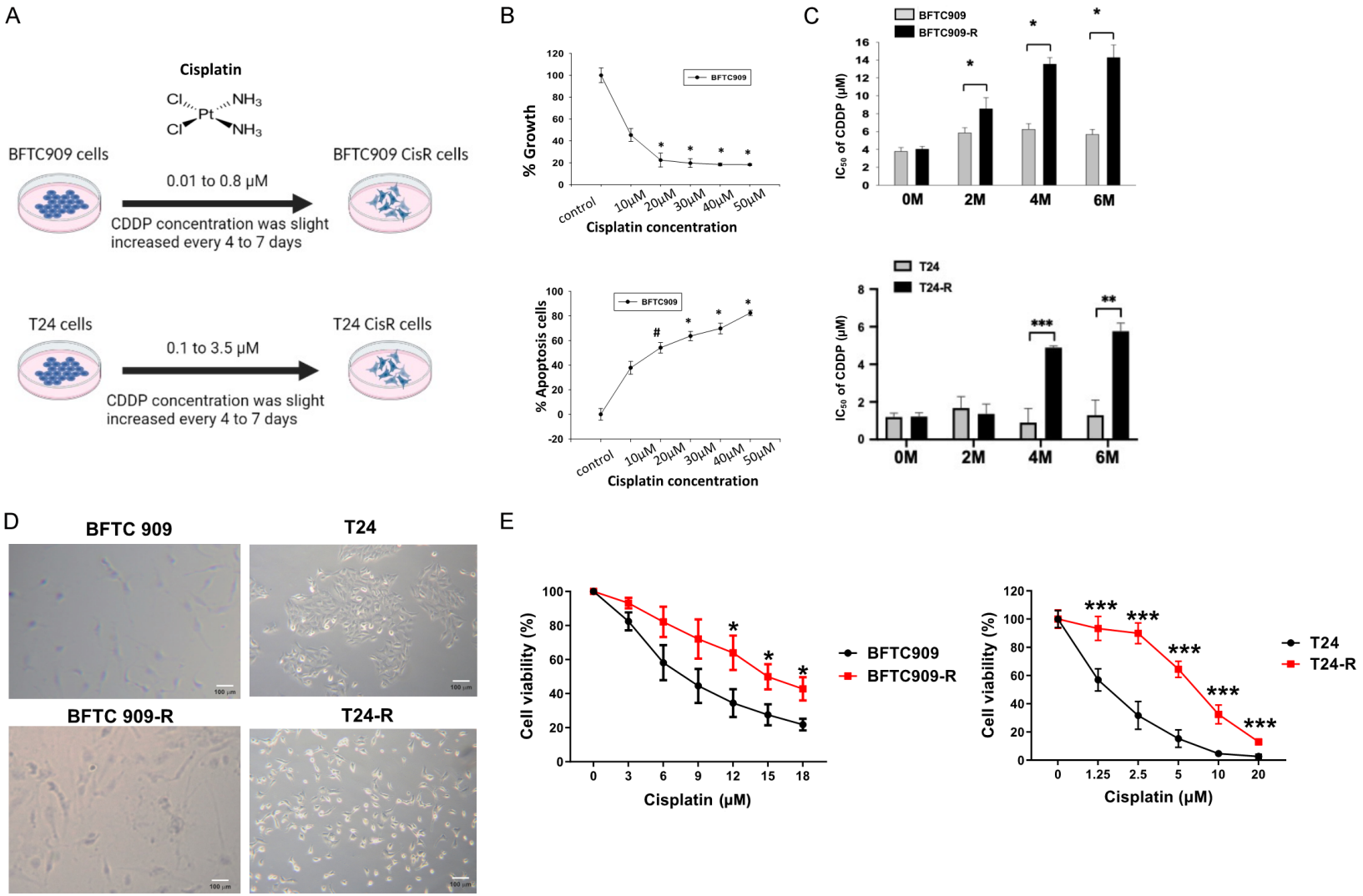

Figure 1. Induction of cisplatin-resistance in urothelial carcinoma cell lines. A. Schematic representation of the process used to establish CDDP-resistant BFTC909 (BFTC909-R) and T24 (T24-R) cells. B. The proportion of cell apoptosis increased, showing an inverse correlation with cell growth as the concentration of CDDP was elevated. C. The IC_{50} value of BFTC909, BFTC909-R, T24 and T24-R cells treated with CDDP at 2, 4, 6 months were determined by using the MTT assay. D. The morphology of BFTC909, BFTC909-R, T24, and T24-R cells was observed under a microscope after 2 days of CDDP treatment. E. Cell viability of BFTC909, BFTC909-R (left panel), T24, and T24-R (right panel) cells treated with different doses of CDDP was assessed using the MTT assay. Statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

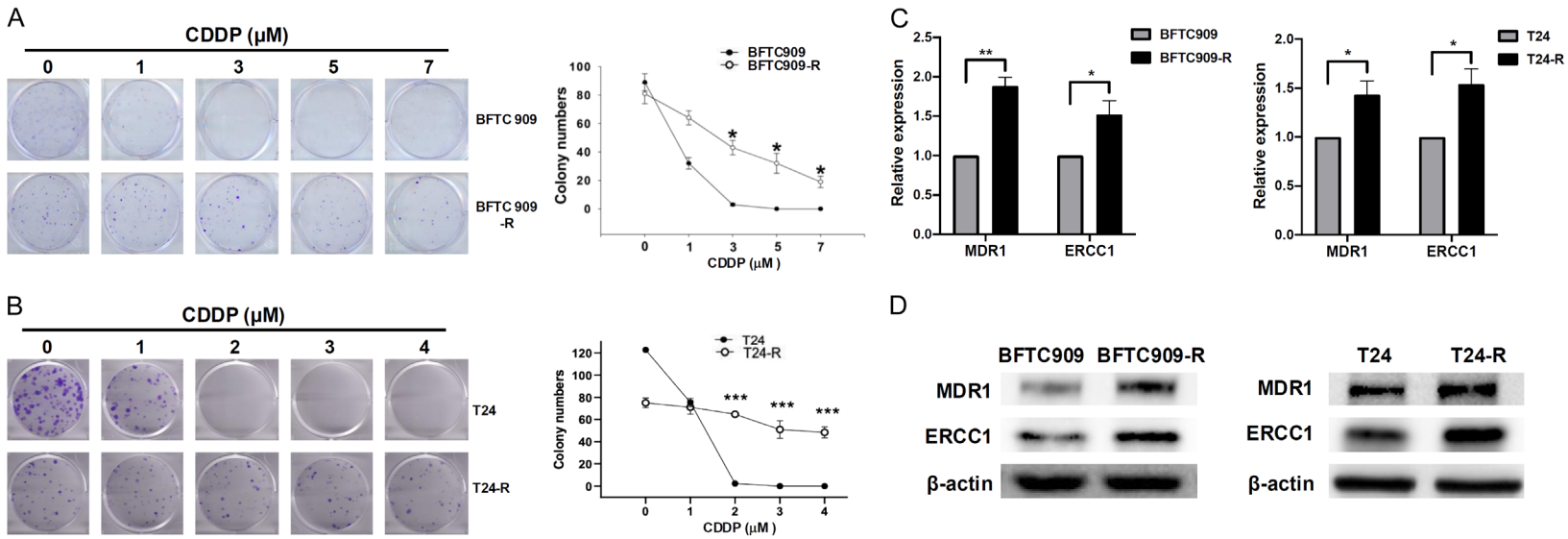

Figure 2. Colony formation and resistance marker expression were assessed in UC cells. The sensitivity of BFTC909 and BFTC909-R cells (A), as well as T24 and T24-R cells (B), to CDDP was evaluated through a colony formation assay. The mRNA (C) and protein (D) expression levels of MDR1 and ERCC1 were determined using qPCR as and western blot, respectively. **P < 0.01; ***P < 0.001.

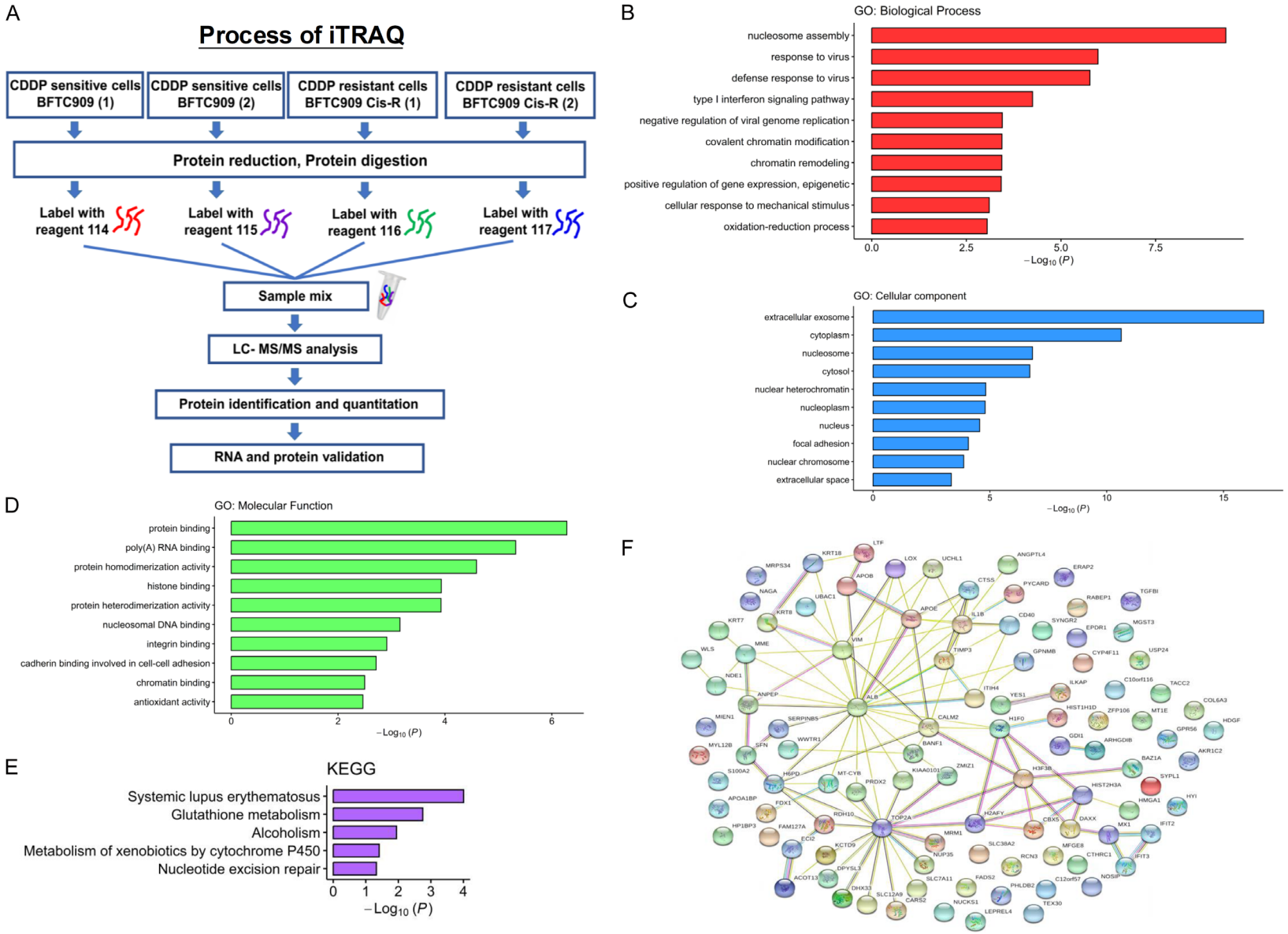

Figure 3. A. Schematic representation of the iTRAQ process. Samples from parental and CDDP-resistant cells (BFTC909 and BFTC909 Cis-R) were labeled with iTRAQ reagents (114, 115, 116, 117) for quantitative proteomics analysis. After labeling, the samples were mixed and subjected to LC-MS/MS analysis, followed by

protein identification and quantification. RNA and protein validation were performed subsequently. B-D. Gene Ontology (GO) analysis of differentially expressed proteins (DEPs). B. Biological processes (BP) with the most enriched DEPs include nucleosome assembly, response to virus, and defense response to virus. C. Cellular components (CC) primarily involve extracellular exosome, cytoplasm, and nucleosome. D. Molecular function (MF) highlights protein binding, poly(A) RNA binding, and protein homodimerization activity as the most enriched categories. E. KEGG pathway analysis reveals systemic lupus erythematosus, glutathione metabolism, and alcoholism as the top pathways enriched in DEPs. F. Protein-protein interaction (PPI) network analysis using STRING software shows the most significantly interacting DEPs, potentially contributing to drug resistance mechanisms.

Table 1. Highest-ranked upregulated and downregulated proteins

Number	Protein name	Gene	MW [kDa]	Sequence covered %	MASCOT score	116/114 Regulation (fold-change)
1	ARF GTPase-activating protein	GIT2	84.49	4.479578	93.143	0.166
2	HLA class I histocompatibility antigen	HLA-B	40.312	35.35912	478.75	0.167
3	Testis-expressed sequence 30 protein	TEX30	25.568	18.06167	60.5876	0.171
4	Cellular tumor antigen p53	TP53	43.625	6.615776	54.37	0.182
5	Protein CLN8	CLN8	32.766	9.440559	81.86	0.184
6	Cell cycle checkpoint protein RAD1	RAD1	31.807	5.673759	38.308	0.185
7	Prostaglandin E synthase	PTGES	17.091	6.578947	44.95	0.192
8	Transmembrane protein 201	TMEM201	72.19	5.405405	119.41	0.235
9	Metalloproteinase inhibitor 3	TIMP3	24.129	13.74408	71.38	0.248
10	Ceramide synthase 6	CERS6	44.861	3.645833	33.13	0.261
11	Tyrosine-protein kinase Yes	YES1	60.763	25.41436	298.163	0.263
12	Histone H3	HIST2H3A	15.394	64.70588	723.664	3.584
13	Annexin A8-like protein 1	ANXA8L1	36.856	44.64832	643.28	3.626
14	Serpin B5	SERPINB5	42.073	19.2	160.987	3.636
15	Beta-taxilin	TXLNB	76.472	2.192982	29.71	3.785
16	Cathepsin S	CTSS	37.471	12.99094	65.296	3.945
17	Metallothionein-1E	MT1E	6.009	67.21311	548.883	3.955
18	14-3-3 protein sigma	SFN	27.757	71.37097	1647.65	4.508
19	Protein S100-A2	S100A2	11.109	27.55102	219.182	5.388
20	Copine-9	CPNE9	61.825	3.074141	72.459	5.407
21	Stonin-2	STON2	101.102	0.994475	38.172	6.008
22	Fatty acid-binding protein	FABP4	15.155	72.59259	87.72	7.37
23	Peptidase M20 domain-containing protein 2	PM20D2	47.746	2.522936	37.55	9.421

protein levels in several UC cell lines. The results indicated that RT4 and UMUC14 exhibited relatively higher levels of YES1 and phosphorylated YES1 (Y537) compared to BFTC909 and T24 cells (**Figure 4C-E**).

YES1 inhibition reduces cisplatin sensitivity in UC cells

To further investigate the relationship between YES1 expression and CDDP resistance, six UC cell lines (BFTC909, T24, J82, RT4, UMUC14, and SV-HUC-1) were treated with varying doses of CDDP. The results revealed that J82 exhibited the highest resistance to CDDP, while UMUC14 was the most sensitive (**Figure 5A**). Additionally, J82 cells had the lowest expression levels of YES1 and its active form, phospho-YES1 (Y537), while UMUC14 cells showed the highest expression levels of both YES1 and phospho-YES1 (**Figure 4D**). To further explore the role of YES1 in CDDP resistance, UMUC14 cells, which had the highest YES1 expression, were treated with the YES1 inhibitor dasatinib. Increasing dasatinib doses led to a proportional decrease in pYES1 expression in UMUC14 cells (**Figure 5B**). Additionally, UMUC14 cells were treated with CDDP, dasatinib, and a combination of both, and cell death distribution was assessed using flow cytometry. CDDP-treated

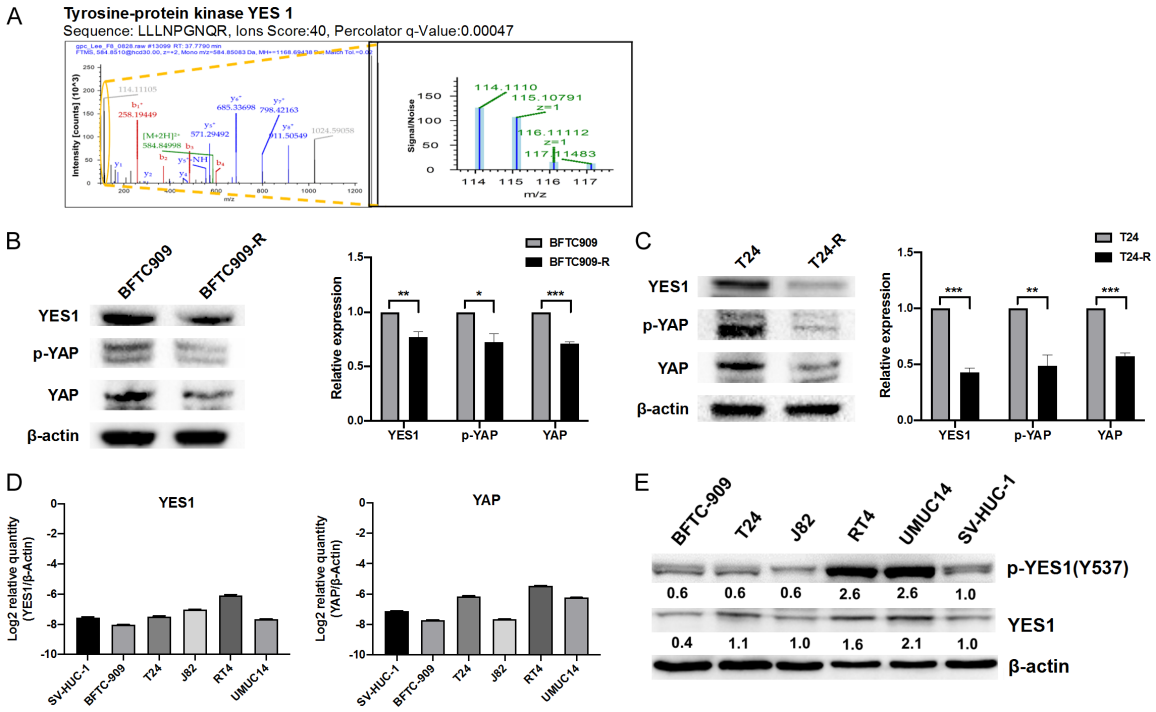

Figure 4. Identification of the YES1 protein by LC-MS/MS analysis. A. The left panel shows the YES1 peptide sequence LLLNPGNQR identified with an ion score of 40 and a q-value of 0.00047, while the right panel illustrates the relative quantification from the iTRAQ experiment. B, C. Western blot shows reduced YES1, p-YAP and YAP expression in cisplatin-resistant (Cis-R) BFTC-909 and T24 cells compared to control (Ctrl) cells. D. Quantification of YES1 and YAP expression across various UC cell lines. E. Western blot analysis of phosphorylated YES1 (pYES1, Y537) and total YES1 expression across different UC cell lines.

cells showed a high cell death rate, but sensitivity decreased when dasatinib was combined with CDDP (**Figure 5C**). Furthermore, the combination of dasatinib and CDDP reduced apoptosis in UMUC14 cells (**Figure 5D**). Taken together, inhibition of YES1 expression by dasatinib induced CDDP resistance in UMUC14 cells.

High expression of YES1 related to improved survival in urothelial carcinoma

To further explore the clinical relationship between YES1 expression and patient survival, we analyzed tumor specimens from UC patients at Kaohsiung Chang Gung Memorial Hospital using IHC staining to assess YES1 levels. All patients in this cohort analysis received platinum-based chemotherapy for metastatic UC. **Figure 6A**, **6B** demonstrated varying YES1 expression levels across patients. Kaplan-Meier analysis revealed that mUC patients with high YES1 expression had improved overall survival and progression-free survival (**Figure 6C**). Additionally, analysis of public datasets (GSE13507 and GSE169455) showed that patients with tumors harboring high YES1 expression had better overall survival (**Figure 6D**). These findings suggest that high YES1 may enhance CDDP sensitivity and could serve as a prognostic marker in UC patients.

Discussion

Platinum-based chemotherapy continues to be the cornerstone of treatment for mUC. Although recent advances in ICIs and ADCs have expanded the therapeutic options for mUC and extended OS, platinum-based chemotherapy remains the most commonly used treatment in real-word settings [3, 5, 6]. A large-cohort study demonstrated that mUC patients treated with platinum-based chemotherapy have longer survival compared to those receiving non-platinum treatments, suggesting that platinum compounds effectively extend the survival of mUC paitents [17, 18]. The initial success of cisplatin-based chemotherapy in treating mUC is often limited by the rapid onset of drug resis-

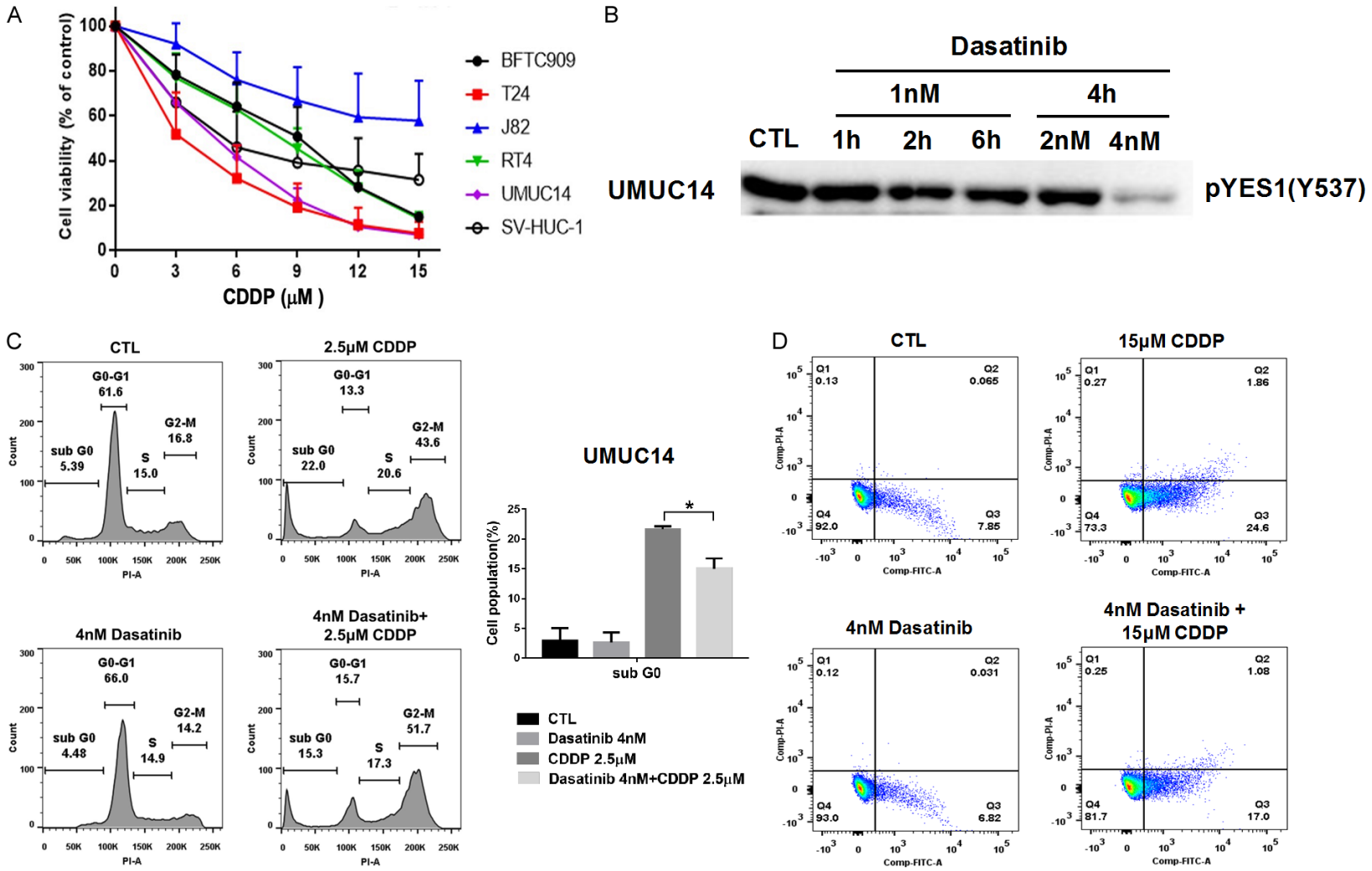

Figure 5. Effect of CDDP and dasatinib in UC cells viability. A. Viability of different UC cell lines after CDDP treatment. B. Western blot shows reduced pYES1 (Y537) levels in UMUC14 cells treated with dasatinib. C. Cell cycle analysis reveals an increased sub-G0 population with the combined treatment of dasatinib and CDDP, indicating enhanced apoptosis. D. Apoptosis is significantly elevated in UMUC14 cells with the combination of CDDP and dasatinib compared to individual treatments.

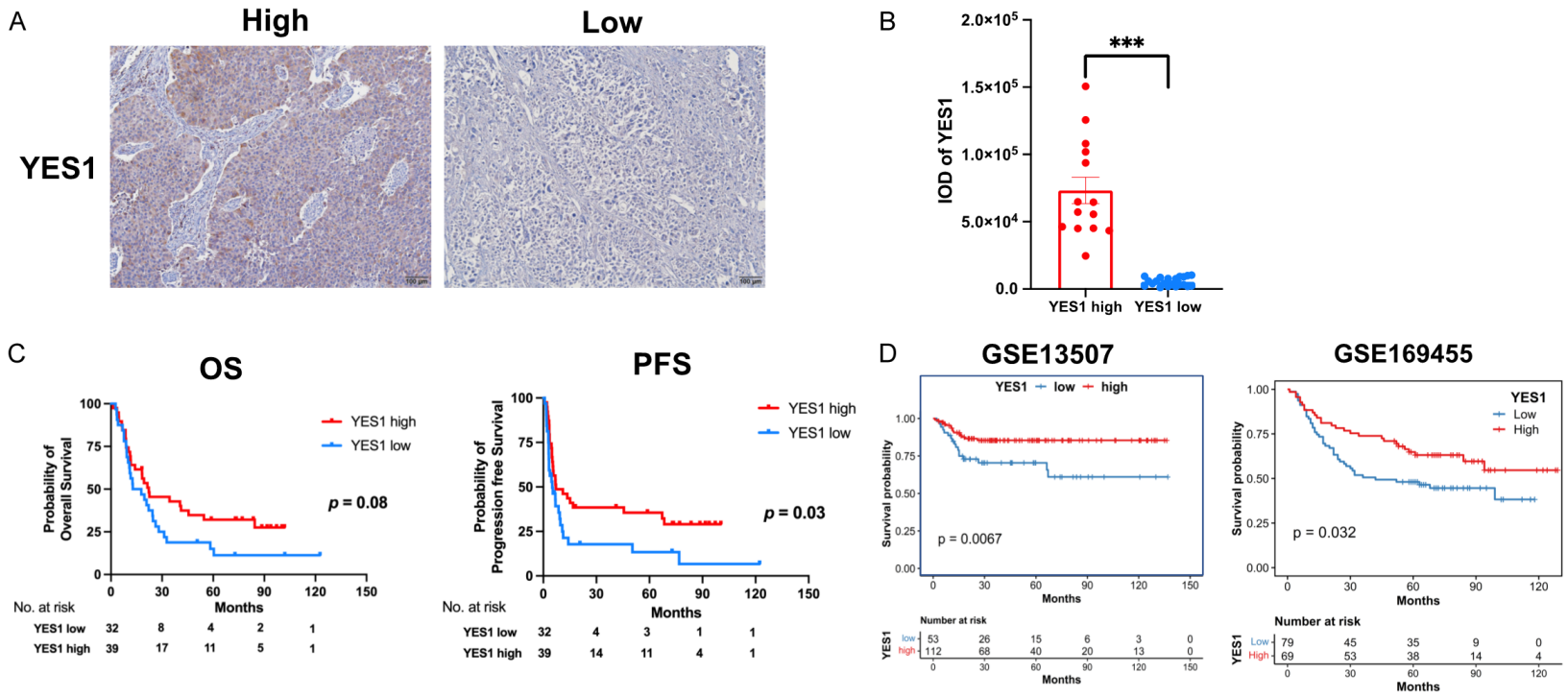

Figure 6. The correlation between YES1 expression in tumor samples and the probability of patient survival. A. Immunohistochemical (IHC staining reveals high and low expression of YES1 in urothelial carcinoma (UC) tissues. B. YES1 expression levels were quantified using Image J software equipped with the IHC Toolbox plugin. C. Kaplan-Meier survival curves indicate that high YES1 expression is associated with longer progression-free survival (PFS, P = 0.03), while overall survival (OS) shows a trend toward significance (P = 0.08). D. Validation with GSE13507 and GSE169455 data sets reinforces the link between higher YES1 expression and better survival rates (P = 0.0067 and P = 0.032, respectively). IOD: Integrated Optical Density. ***P < 0.001. Scale bar, 100 μM.

tance. As a result, research is focused on understanding the mechanisms behind this resistance and enhancing cisplatin's efficacy by combining it with new treatments to prolong its effectiveness. In this study, we induced cisplatin-resistant UC cells in vitro and investigated the differences in protein expression compared to their parental cells using the iTRAQ assay. We demonstrated that YES1 expression is downregulated in cisplatin-resistant BFTC909-R cells compared to parental BFTC909 cells. Inhibition of YES1 with dasatinib in the highly expressing UMUC14 cells resulted in decreased cisplatin sensitivity, suggesting that YES1 contributes to the regulation of chemoresistance in UC.

YES1 belongs to the SRC family of non-receptor tyrosine kinases. This family consists of c-SRC, FYN, LYN, FGR, BLK, HCK, LCK, FRK, and YES1, all of which are essential regulators of several signal transduction pathways [10, 19]. Notably, YES and LYN directly interact with EGFR and regulate its nuclear translocation, suggesting a central role in the EGFR signaling pathway [20, 21]. Several preclinical studies have highlighted the association between YES1 and the resistance mechanisms to targeted therapies in solid cancers. YES1 has been identified as a key tyrosine kinase regulating the EGFR inhibitors response in non-small cell lung cancer (NSCLC). Fan et al. demonstrated that EGFR mutant lung cancer cells with overexpressed YES1 exhibited resistance to all EGFR inhibitors [14]. This resistance was reversed by knocking down YES1 using siRNA, indicating that YES1 amplification mediates acquired resistance to EGFR inhibitors in lung cancer. Furthermore, overexpression of YES1 was observed in both T-DM1-resistant and neratinib-resistant HER2 positive breast cancer cells [13, 15]. Targeting YES1 with dasatinib and osimertinib has shown a strong antitumor response in NSCLC xenografts in previous studies, reversing resistance by inhibiting YES1 phosphorylation [22]. Several ongoing clinical trials are currently investigating the combination of dasatinib with EGFR inhibitors for treating EGFR mutant NSCLC.

The well-established link between YES1 and EGFR inhibitors contrasts with the uncertain role of YES1 in chemotherapy resistance in solid tumors. Previous studies have suggested that YES1 upregulation may be linked to chemotherapy resistance to paclitaxel and 5-fluorouracil (5-FU) [16, 23, 24]. Wang et al. showed that YES1 and its phosphorylation during mitosis contribute to paclitaxel resistance, with cyclin-dependent kinase 1 (CDK1) phosphorylating YES1, leading to mitotic arrest and apoptosis in ovarian cancer during paclitaxel treatment [23]. Touil et al. found that 5-FU resistant colon cancer cells overexpress YES1 and exhibit stem cell-like traits, with highly elevated YES1 and YAP levels in liver metastases correlating with poor overall survival [16]. The relationship between YES1 and cisplatin resistance has been minimally explored. Zhou et al. demonstrated that miR-133a reduces cisplatin resistance in ovarian cancer cells by directly targeting the 3'UTR of YES1, leading to its downregulation [25]. However, this finding was not corroborated in human specimens. In contrast, a retrospective analysis of 132 epithelial ovarian cancer (EOC) patients revealed that elevated YES1 expression is significantly correlated with improved OS and PFS, along with greater sensitivity to platinum-based chemotherapy [26]. Our data also suggest that YES1 downregulation may contribute to cisplatin resistance by disrupting the nuclear localization of YAP, a key regulator of cell proliferation and apoptosis. In cisplatin-resistant UC cells, lower YES1 expression was associated with decreased YAP levels, which may enable these cells to evade the cytotoxic effects of chemotherapy by entering a quiescent state.

Despite the promising results of this study, several limitations need to be acknowledged. First, while the in vitro models provide valuable insights into the role of YES1 in cisplatin resistance, they may not fully capture the complexity of the tumor microenvironment in vivo. Second, although the clinical data indicate a strong correlation between YES1 expression and cisplatin sensitivity, the retrospective nature of the analysis limits the ability to establish definitive causality. Additionally, while we observed a link between YES1 expression and YAP regulation, the precise molecular mechanisms by which YES1 influences YAP activity in the context of chemotherapy resistance remain unclear. Further mechanistic studies are required to elucidate the downstream signaling pathways involved. Lastly, the use of dasatinib, a broad-spectrum SRC kinase inhibitor, in our experiments may affect the speci-

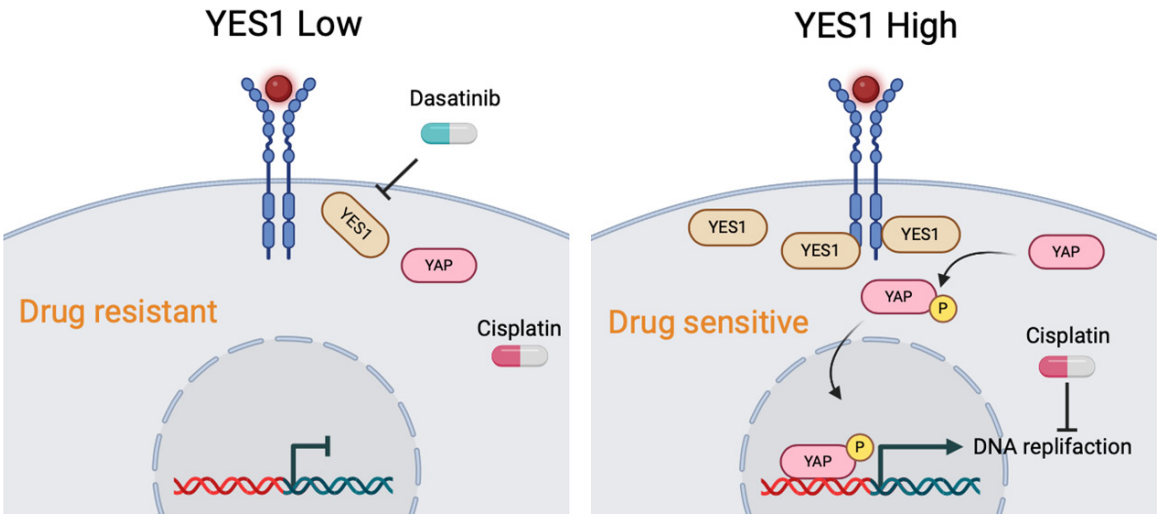

Figure 7. The schematic illustrates the role of YES1 in cisplatin-resistant and sensitive UC cells. When YES1 levels are low o11r inhibited by dasatinib, cisplatin becomes less effective due to a reduction in the DNA replication machinery (left panel). In contrast, high YES1 expression promotes DNA replication through YAP1 activation, allowing cisplatin to efficiently block DNA replication and induce cell death (right panel). This diagram was created using BioRender.com.

ficity of our findings, potentially limiting the interpretation of its impact on cisplatin sensitivity.

Conclusion

In summary, our study highlights the crucial role of YES1 in regulating cisplatin resistance in UC through its interaction with the YAP signaling pathway. The downregulation of YES1 in cisplatin-resistant cells suggests it as a key factor influencing chemotherapy response, with clinical data showing that high YES1 expression correlates with improved survival outcomes in UC patients treated with cisplatin. Further exploration of the molecular mechanisms behind YES1 and the development of selective YES1 inducer could lead to new therapeutic approaches to combat chemoresistance in UC (**Figure 7**).

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Disclosure of conflict of interest

None.

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