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GULP1 as a Downstream Effector of the Estrogen Receptor-β Modulates Cisplatin Sensitivity in Bladder Cancer

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Abstract. *Background/Aim: Precise molecular mechanisms underlying resistance to cisplatin-based chemotherapy remain unclear, while the activity of estrogen receptor-β (ERβ) has been suggested to be associated with chemosensitivity in urothelial cancer. We aimed to determine if GULP1, an adapter protein known to facilitate phagocytosis, could represent a downstream effector of ERβ and thereby modulate cisplatin sensitivity in bladder cancer. Materials and Methods: GULP1 expression and cisplatin cytotoxicity were compared in bladder cancer lines. Immunohistochemistry was used to determine the expression of GULP1 and ERβ in two sets of tissue microarray (TMA) consisting of transurethral resection specimens. Results: The levels of GULP1 expression were considerably higher in ERβ-knockdown sublines than in the respective control ERβ-positive sublines. Estradiol treatment reduced GULP1 expression in ERα-negative/ERβ-positive lines, which was restored by the anti-estrogen tamoxifen. Chromatin immunoprecipitation assay revealed the binding of ERβ to the GULP1 promoter in bladder cancer cells. Moreover, GULP1 knockdown sublines were significantly more resistant to cisplatin treatment, but not to other chemotherapeutic agents, including gemcitabine, methotrexate, vinblastine, and doxorubicin. In the first set of TMA (n=129), the expression of ERβ and GULP1 was inversely correlated (p=0.023), and ERβ(–)/GULP1(+) in 51 muscle-invasive tumors was associated with significantly lower risk of disease progression and cancer-specific mortality. Similarly, in the second set (n=43), patients with ERβ(–)/GULP1(+) muscle-invasive disease were significantly (p=0.021) more likely to be responders to cisplatin-based neoadjuvant chemotherapy before radical cystectomy. Conclusion: ERβ activation was found to reduce the expression of GULP1 as a direct downstream target in bladder cancer cells, resulting in the induction of cisplatin resistance.*

Urothelial carcinoma in the urinary bladder is a commonly diagnosed malignancy, especially among men, and the global number of bladder cancer-related deaths has increased; *e.g.*, 165,100 in 2012 (1) to 220,000 in 2022 (2). Clinically, there exist two distinct forms of bladder cancer, non-muscle-invasive and muscle-invasive diseases that are associated with the risk of developing postoperative recurrence occasionally with invasive tumor and metastatic disease, respectively. In particular, overall oncologic outcomes of patients with lymph node metastasis [*i.e.*, 5-year survival rate of 39.5% (3)] or distant metastasis [*i.e.*, 8.8% (3)] remain unfavorable. Urothelial carcinoma also develops in the upper urinary tract, which is often [*e.g.*, 60% (4)] invasive at the time of initial diagnosis.

New modalities, such as immune checkpoint blockade and other targeted therapies, have recently been employed for the treatment of urothelial cancer (5-9). Nonetheless, cisplatin (CDDP)-based systemic chemotherapy, such as "GC" [gemcitabine (GEM) + CDDP] and "MVAC" [methotrexate (MTX) + vinblastine (VBL) + adriamycin (doxorubicin; DXR) + CDDP], remains the standard of care for locally advanced or metastatic urothelial carcinoma. Remarkably, a considerable number of patients fail to respond to such combination

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chemotherapy in neoadjuvant and adjuvant settings (4-8, 10-12). Accordingly, novel strategies for enhancing and/or predicting chemosensitivity should offer considerable improvements in the management of advanced urothelial cancer.

The precise molecular mechanisms responsible for CDDP resistance are still not fully understood (13, 14). Meanwhile, a growing body of evidence suggests an important role of sex hormone receptors, including androgen receptor (AR) (15) and estrogen receptors (ERs) (16), particularly ERβ, in the development and progression of urothelial cancer, as well as the modulation of conventional non-surgical therapy for bladder cancer (17). Specifically, activation of AR (18, 19) or ERβ (20, 21) signaling has been implicated in inducing CDDP resistance in bladder cancer, while its underlying mechanisms remain largely uncharacterized. More recently, our data suggested that GULP1, an adaptor protein known to facilitate phagocytosis (22), could modulate sensitivity to CDDP therapy (23). In the present study, we investigated whether GULP1 could function as a downstream effector of ERβ in bladder cancer cells and thereby modulate CDDP sensitivity, which might lead to the development of a strategy for chemosensitization.

Materials and Methods

Antibodies and chemicals. We obtained anti-ERβ (H-150 or B-3), anti-GULP1 (E-4), and anti-GAPDH (6c5) antibodies from Santa Cruz Biotechnology (Dallas, TX, USA). CDDP, 17β-estradiol (E2), and tamoxifen (TAM) were from Sigma-Aldrich (St. Louis, MO, USA), whereas GEM, MTX, VBL, and DXR were from Cayman Chemical (Ann Arbor, MI, USA).

Cell lines. Human bladder urothelial carcinoma cell lines, UMUC3, 5637, and 647V were originally obtained from the American Type Culture Collection (Manassas, VA, USA) and thereafter authenticated by the institutional core facility using GenePrint 10 System (Promega, Madison, WI, USA). Sublines stably expressing ERβ-short hairpin RNA (shRNA) [*i.e.*, UMUC3-ERβ-shRNA (24), 5637-ERβ-shRNA (24)], GULP1-shRNA [*i.e.*, UMUC3-GULP1-shRNA (23), 647V-GULP1-shRNA (23)], or control-shRNA [*i.e.*, UMUC3-control-shRNA (25), 5637-control-shRNA (24), 647V-control-shRNA (24)] were established in our previous studies. These parental lines and their stable sublines were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (both 100 units/ml), and then cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for the experimental treatment with E2/TAM or 5% FBS for other experiments at least 24 h before actual assays.

Western blotting. Total proteins were extracted from the cells collected with RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Halt™; Thermo Fisher Scientific), and the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) was used for the determination of protein concentration. Equal amounts of proteins (30 μg) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane electronically, blocked with 0.03-0.3% Blotting-Grade Blocker (Bio-Rad), and incubated with a primary antibody [*i.e.*, ERβ (dilution 1:100), GULP1 (dilution 1:100), GAPDH (dilution 1:1,000)] at 4˚C overnight and a HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Chemiluminescent signals were generated by a Clarity Western ECL Substrate (Bio-Rad) and detected by ChemiDOC™ MP (Bio-Rad).

Chromatin immunoprecipitation (ChIP). We first performed a bioinformatic search [LASAGNA-Search 2.0. Available online at https://biogrid-lasagna.engr.uconn.edu/lasagna_search (26)] for identifying potential ERβ binding sites in the promoter region of *GULP1* and found a target site (Figure 1C). A ChIP assay was then performed, using a Magna ChIP kit (Sigma-Aldrich) according to the manufacturer's recommended protocol with minor modifications, as we previously described (23, 24). Briefly, UMUC3 cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and the cell lysates were sonicated in nuclear buffer (four 30-s pulses, output 3.0, duty cycle 30% in ice with 120-s rest between pulses; Branson Sonifier 450). Soluble chromatin was immunoprecipitated with an anti-ERβ antibody or normal mouse IgG (sc-2025, Santa Cruz Biochemistry) directly conjugated with Protein A magnetic beads (Thermo Fisher Scientific). Immunoprecipitated DNA was eluted and reverse cross-linked, and DNA was extracted and purified using a spin filter column (Thermo Fisher Scientific). DNA samples were eventually analyzed by PCR, using the following primer set: forward, 5'-CCAGAGATTAAGGCCGAGTTAAA-3'; reverse, 5'-GCCCGGACAGAAGCAAA-3'. The PCR products electrophoresed on 1% agarose gel and stained with ethidium bromide were visualized using Gel Doc XR+ (Bio-Rad).

Cell proliferation. The MTT (3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide) assay was used to assess the cell viability. Cells (5×10^3/well) seeded in 96-well tissue culture plates were cultured for 48 h and then incubated with 0.5 mg/ml of MTT (Sigma-Aldrich) for 3 h at 37˚C. MTT was dissolved by dimethyl sulfoxide, and the absorbance at 570 nm with background subtraction at 630 nm was measured.

Immunohistochemistry. Two sets of bladder tissue microarray (TMA) consisting of transurethral resection specimens, including 129 cases with urothelial neoplasm showing various tumor grades and stages, as well as 43 cases with high-grade muscle-invasive urothelial carcinoma subsequently undergoing CDDP-based neoadjuvant therapy prior to radical cystectomy, were previously constructed upon appropriate approval by the institutional review boards (21, 28). Immunostaining was performed on 5 μm sections from the TMA, using a primary antibody to ERβ or GULP1, as we previously described (21, 23, 28). Immunoreactivity was considered positive when at least 1% of tumor cells showed moderate or strong positivity or more than 10% of tumor cells showed at least weak positivity.

Statistical analysis. Student's *t*-test and Chi-square test were used to evaluate numerical data and categorized variables, respectively. Time-to-event estimates of progression-free survival and cancer-specific survival were calculated by the Kaplan-Meier method and compared by the log-rank test. All statistical analyses were performed using Excel (Microsoft, Redmond, WA, USA) or Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered to be statistically significant.

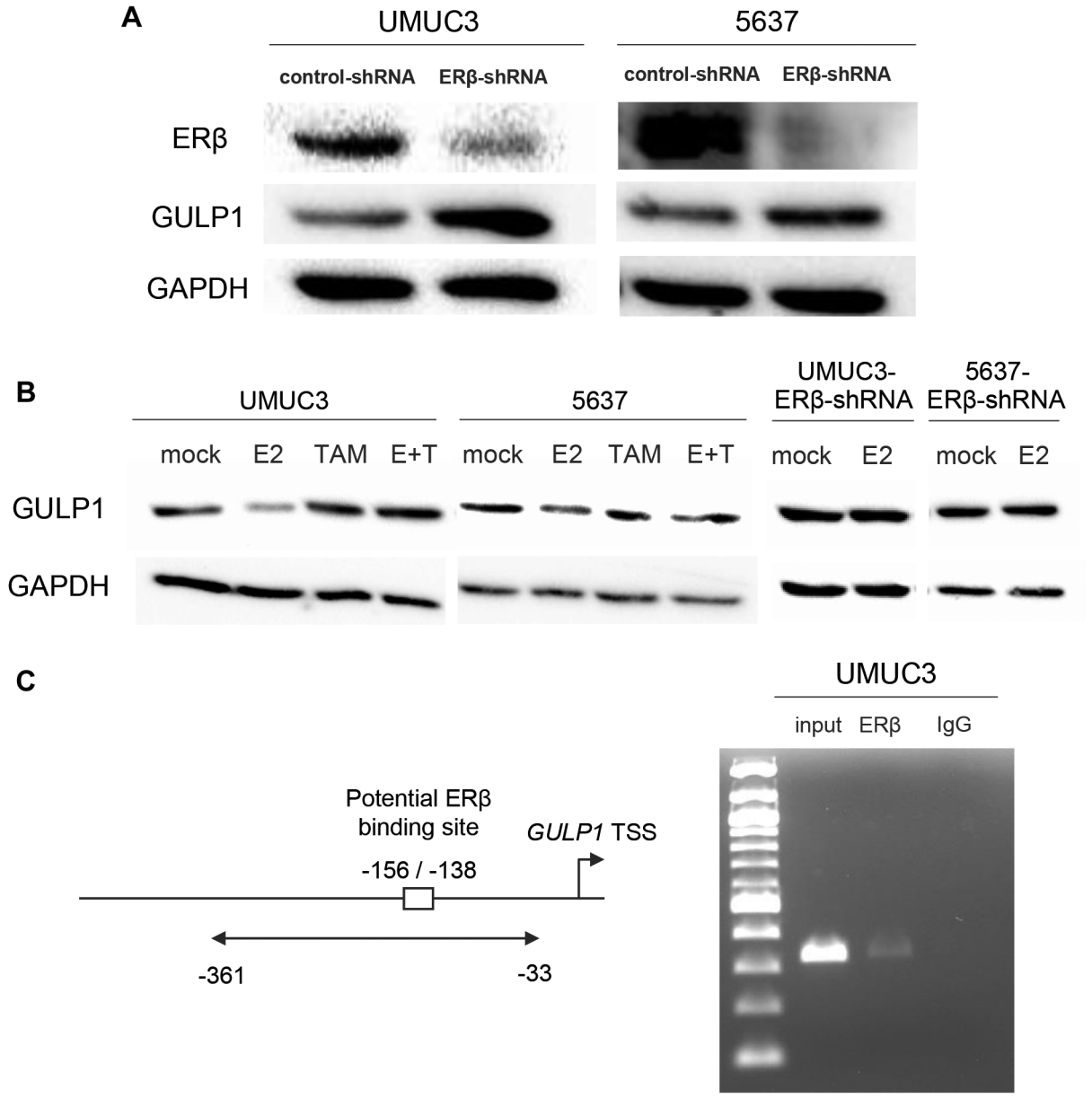

Figure 1. *Associations between estrogen receptor-β (ERβ) signaling and GULP1 expression in bladder cancer cells. (A) Western blotting of ERβ and GULP1 in UMUC3-control-shRNA vs. UMUC3-ERβ-shRNA and 5637-control-shRNA vs. 5637-ERβ-shRNA. (B) Western blotting of GULP1 in UMUC3, 5637, UMUC3-ERβ-shRNA, and 5637-ERβ-shRNA cultured for 24 h with ethanol (mock), E2 (10 nM) and/or tamoxifen (TAM; 1 μM), as indicated. GAPDH served as a loading control. (C) The ChIP assay, using UMUC3 cell lysates immunoprecipitated with an anti-ERβ antibody (or IgG as a negative control). DNA fragments were PCR amplified with a set of GULP1 promoter-specific primers, and the PCR products (i.e., 328 bp for the binding site) were electrophoresed on 1% agarose gel. A fraction of the mixture of protein-DNA complex (i.e., 1% of total cross-linked, reserved chromatin prior to immunoprecipitation) was used as "input" DNA.*

Results

Crosstalk between ERβ and GULP1. We previously demonstrated that human bladder cancer cell lines, including UMUC3, 5637, and 647V, possessed functional ERβ but did not express ERα (24, 27). We first compared the levels of GULP1 expression in control ERβ-positive and ERβ knockdown sublines. Western blotting detected GULP1 signals in all of these sublines, and its levels were considerably higher in ERβ knockdown cells than in control cells (Figure 1A). We further assessed the effects of estrogen (*i.e.*, E2) and anti-estrogen (*i.e.*, TAM) on GULP1 expression. In ERβ-positive

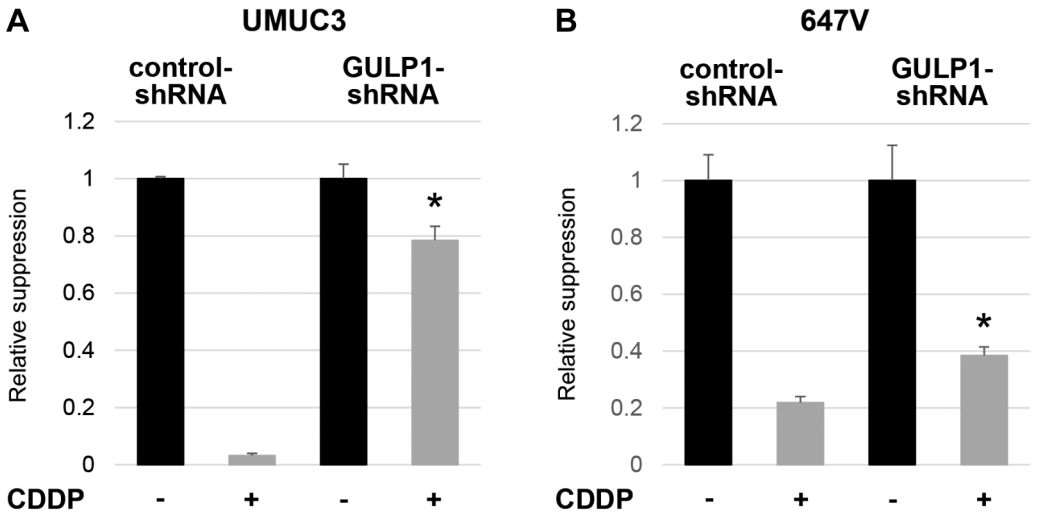

Figure 2. *Effects of GULP1 knockdown on cytotoxicity of cisplatin (CDDP) in bladder cancer cells. MTT assay in UMUC3-control-shRNA and UMUC3-GULP1-shRNA sublines (A), as well as in 647V-control-shRNA and 647V-GULP1-shRNA sublines (B), cultured for 48 h in the absence or presence of 5 μM CDDP. Cell viability is presented relative to that of each subline without drug treatment from triplicate experiments. *p<0.05 (vs. control-shRNA with CDDP).*

cell lines, E2 treatment considerably reduced GULP1 expression over the mock treatment, which was restored by TAM treatment (Figure 1B). However, E2 did not significantly change the levels of GULP1 expression in ERβ-knockdown cells. Thus, the expression of GULP1 was inversely correlated with the expression/activity of ERβ in bladder cancer cells.

Then, ChIP assay was performed upon the identification of a putative ERβ binding site in the promoter region of *GULP1 via* a bioinformatics-driven search (Figure 1C). DNA fragments from UMUC3 cells immunoprecipitated with an anti-ERβ antibody were amplified by PCR with a set of primers specific for the *GULP1* promoter. The PCR products for the potential binding site could be visualized from those precipitated by the ERβ antibody, but not control precipitations.

Impact of GULP1 on sensitivity to chemotherapeutic agents. We next assessed the impact of GULP1 expression on the cytotoxic effects of CDDP, as well as other chemotherapeutic agents clinically used in combination with CDDP, in bladder cancer cells. We compared the cytotoxicity of these drugs in control *versus* GULP1 knockdown cells. The MTT assay performed in these sublines treated with 5 μM CDDP showed that UMUC3-GULP1-shRNA (22% decrease; Figure 2A) and 647V-GULP1-shRNA (62% decrease; Figure 2B) cells were significantly more resistant to CDDP, compared with UMUC3-control-shRNA (97% decrease) and 647V-control-shRNA (79% decrease) cells, respectively. By contrast, there were no significant differences in the effects of GEM (Figure 3A), MTX (Figure 3B), VBL (Figure 3C), or DXR (Figure 3D) between control-shRNA and GULP1-shRNA sublines.

Clinical impact of ERβ/GULP1 expression in bladder cancer. We previously performed immunohistochemistry for ERβ (21, 28) and GULP1 (23) both in two separate sets of bladder TMA (Figure 4A). We then re-analyzed these data.

In the first set of TMA consisting of 129 urothelial neoplasm specimens, ERβ and GULP1 were positive in 64 (49.6%) and 96 (74.4%) of the cases, respectively. Consistent with their expression levels in cell lines described above, the immunoreactivity of ERβ *versus* GULP1 in these tumors was inversely correlated (p=0.023; Table I). We then performed Kaplan-Meier analysis coupled with the log-rank test to assess possible associations between ERβ/GULP1 expression in 51 muscle-invasive bladder tumors and oncologic outcomes after radical cystectomy. Patients with ERβ-negative/GULP1-positive tumor had a significantly lower risk of disease progression (p=0.032) or cancer-specific mortality (p=0.039) than those with ERβ-positive/GULP1-negative tumor (Figure 4B). Similarly, when the cohort was dichotomized, ERβ-negativity/GULP1-positivity was associated with significantly better progression-free survival (p=0.011) or cancer-specific survival (p=0.015) (Figure 4C).

Another set of TMA consisted of muscle-invasive bladder cancer specimens from 43 patients who had subsequently undergone CDDP-based neoadjuvant chemotherapy prior to cystectomy. When this cohort was dichotomized based on ERβ and GULP1 immunoreactivity, 9 (64.2%) of 14 patients with ERβ-negative/GULP1-positive tumor and 8 (27.6%) of 29 patients with ERβ-positive or ERβ-negative/GULP1-negative tumor were responders to the neoadjuvant therapy (p=0.021; Table II). Thus, GULP1 loss and/or ERβ positivity were strongly associated with chemoresistance.

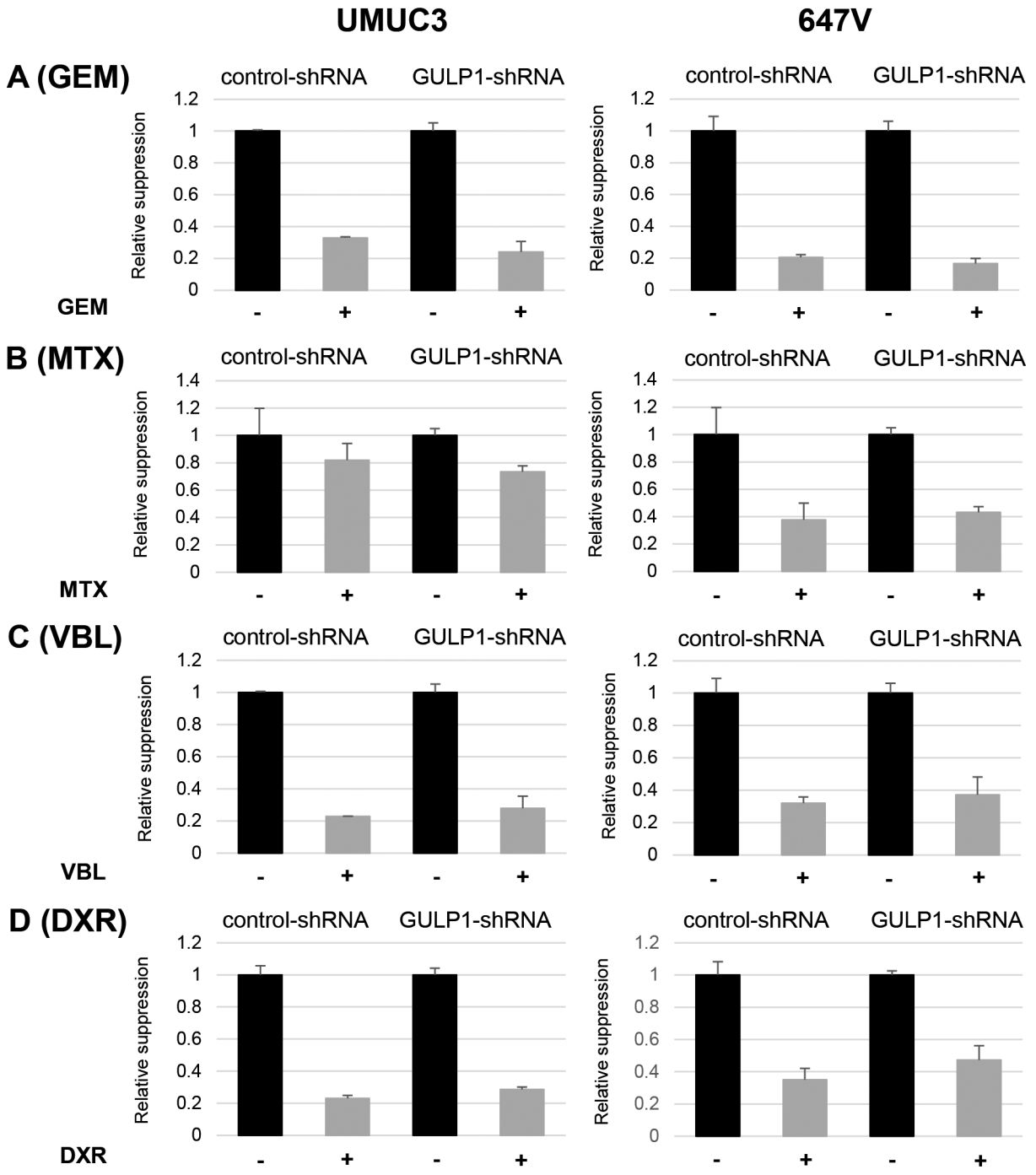

Figure 3. *Effects of GULP1 knockdown on cytotoxicity of other chemotherapeutic drugs in bladder cancer cells. MTT assay in UMUC3-control-shRNA and UMUC3-GULP1-shRNA sublines, as well as in 647V-control-shRNA and 647V-GULP1-shRNA, cultured for 48 h in the absence or presence of 5 μM gemcitabine (GEM) (A), 0.5 μM methotrexate (MTX) (B), 15 μM vinblastine (VBL) (C), or 0.5 μM doxorubicin (DXR) (D). Cell viability is shown relative to that of each subline without drug treatment from triplicate experiments.*

Discussion

Resistance to CDDP-based chemotherapy is often observed in patients with locally advanced or metastatic urothelial cancer. Importantly, its underlying mechanisms remain to be determined. Meanwhile, activation of sex hormone receptors, particularly ERβ, in bladder cancer has been linked to CDDP resistance (17, 20, 21). In addition, an adapter protein GULP1

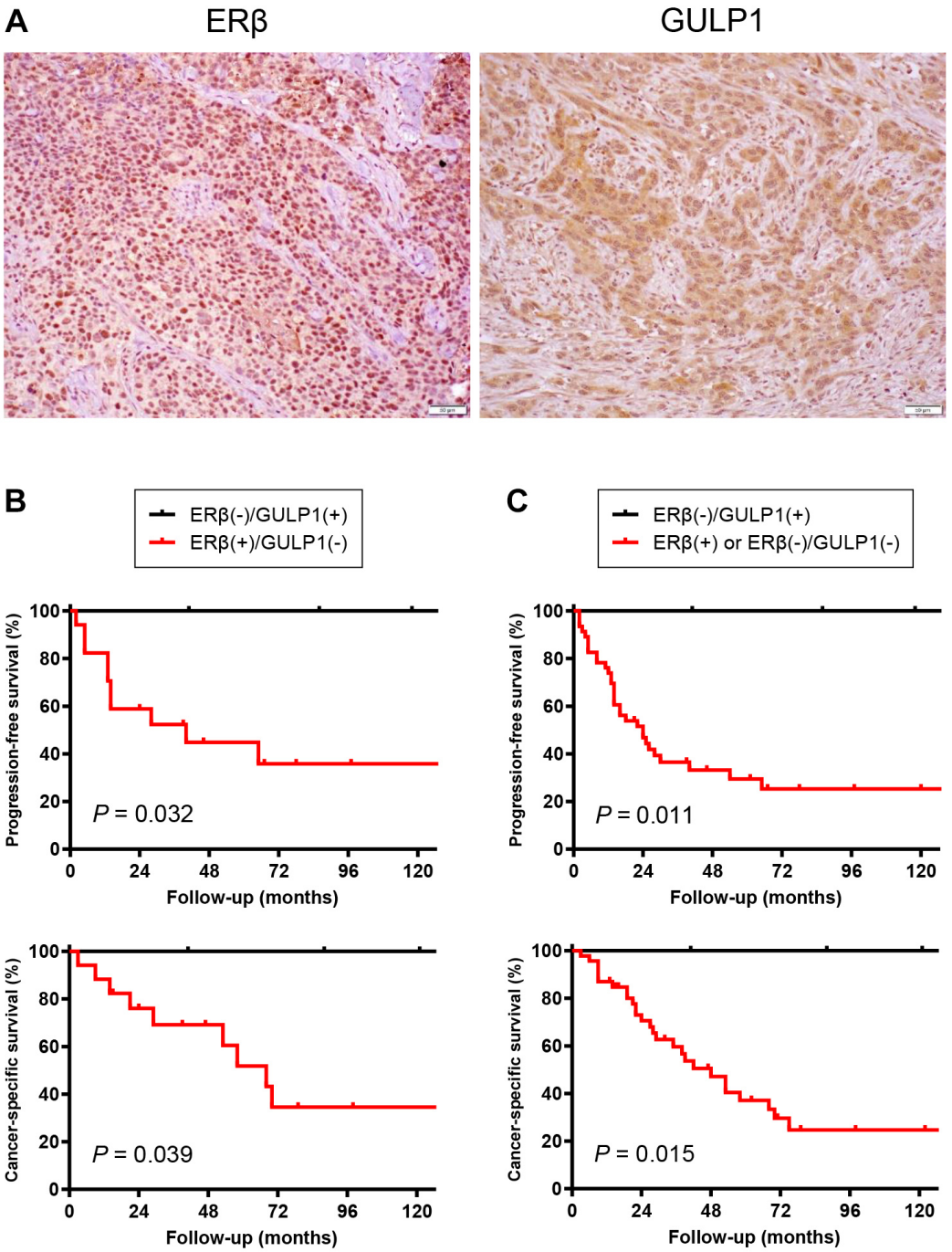

Figure 4. *Immunohistochemistry of estrogen receptor-β (ERβ) and GULP1 in surgical specimens. (A) Representative images of ERβ and GULP1 expression in bladder cancer (original magnification: 200×). Kaplan-Meier curves for progression-free survival and cancer-specific survival in patients with ERβ-negative/GULP1-positive (n=5) vs. ERβ-positive//GULP1-negative (n=17) muscle-invasive tumors (B) or ERβ-negative/GULP1-positive (n=5) vs. ERβ-positive or ERβ-negative/GULP1-negative (n=46) muscle-invasive tumors (C).*

has been shown to enhance CDDP cytotoxicity in bladder cancer cells (23, 29). In the present study, we have determined whether GULP1 functions as a downstream effector of ERβ and thereby modulates chemosensitivity in bladder cancer.

The connection between ERβ signaling and GULP1 remained unknown. We herein demonstrated that the activity of ERβ was inversely associated with the expression of GULP1 in bladder cancer cell lines. Specifically, ERβ

Table I. *Correlation of ERβ and GULP1 expression in bladder tumors.*

	GULP1(–)	GULP1(+)	*p*-Value
ERβ(–)	11	54	0.023
ERβ(+)	22	42	

ERβ, Estrogen receptor-β.

Table II. *Association of ERβ/GULP1 expression with response to neoadjuvant chemotherapy.*

	ERβ(–)/ GULP1(+)	ERβ(+) or ERβ(–)/GULP1(–)	*p*-Value
Responders	9	8	0.021
Non-responders	5	21	

ERβ, Estrogen receptor-β.

knockdown resulted in the up-regulation of GULP1 expression, whereas estrogen treatment down-regulated it, which was blocked by an anti-estrogen. Moreover, using a ChIP assay in bladder cancer cells, we demonstrated the interaction of ERβ with *GULP1* at its promoter region, indicating the direct regulation of GULP1 expression by ERβ. These results indicate that GULP1 represents a direct downstream target of ERβ signaling in bladder cancer cells.

Again, ERβ and GULP1 have been separately suggested to involve CDDP resistance in bladder cancer. However, it remains unclear how ERβ or GULP1 reduces or induces, respectively, sensitivity to CDDP therapy. Remarkably, GULP1 is known to play an important role in apoptotic cell phagocytosis (22), while phagocytosis has been implicated in chemoresistance in non-bladder cancer cells *via* modulating, for example, an anti-phagocytic molecule CD47 (30, 31). We first confirmed that GULP1 knockdown bladder cancer sublines are significantly more resistant to CDDP therapy. We then found that GULP1 knockdown did not considerably affect the cytotoxic effects of other chemotherapeutic agents widely used as combined systemic chemotherapy with CDDP, including GEM, MTX, VBL, and DXR, in patients with bladder cancer. GULP1 may, thus, represent a key downstream effector of ERβ in modulating sensitivity to CDDP therapy in bladder cancer. Nonetheless, further studies are warranted particularly to elucidate underlying molecular mechanisms responsible for ERβ/GULP1-mediated chemoresistance specific for CDDP.

We additionally analyzed our immunohistochemistry data derived from staining for ERβ (21, 28) and GULP1 (23) in two separate sets of TMA consisting of transurethral resection specimens. ERβ had been shown to be significantly up-regulated in high-grade and/or muscle-invasive tumors (28), and ERβ positivity in muscle-invasive tumors had been associated with significantly worse prognosis (28) or unfavorable response to CDDP-based neoadjuvant therapy (21). Similarly, GULP1 overexpression had been found to be associated with a favorable response to CDDP-based chemotherapy (23). By combining these data, we further found that patients with ERβ-negative/GULP1-positive muscle-invasive tumor not only showed significantly higher risks of disease progression and cancer-specific mortality but also were significantly more likely to be responders to CDDP therapy. These findings in surgical specimens further support our *in vitro* data indicating that ERβ and GULP1 signals are involved in modulating CDDP sensitivity in bladder cancer. Meanwhile, in line with our western blotting data in cell lines, the expression of ERβ and GULP1 in bladder cancer samples was inversely correlated.

Conclusion

GULP1 was found to function as a direct downstream target of ERβ in bladder cancer cells. Moreover, ERβ activation and resultant down-regulation of GULP1 expression induced resistance to CDDP therapy. Thus, concurrent anti-estrogen treatment and/or GULP1 activation have the potential of being a means of chemosensitization, particularly in female patients. Moreover, ERβ overexpression and/or GULP1 loss may serve as a predictor of CDDP resistance in patients with bladder cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest or financial ties related to this study.

Authors' Contributions

T. T. and T. M. collected and analyzed data and drafted the manuscript. T. G., G. J., A. S., M. A. E. N., and Y. T. collected data. H. M. conceived, designed, and supervised the study and analyzed data. All Authors read and approved the final manuscript for submission.

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