Expression of Class III Beta-tubulin Predicts Prognosis in Patients with Cisplatin-resistant Bladder Cancer Receiving Paclitaxel-based Second-line Chemotherapy

YASUYOSHI MIYATA, TOMOHIRO MATSUO, YUICHIRO NAKAMURA, TAKUJI YASUDA, KOJIRO OHBA, KOSUKE TAKEHARA and HIDEKI SAKAI

Department of Urology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Abstract. Background/Aim: Class III beta-tubulin (TUBB3) expression is recognized as a predictive marker for chemosensitivity to cisplatin- and taxane-based chemotherapies in various malignancies. The aim of this study was to clarify the predictive value of TUBB3 expression for the anticancer effects of first-line cisplatin-based chemotherapy and second-line paclitaxel-based chemotherapy in patients with urothelial cancer (UC). Patients and Methods: We reviewed 116 patients with UC (90 with bladder cancer and 27 with upper urinary tract cancer) treated with first-line cisplatin-based chemotherapy. Among them, 42 patients received a paclitaxel-based regimen as second-line chemotherapy for advanced cisplatin-resistant UC. TUBB3 expression was evaluated using immunohistochemistry, and survival analyses were performed using Kaplan–Meier survival curves and multivariate Cox proportional hazard analysis. Results: TUBB3 was mainly detected in the cytoplasm of cancer cells, and 64 patients (55.2%) were judged as having positive TUBB3 expression. TUBB3 expression was significantly associated with tumour grade (p<0.001). TUBB3 expression was not associated with time to progression after first-line cisplatin-based chemotherapy. However, positive expression of TUBB3 was significantly associated with unfavourable overall survival in patients receiving second-line paclitaxel-based chemotherapy (p=0.021). In addition, a multivariate analysis model including T-stage and metastasis at the beginning of second-line therapy and regimen showed that TUBB3 expression was an independent predictor of poorer survival (hazard ratio(HR)=3.44, 95% confidential interval(CI)=1.15-10.33, p=0.027). Conclusion: TUBB3 expression was identified as a useful predictive factor for survival after second-line paclitaxel-based therapy in patients with cisplatin-resistant UC. Our results are useful for determining treatment strategies for such patients.

Bladder cancer (BC) is one of the most common malignancies, especially in industrialized countries. At initial diagnosis, 75% of patients have non-muscle-invasive bladder cancer, and the remaining 25% have muscle-invasive bladder cancer (1). Unfortunately, nearly one-third of patients with non-muscle invasion subsequently have regional invasion or metastasis (2). Although upper tract urothelial cancer (UC) is relatively rare, the prognosis is poor. In fact, the 5-year survival rate of patients with pT4 or metastasis of upper tract UC is only 10% (3). For such patients with advanced UC, chemotherapy is the most common and effective method to improve patient outcome. Therefore, information on predictive factors for the anticancer effects of chemotherapy in patients with UC is important for planning their treatment strategy.

Microtubules are cytoskeletal proteins built of α- and β-tubulin heterodimers (4). They are well-studied regulators of various cellular mechanisms, including maintenance of cell shape, intracellular transport, and cell mobility during mitosis and meiosis (5). Class III β-tubulin (TUBB3) is a β-tubulin isotype that has been reported to play important roles in malignant behaviour and prognosis of various types of malignancies (6,7). Furthermore, many investigators have paid attention to the relationships between TUBB3 expression and efficacy of a variety of chemotherapeutic agents in patients with advanced stage malignancies. For example, in vivo and in vitro studies demonstrated that TUBB3 expression was associated with chemoresistance to cisplatin and taxane-based agents.
taxanes in ovarian, breast, and lung cancer (8-10). In addition to its relationship with taxanes, several investigators reported that TUBB3 affected the response to DNA-damaging agents, including platinum-based agents, in lung cancer (11, 12). These reports support the opinion that TUBB3 overexpression is associated with poor outcome in patients treated with taxanes or platinum-based agents. However, other studies have shown that high expression of TUBB3 predicted better response to taxane-based chemotherapy in several types of cancer (13,14). Likewise, there is a report that TUBB3 expression was not associated with chemosensitivity to cisplatin in non-small cell lung cancer (15). Thus, the predictive value of TUBB3 expression for chemo- sensitivity is not fully understood.

A recent report showed that TUBB3 overexpression is positively associated with malignant aggressiveness in BC, via increased genetic instability, p53 alterations, and rapid cell proliferation (16). In addition, another report also showed that TUBB3 overexpression in BC cells was a significant predictor of recurrence-free survival in patients treated with radical cystectomy (17). Thus, relationships between TUBB3 expression and malignant aggressiveness, pathological features, and prognosis in patients with BC have already been investigated. However, the predictive value of TUBB3 expression for sensitivity to chemotherapeutic agents in patients with UC is not fully understood, despite platinum- or paclitaxel-based chemotherapy being commonly used to treat UC. A cisplatin-based regimen is recognized as the standard first-line chemotherapy for patients with locally invasive/metastatic UC. Although no second-line regimen for cisplatin-resistant UC has yet been established, several clinical studies have demonstrated that paclitaxel-based chemotherapy is useful for improving the quality of life and prognosis in patients with cisplatin-resistant UC (18-20). In recent years, the efficacy of immune-checkpoint inhibitors was reported in patients with advanced UC (21, 22). There is general agreement that this new tool is an epoch-changing therapeutic strategy for these patients. However, immune-checkpoint inhibitors are currently extremely expensive and have some severe side-effects. Therefore, we believe that effort should be focused on identifying those patients who are most likely to benefit from cisplatin- and paclitaxel-based regimens.

Based on these facts, the main aim of this study was to clarify the prognostic roles of TUBB3 expression in the efficacy of a cisplatin- and paclitaxel-based regimen in patients with advanced UC. In this study, the relationship between TUBB3 expression and time to progression (TTP) after first-line cisplatin-based chemotherapy was first investigated. Next, we analyzed the predictive value of TUBB3 expression for overall survival (OS) after second-line paclitaxel-based chemotherapy in patients with cisplatin-resistant UC.

### Patients and Methods

**Patients.** A total of 116 patients with UC (31 men and 85 women; 90 BC and 26 upper tract UC) who were treated with chemotherapy at the Nagasaki University Hospital between 2003 and 2013 were analysed retrospectively. The median age at the beginning of first-line chemotherapy administration was 66 years (interquartile range=60-75 years). Clinicopathological features at the beginning of first-line chemotherapy are shown in Table I. Tumour grade was judged by histopathological examination in biopsy or transurethral resection specimens. T-Stage was diagnosed by imaging analysis, including enhanced computed tomography (CT) or magnetic resonance imaging (MRI). In this study, 18 patients with non-muscle invasive disease were treated with cisplatin-based chemotherapy because the tumour size was large (over 5 cm), the number of tumours was extremely high (over 20), or the patients were unable to undergo radical surgery as they were elderly or had metastatic disease. Metastasis was evaluated with chest radiography, abdominal and pelvic CT, pelvic MRI, and bone scintigraphy. Patients with pure squamous cell carcinoma or adenocarcinoma were excluded. This study protocol and the use of all materials were approved by the Institutional Review Board of Nagasaki University Hospital and written informed consent was obtained.

**Treatment protocols.** A schema of treatment progress is shown in Figure 1. All patients received cisplatin-based chemotherapy as a first-line regimen, and 42 patients underwent paclitaxel-based second-line chemotherapy for advanced UC. Another 11 patients were treated with gemcitabine monotherapy (n=4) or best supportive

### Table I. Patient characteristics and class III beta-tubulin (TUBB3) expression at the beginning of first-line chemotherapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients (%)</th>
<th>TUBB3 expression, n (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5 (43.1)</td>
<td>5 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>2</td>
<td>37 (31.9)</td>
<td>23 (62.2)</td>
<td>14 (37.8)</td>
</tr>
<tr>
<td>3</td>
<td>74 (63.8)</td>
<td>24 (32.4)</td>
<td>50 (67.6)</td>
</tr>
<tr>
<td>T-Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta+1</td>
<td>18 (15.5)</td>
<td>5 (27.8)</td>
<td>13 (72.2)</td>
</tr>
<tr>
<td>T2</td>
<td>45 (38.8)</td>
<td>29 (64.4)</td>
<td>16 (35.6)</td>
</tr>
<tr>
<td>T3</td>
<td>42 (36.2)</td>
<td>23 (54.8)</td>
<td>19 (45.2)</td>
</tr>
<tr>
<td>T4</td>
<td>11 (9.5)</td>
<td>7 (63.6)</td>
<td>4 (36.4)</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence</td>
<td>24 (20.7)</td>
<td>10 (41.7)</td>
<td>14 (58.3)</td>
</tr>
<tr>
<td>Presence</td>
<td>92 (79.3)</td>
<td>42 (45.7)</td>
<td>50 (54.3)</td>
</tr>
<tr>
<td>Chemotherapy regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVEC</td>
<td>32</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>GC</td>
<td>31</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Intra-arterial</td>
<td>53</td>
<td>24</td>
<td>29</td>
</tr>
</tbody>
</table>

MVEC: Methotrexate, vinblastine, epirubicin and cisplatin; GC: combination of gemcitabine and cisplatin.
For cisplatin-based chemotherapy, methotrexate, vinblastine, epirubicin, and cisplatin (MVEC) regimen, gemcitabine and cisplatin (GC) regimen, and intra-arterial infusion of cisplatin were administered to 32 (27.6%), 31 (26.7%), and 53 patients (45.7%), respectively. The MVEC regimen consisted of methotrexate at 30 mg/m² on days 1, 15, and 22; vinblastine at 3 mg/m² on days 2, 15, and 22; epirubicin at 30 mg/m² on day 2; and cisplatin at 70 mg/m² on day 2, administered by intravenous infusion over a 28-day cycle. GC therapy consisted of gemcitabine at 1,000 mg/m² on days 1, 8, and 15; and cisplatin at 70 mg/m² on day 2, and was also administered by intravenous infusion over a 28-day cycle. MVEC or GC regimens were continued until disease progression, unacceptable toxicity, or patient decision. In our study population, all patients receiving first-line MVEC or GC regimens received at least two cycles, and the median number of treatment cycles was four. In regard to intra-arterial therapy, cisplatin at 70 mg/m² and doxorubicin at 30 mg/m² (1988-1994) or epirubicin at 30 mg/m² (1993-present) were introduced in a 30- to 40-min sequence. Two to four cycles of intra-arterial chemotherapy were administered (median=2).

For second-line therapy, paclitaxel plus gemcitabine therapy (n=34) and paclitaxel plus carboplatin (n=8) were administered. Gemcitabine at 700 mg/m² and paclitaxel at 70 mg/m² were administered by intravenous infusion on days 1 and 8 of each 28-day cycle. Paclitaxel plus carboplatin therapy consisted of paclitaxel at 100 mg/m² on days 1 and 8, and carboplatin at an area under the curve of 5 on day 1, administered by intravenous infusion over a 28-day cycle. Second-line treatment was continued until unacceptable toxicity or patient decision.

Immunohistochemistry. Five micrometre-thick sections were deparaffinized in xylene and rehydrated in ethanol. All sections were subjected to antigen retrieval and then immersed in hydrogen peroxide to block endogenous peroxidase activity. The antibody to TUBB3 was obtained from Abcam PLC (Cambridge, UK). Sections were incubated with the primary antibody at 4°C overnight. Then the sections were washed extensively and treated with peroxidase using the labelled polymer method with DAKO EnVision™ Peroxidase (Dako Corp., Carpinteria, CA, USA). The peroxidase reaction was visualized using the liquid 3,3'-diaminobenzidine (DAB) substrate kit (Zymed Laboratories Inc., San Francisco, CA, USA). Sections were counterstained with haematoxylin.

Staining intensity was graded on a scale of 0 to 2 (no staining=0, weak=1, and strong=2). In addition, the extent of staining was scored as 0: none, 1: 1-10%, 2: 11-50%, or 3: 51-100%, according to the percentage of positively stained cancer cells. This proportional score was multiplied by the intensity score to obtain a semi-quantitative score, and specimens with scores of 4-6 were judged as having high expression of TUBB3, as described previously (15). Slides were examined using an E-400 microscope (Nikon, Tokyo, Japan) to produce digital images, which were examined using a computer-aided image analysis system (Win ROOF version 5.0; MITANI, Fukui, Japan). Slides were evaluated twice at different times by two investigators (YM and TM), who were blinded to the clinicopathological features and survival data.

Survival analyses. TTP and OS were calculated from the date therapy started to the date of disease progression and death, respectively. TTP after first-line cisplatin-based chemotherapy and OS after second-line paclitaxel-based chemotherapy were analysed.
in 83 and 42 patients, respectively. Patients who were treated by radical operation were excluded for TTP analyses after therapy. OS after second-line paclitaxel-based therapy was analysed in patients with cisplatin-resistant UC.

**Statistics.** Student’s t-test was used for comparisons involving continuous variables. The chi-square test was used to compare categorical data. Differences in survival were assessed using Kaplan–Meier curves, the log-rank test, and Cox regression analysis, and are expressed using hazard ratios (HRs), 95% confidence intervals (95% CIs), and p-values. All statistical analyses were two-sided and performed in StatView for Windows (version 5.0; Abacus Concepts, Inc., Berkeley, CA, USA). p-Values of less than 0.05 were considered representative of statistical significance.

**Results**

**TUBB3 expression and correlations with clinicopathological features.** A representative example of TUBB3 expression is shown in Figure 2. TUBB3 was mainly found in the cytoplasm of cancer cells, and 64 cases (55.2%) were judged as being positive for TUBB3 expression. TUBB3 positivity was similar between samples from BC and upper UC (49 patients/54.4% and 15 patients/57.7%, respectively; p=0.769).

Relationships between TUBB3 expression and grade, stage, and regimen are shown in Table I. The percentage of TUBB3-positive grade 3 tumors (50/74=67.6%) was significantly higher (p<0.001) than grade 1 (0/5=0.0%) and grade 2 (14/37=37.8%) tumors. A similar trend was found for T-stage; however, it did not reach significance (p=0.062; Table I). No significant relationship was found between TUBB3 level and metastasis (p=0.727). There was no correlation between TUBB3 expression and first-line chemotherapy regimen.

**Correlation with progression after cisplatin-based chemotherapy.** As shown in Figure 3A, TUBB3 expression was not significantly associated with TTP after first-line cisplatin-based chemotherapy (p=0.796). When a similar analysis was performed separately for patients with BC and upper tract UC, no significant prognostic role was found for either (p=0.704 and p=0.816, respectively). Similarly, the predictive value of TUBB3 expression was not influenced by first-line chemotherapy regimen (MVEC, p=0.489; GC, p=0.852; intra-arterial therapy, p=0.229).

**Correlation with survival after second-line paclitaxel-based therapy.** In this study, 42 patients received second-line paclitaxel-based chemotherapy. At the beginning of second-

<table>
<thead>
<tr>
<th>Factor</th>
<th>HR</th>
<th>95% CI</th>
<th>p-Value</th>
</tr>
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<tbody>
<tr>
<td>T-Stage at second-line therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>0.77</td>
<td>0.25-2.36</td>
<td>0.643</td>
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<tr>
<td>T4</td>
<td>0.78</td>
<td>0.21-2.83</td>
<td>0.701</td>
</tr>
<tr>
<td>Metastasis at second-line therapy</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Presence</td>
<td>0.88</td>
<td>0.38-2.06</td>
<td>0.767</td>
</tr>
<tr>
<td>Second-line regimen</td>
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<td></td>
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</tr>
<tr>
<td>Paclitaxel+gemcitabine</td>
<td>0.48</td>
<td>0.17-1.36</td>
<td>0.166</td>
</tr>
<tr>
<td>TUBB3 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>3.44</td>
<td>1.15-10.33</td>
<td>0.027</td>
</tr>
</tbody>
</table>

TUBB3: Class III beta-tubulin; HR: hazard ratio; CI: confidence interval.
line chemotherapy, light (19.0%), 14 (33.3%), and 20 (47.6%) patients were diagnosed as having stage T2, T3, and T4 tumour, respectively. Twenty-seven patients (64.3%) had metastases. All patients were judged to have unresectable tumours. High expression of TUBB3 was significantly associated with unfavourable OS in patients receiving second-line paclitaxel-based chemotherapy ($p=0.013$; Figure 3B). A multivariate analysis model including T-stage and metastatic status at the beginning of second-line chemotherapy and regimen of second-line therapy demonstrated that TUBB3 expression was independently associated with OS after second-line paclitaxel-based chemotherapy ($HR=3.44$, 95% CI=1.15-10.33, $p=0.027$; Table II).

**Discussion**

Our results demonstrated that high expression of TUBB3 was linked to poor outcome of second-line paclitaxel-based chemotherapy in patients with cisplatin-resistant UC. In addition, its expression was identified as an independent predictor in multivariate analysis models including tumour grade and stage. Unfortunately, as stated in the introduction, there are currently no established second-line chemotherapy regimens for cisplatin-resistant UC. However, there are several reports that paclitaxel-based chemotherapy has had some effectiveness in terms of improvement of quality of life and prognosis in patients with cisplatin-resistant UC (18, 23). In recent years, immune-checkpoint inhibitors have been reported as a novel treatment modality for patients with advanced UC, including platinum-treated advanced UC (21, 22). Many investigators and urologists have a great hope of increasing survival with these new agents in patients with advanced and cisplatin-resistant UC; however, more careful evaluation of cost-effectiveness, useful biomarkers, and safety are essential for standardization (21). Therefore, we believe that paclitaxel-based chemotherapy can immediately become a useful treatment option for patients with cisplatin-resistant UC. However, it is difficult to select patients most likely to benefit from paclitaxel-based chemotherapy. Thus, our finding that TUBB3 expression is a strong predictor for survival after second-line PTX-based chemotherapy is important information when discussing treatment strategies in cisplatin-resistant UC.

In contrast to second-line paclitaxel-based chemotherapy, our results showed that TUBB3 expression was not associated with TTP after first-line cisplatin-based chemotherapy. Cisplatin-based regimens are well-known as the most effective and standard method of first-line chemotherapy in patients with UC. On the other hand, cisplatin has relatively strong side-effects, such as nausea, bone marrow suppression, and alopecia. Therefore, prediction of the anticancer effects of cisplatin-based chemotherapy is important in planning treatment strategies. In several malignancies, increased expression of TUBB3 was reported to be significantly associated with poor prognosis and survival in patients receiving platinum-based chemotherapy (24, 25). Therefore, we expected that TUBB3 expression would be significantly associated with the anticancer effect of cisplatin-based chemotherapy in patients with advanced UC; however, our results did not support this hypothesis. Unfortunately, there is little information on the relationship between TUBB3 expression and the anticancer effects of cisplatin-based chemotherapy in patients with UC. On the other hand, some studies have shown that TUBB3 expression was not associated with prognosis in the adjuvant setting in non-small cell lung cancer (15). Ultimately, we suggest that the clinical prognostic role of TUBB3 expression for first-line cisplatin chemotherapy in patients with UC is minimal, and more detailed studies are necessary.

In this study, TUBB3 expression was significantly associated with tumor grade, but not T-stage or metastatic status. In various types of other malignancies, TUBB3 expression was positively associated with malignant aggressiveness, including grade, invasion, and metastasis (6, 7). Other investigators also reported that TUBB3 overexpression was associated with high grade in BC (17). We cannot explain the reason for the discrepancy between UC and other malignancies regarding the pathological significance of TUBB3. However, several other studies on a variety of malignancies have also repeated that TUBB3 expression is not associated with stage or metastatic status (7, 26-28). Finally, we speculate that methodological differences, such as antibodies used, method of evaluation, and background of patients, may contribute to this discrepancy, and that the pathological roles of TUBB3 in invasion and metastasis are minimal in UC. In addition, others have theorized that the malignant behaviour and pathological significance of TUBB3 is dependent on the cancer microenvironment (6, 29).

The major limitation of this study is that the sample size was relatively small. However, to our knowledge, this is the first study on the prognostic roles of TUBB3 for survival after second-line paclitaxel-based chemotherapy in patients with cisplatin-resistant UC. Another limitation is that the dose and schedule of the different regimens were different from patient to patient, according to tolerability and toxicity profile, because this was a retrospective analysis. However, no patients were treated with a remarkably different protocols because all treatments were performed at a single institution. We believe that these results are important for the discussion and determination of treatment strategies in patients with advanced UC, although more detailed studies are necessary to draw a firm conclusion on the predictive value of TUBB3 expression.
Conclusion

Our results demonstrated that TUBB3 expression is a useful predictive factor for OS after second-line paclitaxel-based chemotherapy, but not for TTP after first-line cisplatin-based chemotherapy in patients with UC. Currently, paclitaxel-based second-line chemotherapy for cisplatin-resistant UC is a useful therapeutic tool. Therefore, our results are important for determining the optimal treatment strategies in these patients.

Conflicts of Interest

The Authors declare that they have no competing interests in regard to this study.

References


