Expression of Estrogen Receptor Beta Predicts Oncologic Outcome of pT3 Upper Urinary Tract Urothelial Carcinoma Better Than Aggressive Pathological Features

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Upper urinary tract urothelial carcinoma (UT-UC) is rare and treatment options or prognostic markers are limited. There is increasing evidence indicating that urothelial carcinoma may be an endocrine-related cancer. The aim of this study was to analyze the prognostic effect of estrogen receptor beta (ERβ) on the outcome of UT-UC. From 2005 to 2012, this study included 105 patients with pT3 UT-UC. Perioperative factors, pathological features, and ERβ immunostaining were reviewed and prognostic effects were examined by multivariate analysis. This study divided patients into either the ERβ-high (n = 52) or ERβ-low (n = 53) group and analyzed their oncologic outcomes. All pathological features except infiltrating tumor architecture (significantly higher incidence in ERβ-low group, \( p = 0.004 \)) are symmetric in both groups. Low ERβ expression was significantly correlated with local recurrence and distant metastasis in univariate analysis (\( p = 0.035 \) and 0.004, respectively) and multivariate analysis (\( p = 0.05 \) and 0.008, respectively). Cell line study also proved that knock down of ERβ cause less UTUC proliferation and migration. In addition, ERβ agonist also enhanced the cytotoxic and migration inhibition effect of cisplatin and ERβ antagonist cause the UTUC cell more resistant to cisplatin. This result may help identify patients in need of adjuvant therapy or develop potential targeted therapy.

Upper urinary tract urothelial carcinoma (UT-UC) is relatively rare, accounting for about 5% of all urothelial carcinomas. The current standard treatment choice for locally advanced UT-UC is still radical nephroureterectomy, but the cancer-specific outcome is relatively poor. Systemic therapy might improve overall survival in patients with locally advanced disease but there is limited evidence for the benefit of neoadjuvant or adjuvant chemotherapy as most patients have renal insufficiencies and are ineligible for chemotherapy. Therefore, further research to improve oncologic outcome (local recurrence and distant metastasis in univariate analysis (\( p = 0.035 \) and 0.004, respectively) and multivariate analysis (\( p = 0.05 \) and 0.008, respectively). Cell line study also proved that knock down of ERβ cause less UTUC proliferation and migration. In addition, ERβ agonist also enhanced the cytotoxic and migration inhibition effect of cisplatin and ERβ antagonist cause the UTUC cell more resistant to cisplatin. This result may help identify patients in need of adjuvant therapy or develop potential targeted therapy.

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Figure 1. (a) 40X microscopic view of high ERβ expression specimen. (b) 100X microscopic view of high ERβ expression specimen.

|                         | ERβ (−) | ERβ (+) | p value |
|-------------------------|---------|---------|---------|
| No.                     | 53      | 52      |         |
| Follow duration         | 39.5 ± 34.3 | 34.6 ± 23.9 | 0.404   |
| Age                     | 68.6 ± 10.8 | 68.6 ± 10.0 | 0.975   |
| Smoking                 | 7(13.2%) | 7(13.5%) | 0.969   |
| Bladder cancer history  | 3(5.7%)  | 8(15.4%) | 0.119   |
| Infiltrating tumor      | 22(41.5%) | 9(17.3%) | 0.004   |
| LN positive             | 2(3.8%)  | 4(7.7%)  | 0.414   |
| LVI                     | 27(50.9%) | 20(38.5%) | 0.144   |
| CIS                     | 16(30.2%) | 20(38.5%) | 0.452   |
| SCC diff.               | 19(35.8%) | 18(34.6%) | 0.782   |
| TN                      | 17(32.1%) | 27(51.9%) | 0.060   |
| Multifocal tumor        | 9(17.0%)  | 9(17.3%)  | 0.965   |
| High grade              | 52(98.1%) | 52(100%)  | 0.320   |
| Bladder recurrence      | 9(17.0%)  | 11(21.2%) | 0.653   |
| Local recurrence        | 24(45.3%) | 14(26.9%) | 0.035   |
| Distant metastasis      | 26(49.1%) | 12(23.1%) | 0.004   |
| Cancer specific mortality| 17(32.1%) | 6(11.5%)  | 0.008   |

Table 1. Patient characteristics. Abbreviation: TN = Tumor necrosis, CIS = Carcinoma in situ, LVI = Lymphovascular invasion, SCC diff. = Squamous differentiation, LN = Lymph node.
Patients and Methods

Study population and selection criteria. From 2005 to 2012, 188 patients with locally advanced UT-UC (pT3) underwent radical nephroureterectomy at our institution. The study excluded 73 patients with concurrent pT3 renal pelvis and ureteral urothelial carcinoma and 10 patients with tissue loss in our specimen bank. Finally, 105 patients with solitary renal pelvis or ureteral pT3 urothelial carcinoma were included in the study. The study was approved by Kaohsiung Chang Gung Medical Center Institutional Review Board (IRB number: 102–2331B) and the method we use is in accordance with the approved guidelines. Informed consents were obtained preoperatively.

Pathological features and clinical outcome assessment. Perioperative data were obtained from patient charts. Data on commonly known prognostic pathological features, including lymphovascular invasion, carcinoma in situ, squamous differentiation, and tumor necrosis were recorded[10]. The follow-up protocol at our institution is postoperative fiber-cystoscopy and renal echo every 3 months during the first 2 years, every 6 months during the third to fifth year, and every year thereafter during the follow-up period. Abdominal computed tomography was performed annually to assess local or regional recurrence of the tumor and lymph node status. Local recurrence is defined as disease occurrence in the ipsilateral retroperitoneal space, and distant metastasis is defined as disease occurrence outside the residual urinary tract system and ipsilateral retroperitoneal space, such as in other organs or in the contralateral retroperitoneal space.

Figure 2. (a) ER3 effect on local recurrence free survival for pT3 UT-UC. (b) ER3 effect on distant metastasis free survival for pT3 UT-UC.
Immunohistochemistry and patient grouping. We have tested the AR and ER-alpha in thirty randomized fifty T3 UTUC specimens initially. However, there is little AR and ER alpha expression in our series and this result might suggest that AR and ER-alpha may not occupy an important role in prediction of clinical prognosis in our series (Supplement file, Fig. 1). Therefore we choose ERβ as our study target. Immunostaining for estrogen receptor beta (ERβ) was performed on a fully automated Bond-Max system (Leica Microsystems).

| Local recurrence | Distant metastasis |
|------------------|--------------------|
|                  | Univariate p value | Multivariate p value | Univariate p value | Multivariate p value |
| ERβ Low vs High  | 0.035              | 0.05 (HR = 2.6, 95%CI = 1.0–6.9) | 0.004              | 0.008 (HR = 4.8, 95%CI = 1.5–15.4) |
| Tumor type       |                    |                      |                    |
| Infiltrating vs papillary | 0.033              | 0.05 (HR = 2.6, 95%CI = 1.0–7.9) | 0.092              |
| Nodal status     |                    |                      |                    |
| Positive vs Negative | 0.468              | 0.013              | 0.998              |
| Lymphovascular invasion | 0.103              | 0.014              | 0.064              |
| Carcinoma in situ |                    |                      |                    |
| Present vs Absent | 0.399              | 0.399              |
| SCC differentiation |                    |                      |                    |
| Present vs Absent | 0.554              | 0.868              |
| Tumor necrosis   |                    |                      |                    |
| Present vs Absent | 0.975              | 0.975              |
| Tumor multifocality |                    |                      |                    |
| Multiple vs Solitary | 0.782              | 0.415              |
| Tumor grade      |                    |                      |                    |
| High vs Low       | 0.182              | 0.449              |
| Gender            |                    |                      |                    |
| Female vs Male    | 0.184              | 0.082              |
| Age > 70 vs <= 70 | 0.301              | 0.531              |
| Smoking           |                    |                      |                    |
| Yes vs No         | 0.524              | 0.080              |
| Previous bladder cancer | 0.189              | 0.008              | 0.997              |

Table 2. Multivariate analysis for prognostic factors about T3 UT-UC recurrence.

Figure 3. Wound healing assay revealed ERβ knock down UTUC cells were more proliferative in 24 hours observation.
with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogen for 5 minutes at room temperature; and (8) hematoxylin counterstaining for 5 minutes. Slides were mounted and examined by light microscopy. Representative conventional tumor sections were selected to examine the pattern of ERβ presentation instead of tissue microarray (Fig. 1). Pathologist M.T.S., who was blinded to the oncologic outcome, scored the percentage of ERβ expression in UTUC representative slide and we choose 10% as the cut-off value. The percentage of ERβ expression more than 10% was thought to be high ERβ expression.

Cell line study. We use BFTC-909 cell line (a cell line from renal pelvis patient), which is an ERβ expression cell line, for cancer behavioral analysis (Supplement file, Fig. 2). This cell line was cultured in Dulbecco’s modified Eagle’s medium, containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO2. ERβ was knock down adequately by Origene ER-beta shRNA transfection (Supplement file, Fig. 3). Wound healing assay was performed to test the proliferation ability of cell line. Cell monolayers were then wounded by sterile pipette tips (1 ml) that generated a gap. Wounded monolayers were then washed three times with PBS to remove cell debris and incubated in 10% CD FBS medium for 24-hour observation. Migration trans-well assay was performed to assess the aggressiveness of cell line. In the upper layer of a cell permeable membrane, and a solution containing the test agent is placed below the cell permeable membrane. Following an incubation period (37 °C, 5% CO2, 24 hours), the cells that have migrated through the membrane are stained and counted. We use DPN (diarylpropionitrile, 10 nM) as ERβ agonist and PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol, 150 nM) as ERβ antagonist. The cell migration number was counted after cisplatin(20 μM) treatment, DPN, and PHTPP for migration inhibition evaluation.

Statistical analysis. SPSS version 17 software was used for all statistical analyses. The chi-square test and independent t-test were used for intergroup comparisons. The Kaplan–Meier method with log-rank test was used for time-to-event analysis. Multivariate Cox regression analysis was used to assess the independent roles of perioperative factors on systemic recurrence or cancer-specific death. A p value of ≤0.05 was defined to be statistically significant.

Results
This retrospective study included samples collected between 2005 and 2012 from 105 patients with locally advanced UT-UC (pT3) and adequate quality of tissue for immunohistochemical stain, divided patients into ERβ-high (n = 52) or ERβ-low (n = 53) groups and then analyzed their oncologic outcomes. Table 1 reveals patient characteristics and all pathological features except infiltrating tumor architecture, which had a significantly higher incidence in the ERβ-low group (p = 0.004), were identical in both groups. Upper urinary tract...
urothelial carcinoma with low ERβ expression had worse local recurrence and distant metastasis-free survival compared with high ERβ expression ($p = 0.05$ and $0.02$ respectively, Fig. 2). The results of multivariate analysis are listed in Table 2. All commonly known prognostic pathological factors were included for examination. Low ERβ expression was significantly correlated with local recurrence and distant metastasis in univariate analysis ($p = 0.035$ and $0.004$ respectively) and multivariate analysis ($p = 0.05$ and $0.008$ respectively).

The result of cell line validation showed that knock down of ERβ cause aggressive UTUC cancer cell proliferation behavior by wound healing assay (Fig. 3). The migration assay also revealed more aggressive UTUC cancer
Cell migration if ERβ was knocked down (Fig. 4). The cytotoxic and migration-inhibiting effect of ERβ on UTUC cell line was observed and compared by cisplatin treatment. ERβ agonist enhanced the cisplatin effect while ERβ antagonist cause UTUC cell more resistant to cisplatin treatment (Fig. 5).

Discussion

UT-UC is rare and the treatment options are limited. The TNM staging for UT-UC is relatively simple and there are fewer markers for sub-classification of advanced stage UT-UC[31]. The current standard treatment for UT-UC is nephroureterectomy with bladder cuff excision. Much effort has been focused on prognostic pathological features or sub-classification of the current TNM staging system[3–5]. The subclassification of locally advanced UT-UC is a clinically important issue. Unlike localized UT-UC (pT0–2), patients with locally advanced stage pT3 UT-UC are thought to experience much higher disease recurrence, even after radical surgery. However, variant prognoses are still noted in pT3 UT-UC in clinical practice. Further sub-classification of such locally advanced stage disease will be helpful to identify patients with need of early adjuvant therapy. Therefore, patients with pT3 UT-UC were selected to identify markers predictive for oncologic outcome in this study.

The most common challenge of advanced UT-UC is the high prevalence of renal insufficiency. Ineligibility for cisplatin-based chemotherapy due to impaired renal function, especially after nephroureterectomy, leads to treatment options limited. The most common chemotherapeutic regimen for cisplatin-based chemotherapy due to impaired renal function, especially after nephroureterectomy, leads to treatment options limited. The current standard treatment for UT-UC is nephroureterectomy with bladder cuff excision. Much effort has been focused on prognostic pathological features or sub-classification of the current TNM staging system[3–5]. The subclassification of locally advanced UT-UC is a clinically important issue. Unlike localized UT-UC (pT0–2), patients with locally advanced stage pT3 UT-UC are thought to experience much higher disease recurrence, even after radical surgery. However, variant prognoses are still noted in pT3 UT-UC in clinical practice. Further sub-classification of such locally advanced stage disease will be helpful to identify patients with need of early adjuvant therapy. Therefore, patients with pT3 UT-UC were selected to identify markers predictive for oncologic outcome in this study.

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**Author Contributions**

L.H.L wrote the main manuscript text and design this study. M.T.S. set IHC protocol and completed all the pathological review. E.M.T. and C.S.L. were involved in this study design. N.L.L. and Y.H.C. prepared the IHC stain. E.M.T. and P.H.C. was involved in this study design and critically revised the final manuscript. All authors reviewed the manuscript.

**Additional Information**

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