Ceruloplasmin Levels in Cancer Tissues and Urine Are Significant Biomarkers of Pathological Features and Outcome in Bladder Cancer

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Abstract. *Background/Aim:* A previous report showed that immune complex-ceruloplasmin (CP) in urine is associated with carcinogenesis and malignant behavior in bladder cancer (BC). We investigated the pathological significance and prognostic roles of urine and tissue levels of CP protein in BC patients. *Materials and Methods:* Urine CP levels were measured using an enzyme-linked immunosorbent assay in 97 patients. CP expression in BC tissues was evaluated by immunohistochemical analysis in 176 patient samples. *Results:* Urine CP levels were positively associated with tumor grade and pT stage in non-muscle invasive BC (NMIBC). CP expression in BC tissues was positively associated with tumor growth and progression. Multivariate analysis demonstrated that high urine CP levels was an independent predictor of recurrence in the urinary tract in NMIBC (hazard ratio=2.87, $p=0.016$). *Conclusion:* CP-related markers, especially urine CP levels, are useful biomarkers of malignant potential and prognosis in NMIBC.

Urothelial carcinoma (UC) is one of the most common urological cancers, and 90%-95% of UC cases are bladder cancers (BCs) (1). BC is divided into two categories: muscle-invasive bladder cancer (MIBC) and non-MIBC (NMIBC). The standard treatment for patients with non-

metastatic MIBC depends on age, comorbidities, and the patient's wishes, but radical cystectomy with perioperative chemotherapy is usually recommended (2, 3). In contrast, for patients with metastatic disease, systemic chemotherapy is performed regardless of the muscle invasion status (3). However, these treatments have major disadvantages, including a reduction in the quality of life, an increase in complications, and the occurrence of adverse events (4). In contrast to MIBC, patients with NMIBC are mainly treated with transurethral resection with a minimal decrease in quality of life. However, nearly half of NMIBC patients develop urinary tract recurrence despite complete resection (5). In addition, approximately one-third of NMIBC patients subsequently demonstrate invasion and/or metastasis (6). Thus, there are still various problems to be solved to improve the prognosis of patients with BC. In fact, many new treatment strategies have been proposed to improve outcomes and suppress adverse events in these patients (7, 8). Furthermore, the identification of better predictive markers for BC recurrence and metastasis is important, and many investigators pay special attention to this issue (9-11).

In recent years, immune complex including ceruloplasmin (IC-CP) has been detected in higher quantities in the urine of BC patients compared to unaffected individuals and patients with non-malignant diseases such as urinary tract infections and stones (12). In addition, this study also showed that urine IC-CP was significantly associated with pathological features of BC and risk of urinary tract recurrence (12). On the other hand, our original method for detecting IC-CP, which was termed "immune complexome analysis," is a qualitative rather than a quantitative analysis that cannot absolute values (13, 14). In other words, immune complexome analysis detects the presence or absence of immune complexes consisting of CP and its antibody, formed as a result of immune reactivity, but does not measure CP levels.

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An important requirement for a tumor marker is to be measurable using easy and versatile methods. Therefore, we measured CP levels in urine samples of patients with BC using enzyme-linked immunosorbent assay (ELISA). In addition, expression levels of CP protein in BC tissues were evaluated by using immunohistochemical analyses. Finally, the pathological significance and prognostic roles of urine and tissue levels of CP were analyzed. Furthermore, we used our analyses to find differences between NMIBC and MIBC.

Materials and Methods

Patients. All patients were diagnosed with urothelial carcinoma of the urinary bladder by routine histopathological examination. Urine samples were collected from 97 BC patients (cohort 1), which are the same as those in our previous report (12). In this cohort, we investigated the relationships between urine CP levels and the presence of IC-CP, clinicopathological features, and outcome. Moreover, to clarify the pathological significance and prognostic roles of CP protein in BC tissues, its expression was measured in 176 BC patients (cohort 2). Cohort 2 included 131 men and 45 women, and their mean age/SD was 69.3/11.4 years. In this study, T stage (of the TNM staging system) was divided into two groups: low T stage (pTa+1) and high T stage (T2-4) for statistical analysis.

Enzyme-linked immunosorbent assay. Urine CP levels were measured according to our previous report (15). In short, all urine samples were centrifuged at $3,000 \times g$ for 10 min, and the supernatants were separated and stored at -80°C until testing. After defrosting, urinary CP levels were measured using a commercial ELISA kit (R&D systems, Minneapolis, MN, USA), as described in the manufacturer's instructions. Absorbance was detected at 450 nm using a microplate reader (Thermo LabSystems Multiskan RC; Artisan Technology Group, Champaign, IL, USA). For survival analyses, urine CP levels were divided into two groups (high and low) according to the median levels.

Immunohistochemistry. Sections of 5 μm thickness were prepared from formalin-fixed paraffin-embedded specimens, deparaffinized in xylene and rehydrated in ethanol. All sections were subjected to antigen retrieval and then immersed in hydrogen peroxide. Sections were incubated with an anti-ceruloplasmin antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C overnight. The sections were then treated with peroxidase using the labeled polymer method with Dako EnVision+™ Peroxidase (Dako Corp., Carpinteria, CA, USA). The peroxidase reaction was visualized using a liquid DAB substrate kit (Zymed Laboratories Inc., San Francisco, CA, USA). The sections were then counterstained with hematoxylin. In this study, the immunoreactive score (IRS) was used to evaluate CP expression, and was calculated based on the range of percentages of stained cells (0-5.0%=0, 5.1-25%=1, 25.1-50.0%=2, 50.1-75%=3, and 75.1%-100%=4) and staining intensity (none=0, weak=1, moderate=2, and strong=3). 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). For statistical analyses, an $\text{IRS} > 3$ was considered a positive result.

Statistical analyses. Histograms were prepared to evaluate the normality of each variable. The results of urine CP levels were expressed as a median and interquartile range (IQR). However, data on CP expression in BC tissues were expressed as mean $\pm$ SD because they were normally distributed. Accordingly, the Mann-Whitney *U*-test or Student's *t*-test were used to analyze continuous variables of urine CP levels or CP expression, respectively. The chi-square test was used for categorical comparisons of the CP expression. For survival analyses, Kaplan-Meier survival curves with log-rank tests and Cox proportional hazards analyses [described as hazard ratios (HRs) with 95% confidence intervals (Cis), together with *p*-values] were performed. Statistical analyses were performed on a personal computer using StatView for Windows (version 5.0; Abacus Concepts, Berkeley, CA, USA), and significance was defined as $p < 0.050$.

Results

Urine ceruloplasmin levels (cohort 1). At first, when the relationship between IC-CP and urine CP levels in 97 BC patients was examined, the CP levels in urine samples with IC-CP were significantly higher than those without IC-CP (1325.4/961.3-2031.5 versus 316.1/128.2-793.5 ng/ml; $p < 0.001$). As shown in Figure 1, urine CP levels in BC patients (660.8/221.0-1396.8 ng/ml) were significantly higher ($p < 0.001$) than those in the controls (168.8/75.0-438.7 ng/ml). In all BC patients, urine CP levels in high-grade tumors tended to be higher than those in low-grade tumors (827.1/259.0-1380.7 versus 361.6/184.2-1450.5 ng/ml); however, the difference was not statistically significant ($p = 0.247$; Table I). Likewise, urine CP levels in MIBC and metastatic tumors were higher than those in NMIBC and non-metastatic tumors; however, there was no significant difference ($p = 0.956$ and 0.734 , respectively; Table I). Furthermore, multiple comparison analysis on each T stage (Ta-T4) showed that there was no significant difference among each T stage, although median urine CP levels of Ta and T4 were lower than those of other T stages (Table I and Figure 2). Next, in patients with NMIBC, urine CP levels in high-grade tumors (990.7/336.7-2419.3 ng/ml) were significantly higher ($p = 0.021$) than those in low-grade tumors (339.5/183.0-1055.9; Table I). In addition, a similar significant difference was found between Ta and T1 disease in the Mann-Whitney *U*-test ($p = 0.015$; Table I).

Ceruloplasmin expression in BC tissues (cohort 2). Representative examples of CP immunoreactivity in normal urothelium, NMIBC, and MIBC tissues are shown in Figure 3A-C. In normal urothelial cells, moderate to strong expression was relatively rare (Figure 3A). CP was mainly detected in the cytoplasm of cancer cells (Figure 3B and C). In particular, invasive cancer cells showed a strong expression of CP (Figure 3C). Finally, 59 BC tissues (33.5%) were judged as positive, and this ratio was remarkably higher than that in normal urothelial tissues (1/20, 5.0%). The

Figure 1. Urine ceruloplasmin levels in normal controls and bladder cancer patients.

Figure 2. Urine ceruloplasmin levels in each T stage.

relationships between pathological features and CP expression are shown in Table II. The absolute value of the IRS was positively associated with grade, muscle invasion, and metastasis ($p < 0.001$; Table II). Multiple comparison analysis showed that significant differences in the IRSs were detected between pTa *versus* T2 or T3 and pT1 *versus* T2 or T3 (Figure 4). The IRS in T4 was higher than that in Ta; however, the difference was not significant ($p = 0.052$). When similar analyses were performed between two divided parameters (negative and positive expression of CP), the χ^2 -test demonstrated that CP expression was positively correlated with all pathological features, including the T stage (Table II).

Survival analyses. First, we investigated the predictive value of urine CP levels for urinary tract recurrence in cohort 1. In

Figure 3. Representative examples of ceruloplasmin expression in the normal urothelium (A), non-muscle invasive disease (B), and muscle invasive cancer cells (C). Magnification: $\times 200$.

this cohort, survival analyses for subsequent extra-urinary tract recurrence and overall survival were not performed because the follow-up periods were relatively short

Table I. Urine ceruloplasmin levels (cohort 1) and pathological features.

	N/%	Median/interquartile range	p-Value
Entire patients	97/100		
Grade			0.247*
Low	37/38.1	361.6/184.2-1,450.5	
High	60/61.9	827.1/259.0-1,380.7	
T stage			NS**
Ta	40/42.6	345.5/157.8-1,055.9	
T1	27/28.7	1,108.8/336.7-2,419.3	
T2	12/12.8	800.9/184.0-1,187.7	
T3	11/9.6	966.6/458.2-1,303.7	
T4	7/6.4	507.5/304.0-1,452.3	
Muscle invasion			0.956*
Absence	67/71.3	641.7/186.8-1,776.1	
Presence	30/28.7	802.7/261.5-325.4	
Metastasis			0.734*
Absence	82/85.1	667.0/227.9-1,441.8	
Presence	15/14.9	507.5/214.4-1,303.7	
NMIBC patients	67/100		
Grade			0.021*
Low	36/53.7	339.5/183.0-1,168.9	
High	31/46.3	990.7/359.7-2,419.3	
T stage			0.015*
Ta	40/59.7	345.5/157.8-1,055.9	
T1	27/40.3	1,108.8/336.7-419.3	

NS: Not significant; NMIBC: non-muscle invasive bladder cancer. *Evaluated by Mann-Whitney *U*-test. **Evaluated by multiple comparison analysis.

(mean=21.3; median=16, IQR=9-31, range=4-58 months), and the frequencies of patients with MIBC and metastatic diseases were relatively low. In this study, such analysis was performed only in NMIBC patients who had undergone potentially curative transurethral resection (n=67) because most MIBC patients were treated with radical cystectomy. When NMIBC urine CP levels were divided into high and low groups according to the median level (461.7 ng/ml), Kaplan–Meier survival curves showed that urinary tract recurrence-free survival periods in the high urine CP level group were significantly worse ($p=0.009$) compared to those in the low urine CP group (Figure 5A).

In cohort 2, Kaplan–Meier survival curves of patients with negative CP expression and those with positive expression for urinary tract recurrence were almost similar, without a significant difference ($p=0.846$; Figure 5B). In addition, no significant association was found when the study population was divided into NMIBC and MIBC ($p=0.635$ and 0.376 , respectively). However, as shown in Figures 5C and D, Kaplan–Meier survival curves showed that positive expression of CP was a significant predictive factor for extra-urinary tract recurrence and overall survival ($p=0.049$ and 0.006 , respectively).

Multi-variate analyses for prognosis. Based on the results obtained by uni-variate analyses, the independent predictive

values of urine and tissue CP levels for pathological features were analyzed using multivariate analysis models. As shown in Table III, a high level of urine CP was identified as a significant predictive factor for urinary tract recurrence in patients with NMIBC (HR=2.87, 95%CI=1.22-6.78, $p=0.016$). In contrast, multi-variate analysis models including grade, T stage, and metastasis showed that CP expression in BC tissues was not an independent predictor of extra-urinary tract recurrence and overall survival ($p=0.777$ and 0.434 , respectively; Table III).

Discussion

The present study used urine samples from cohort 1 to demonstrate that CP urine levels were positively associated with pathological features of BC, such as grade and lamina propria invasion, in patients with NMIBC. In addition, a multivariate analysis model using pathological features showed that a high urine CP level was a significant predictive marker for urinary tract recurrence in these patients. CP is a multi-function glycoprotein and has been reported to play crucial roles in various pathological conditions, such as Wilson disease, inflammation, fibrosis, and neurodegenerative disorders (16-18). In addition to its role in non-malignant diseases, CP is a well-known tumor

Table II. Tissue expression of ceruloplasmin (cohort 2) and pathological features.

	N %	Immunoreactive score*	Immunoreactivity**	
			Negative	Positive
Entire				
Grade				
Low	75/42.6	1.56/1.66	57 (76.0)	18 (24.0)
High	101/57.4	2.84/2.40	60 (59.4)	41 (40.6)
<i>p</i> -Value		<0.001	<0.001	
T stage				
Ta	52/29.5	1.27/1.52	42 (80.8)	10 (19.2)
T1	71/40.3	1.76/.59	54 (76.1)	17 (23.9)
T2	25 /14.2	4.04/2.43	8 (32.0)	17 (68.0)
T3	20/11.4	4.20/2.57	9 (45.0)	11 (55.0)
T4	8/4.5	3.50/2.78	4 (50.0)	4 (50.0)
<i>p</i> -Value		See Figure 2	<0.001	
Muscle invasive				
Absence	123/69.9	1.55/1.57	96 (78.0)	27 (22.0)
Presence	53/30.1	4.02/2.49	27 (39.6)	32 (60.4)
<i>p</i> -Value		<0.001	<0.001	
Metastasis				
Absence	156/88.6	1.97/1.91	110 (70.5)	46 (29.5)
Presence	20/11.4	4.85/2.68	7 (35.0)	13 (65.0)
<i>p</i> -Value		<0.001	0.002	

*Data were shown as mean/SD. **Data were shown as N (%).

Figure 4. Immunoreactive scores of ceruloplasmin expression in each T stage.

promoter in various malignancies. For example, serum CP levels are positively associated with carcinogenesis, tumor stage, and recurrence in pancreatic cancer, oral cancer, lung cancer, leukemia, and Hodgkin's lymphoma (19-23). Furthermore, high CP mRNA levels are associated with malignant potential in esophageal cancer, bile duct cancer,

renal cell carcinoma, and adrenocortical carcinoma (24-27). Moreover, the pathological roles of CP have been confirmed in animal experiments and *in vitro* studies using lung and ovarian cancer cell lines (28-30). Regarding CP in urine samples, its level has been reported to be a candidate marker for the diagnosis of HER2-enriched breast cancer (31). Furthermore, in BC patients, serum and urine CP levels were also higher compared to non-malignant patients (32, 33). These findings suggest that CP is a useful diagnostic tool for patients with BC (32, 33). However, the relationships between these CP-related parameters and tumor growth, progression, and outcomes in patients with BC were not mentioned. In recent years, we searched for BC-specific biomarkers from urine samples using our original "immune complexome analysis" method (12). As a result, IC-CP was detected more frequently in BC patients than in healthy controls and patients with non-malignant diseases (12). In addition, the detection of IC-CP was positively associated with malignancy and aggressiveness in patients with BC. However, immune complexome analysis only evaluates whether the immunocomplex form of CP is present or absent and cannot quantify CP levels. Thus, this is the first report on the pathological significance and prognostic role of quantitative urine CP levels in BC patients.

When the presence of urine IC-CP and quantitative urine CP levels were compared in all BC patients, we noticed that IC-CP was significantly associated with grade and T stage,

Figure 5. Kaplan–Meier survival curves for urinary tract recurrence of urine ceruloplasmin levels in non-muscle invasive bladder cancer patients (A). Kaplan–Meier survival curves for urinary tract recurrence (B), extra-urinary tract recurrence (C), and overall survival (D) according to different ceruloplasmin expression levels in bladder cancer tissues.

whereas urine CP level was not (12). In contrast, urine CP level was significantly associated with grade and T stage in patients with NMIBC specifically. In addition, both factors were useful predictors of urinary tract recurrence after complete resection in patients with NMIBC (12). In particular, a high urine CP level was identified as an independent predictive marker for urinary tract recurrence in these patients. Based on these facts, we suggest that urine CP levels reflect malignant aggressiveness in patients with NMIBC, but not in those with MIBC. Based on these findings, we hypothesized that the physical contact between the tumor mass in the bladder lumen and urine stored therein affected urine CP levels but was not influenced by the parts of the tumor invading the muscle. In fact, neither urine CP protein nor urine IC-CP was significantly correlated with

metastasis (12). Furthermore, it has been reported that CP is secreted by lung cancer cells (29). These findings support our hypothesis. In contrast, several investigators have suggested that significant biomarkers in NMIBC differ from those in MIBC (34-36). Unfortunately, our study design provides little definitive information to conclude about this issue; however, our results are important in proposing the clinical usefulness of urine CP as a predictive biomarker in patients with BC.

The present study used cancer tissues from cohort 2 and showed that CP expression was positively associated with all pathological features, including grade, T stage, and metastasis in BC patients. In addition, Kaplan–Meier survival curves showed that positive expression of CP was a significant predictor of extra-urinary tract recurrence and overall survival

Table III. Multi-variate analyses of ceruloplasmin for outcomes.

	HR	95%CI	p-Value
Urine level: high*			
Urinary tract recurrence [†]	2.87	1.22-6.78	0.016
Tissue expression: positive**			
Extra-urinary tract recurrence	1.12	0.51-2.47	0.777
Overall survival	1.32	0.66-2.67	0.434

HR: Hazard ratio; CI: confidence interval. [†]In patients with non-muscle invasive bladder cancer. *Adjusted by high grade and high T stage. **Adjusted by high grade, high T stage, and presence of metastasis.

in BC patients. As mentioned above, many *in vivo* and *in vitro* studies support the hypothesis that CP plays important roles in carcinogenesis, malignant behavior, and outcomes in various malignancies (12, 19-24, 32, 33). However, the clinical significance of CP immunoreactivity in human cancer tissues is not fully understood. In fact, although protein expression of CP has been reported to be associated with tumor growth, invasive potential, lymph node metastasis, and prognosis in lung cancer patients (29), other investigators have reported that expression of CP protein was not significantly associated with T stage and N stage in patients with bile duct cancer (25). In general, the detection of pathogenetic molecules by immunohistochemical methods is relatively easy and low-cost because state-of-the-art technology, expensive instruments, and special techniques are not necessary. From this point of view, our results are important for a more detailed and wider discussion on the clinical usefulness of CP as a biomarker in human cancer tissues.

One of the important limitations of this study is that urine CP level is judged as high according to a median value, although using this value is not always optimal in predicting outcomes. We originally attempted to determine an optimal cut-off value using the receiver operating characteristic curve in our study population. However, we thought that the sample size was too low to derive the reference values for accurate prediction. Additionally, no data on serum CP levels were collected in this study. Another limitation is that our study design cannot clarify why the prognostic roles of urine CP levels and tissue CP expression are different. Moreover, as described in the Results, although urine CP levels are thought to reflect the presence of IC-CP in urine samples, the pathological significance for grade and tumor growth of urine CP levels differ from that of IC-CP in urine samples. Thus, further detailed studies with larger study populations are necessary to discuss the pathological significance and prognostic roles of CP-related variables in BC patients.

In conclusion, the present study showed that CP protein expression in BC tissues was significantly associated with malignant aggressiveness, including tumor growth, invasion, metastasis, metastatic recurrence, and mortality in patients with

BC. However, CP expression in BC tissues was not identified as a significant predictor of these outcomes using multivariate analyses. In contrast, urine CP levels were not significantly associated with any pathological features in all BC patients; however, urine CP levels were positively associated with tumor grade and pT stage in patients with NMIBC. In addition, a high level of urine CP was independently associated with shorter urinary tract recurrence-free periods in the multivariate analysis. Finally, we concluded that CP plays an important role in tumor growth, progression, and survival in BC. In particular, urine CP levels were hypothesized to be a useful predictor of urinary tract recurrence in patients with NMIBC.

Conflicts of Interest

None of the Authors have any conflict of interest to declare regarding this study.

Authors' Contributions

Yuta Mukae performed the experiments and contributed to writing of the manuscript. Yasuyoshi Miyata conceived and designed the experiments, performed data analysis, and contributed to the writing of the manuscript. KA, Tsuyoshi Matsuda, YN, and NA performed the experiments and analysed the data. HI and Tomohiro Matsuo contributed to the sample and clinical data collection and to the writing of the manuscript. HS and KO designed the experiments and supervised the study.

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