

Detection of Novel Urine Markers Using Immune Complexome Analysis in Bladder Cancer Patients: A Preliminary Study

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Abstract. *Background/Aim:* Little is known on urine biomarkers that are associated with malignant behavior in patients with bladder cancer (BC). Our aim was to identify BC-related factors in urine samples using our original method “immune complexome analysis”, based on detecting the immune complex (IC). *Patients and Methods:* Immune complexome analysis was performed using urine samples from 97 BC patients, including 67 with non-muscle invasive BC (NMIBC). *Results:* Eight IC-antigens were recognized as candidates for BC-related factors from 20,165 proteins. IC-serum albumin, -fibrinogen γ chain, -hemoglobin subunit α , -hemoglobin subunit β , -ceruloplasmin, and fibrinogen β chain were significantly associated with either pathological features and/or outcome. IC-ceruloplasmin was most widely associated with pathological features in all BC patients and lamina propria invasion and urinary tract recurrence in NMIBC. *Conclusion:* Based on detection of IC-antigens it was demonstrated that six IC-antigens, especially IC-ceruloplasmin, are potential urine biomarkers in BC.

Bladder cancer (BC) is one of the major urological cancers, and its most representative characteristics include high frequency of cancer cell invasion and recurrence. Indeed, the rates of urinary tract recurrence in non-muscle invasive BC (NMIBC) were reported in more than half of the patients, despite complete tumor removal was performed using transurethral resection (1, 2). In addition, such recurrent tumors often lead to muscle invasion and metastasis, which

causes a decrease in quality of life and patient death. Therefore, understanding the pathological mechanisms of tumor progression and urinary tract recurrence in patients with BC is important to improve outcomes. In previous reports, various factors have been reported as useful predictive markers of clinical outcomes, including recurrence in BC patients (3-5). At present, to predict and diagnose urinary tract recurrence in NMIBC, various new methods and tools are being developed and approved around the world (6, 7). However, they suffer drawbacks for common use in clinical practice because of their high cost, complicated operation procedure, and medical insurance system. Thus, more detailed information obtained by new methods is important to discuss the diagnosis and observation strategies.

Immune complexes (ICs) are products of reactions that involve noncovalent interactions between foreign antigens or autoantigens and antibody molecules (8). Furthermore, ICs are detected in various pathological conditions, such as autoimmune diseases, cancers, and infections, where they play important roles in the etiology and pathology (9). Therefore, comprehensive identification of antigens incorporated into ICs in body fluids might provide new insights into pathophysiology and could form the basis for novel diagnostic, observation, and treatment strategies for various diseases. To elucidate the pathogenesis and development of predictive biomarkers in a certain immune-related disease, the identification of IC-antigens that are formed *in vivo* from biological fluids might be more effective than that of free antigens because ICs are real-time products of an immune response (10, 11). In other words, identification of the IC-antigen profile in patient samples is useful to understand the biological and pathological status of various diseases.

To detect disease-specific antigens from multiple and wide varieties of candidate samples, microarrays are often used in *in vivo* and *in vitro* studies (12). However, the analytical comprehensiveness of this technique is fundamentally limited because the preparation and selection of antigens or antibodies is required, and because only those molecules that are

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Key Words: Immune complexome analysis, urine marker, invasion, recurrence, bladder cancer.

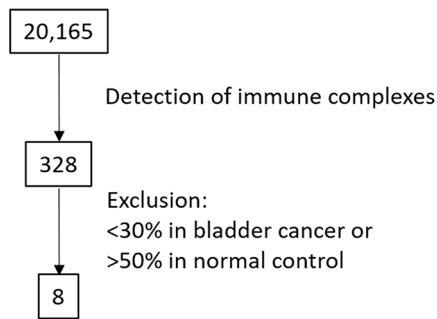


Figure 1. Flow diagram for selection of bladder cancer-related immune complex-antigens.

Table I. Frequencies of patients judged as positive.

IC-antigens. N (%)	BC N=97	UTI N=52	US N=22	Normal N=46
Serum albumin	51 (52.6)	6 (11.5)	11 (50.0)	11 (23.9)
α 1 antitrypsin	51 (52.6)	7 (13.5)	11 (50.0)	8 (17.4)
Fibrinogen γ chain	46 (47.4)	6 (11.5)	12 (54.5)	3 (6.5)
Hemoglobin subunit α	45 (46.4)	6 (11.5)	7 (31.8)	3 (6.5)
Hemoglobin subunit β	45 (46.4)	8 (15.4)	9 (40.9)	7 (15.2)
Complement C3	41 (42.3)	8 (15.4)	9 (40.9)	2 (4.3)
Ceruloplasmin	37 (38.1)	7 (13.5)	6 (27.3)	3 (6.5)
Fibrinogen β chain	36 (37.1)	3 (5.8)	8 (36.4)	3 (6.5)

BC: Bladder cancer; UTI: urinary tract infection; US, urinary stone.

represented on microarrays can be identified. Therefore, in order to comprehensively identify and profile IC-antigens, we previously developed a new method, designated “immune complexome analysis,” in which ICs were separated from human fluid samples by using protein-coated beads that bound the fragment of crystallization (Fc) domain of antibodies, and then the ICs were subjected to tryptic digestion and to nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analysis (10). This method is different from the identification of free antigens by widely used antigen or antibody microarray technologies. In addition, we emphasize that the IC-antigens identified by this method have been directly shown to form antigen-antibody complexes *in vivo* in patients, which form as a result of immune recognition.

Based on these findings, the main purpose of this preliminary study was to clarify the following three issues in patients with BC: [i] characterize IC-antigen profiles in urine samples by using “immune complexome analysis” and identify the specificity of these factors in BC compared to non-tumoral conditions including urinary tract infection (UTI), urinary stone (US), and normal control, [ii] evaluate relationships between such disease-related IC-antigens and pathological features, grade, tumor growth (T stage) and metastasis, and [iii] define predictive value of IC-antigens for urinary tract recurrence in patients with NMIBC.

Patients and Methods

Patients. Ninety-seven patients diagnosed with BC at the Nagasaki University Hospital were analyzed. The specimens that included non-urothelial cancer, such as squamous cell carcinoma, adenocarcinoma, and neuroendocrine carcinoma, were excluded from this study. In this study, T stage was also divided into non-muscle invasive diseases (Ta and T1) and muscle invasive ones (MIBC; T2-4) in all patients, and into non-lamina propria invasion (pTa) and lamina propria invasion (pT1) in 67 patients with NMIBC. As non-tumoral controls, urine samples from UTI (N=52), US (N=22), and normal persons (N=46) were analyzed. In our study

population, all patients with UTI had pyuria (white blood cell>10/high power field) and those with US had hematuria (red blood cell>10/high power field) due to these diseases. The mean \pm SD age of BC, UTI, US, and normal control were 73.2 \pm 10.0, 68.9 \pm 11.6, 64.3 \pm 14.1, and 70.0 \pm 12.8 years, respectively. The mean ages in patients with UTI and US showed a trend to be lower than in those with BC; however, this difference was not significant ($p=0.203$ and 0.058 , respectively). The frequencies of males in BC, UTI, US, and normal controls were 82.5, 46.2, 86.3, and 78.3%, respectively. Thus, there were significantly fewer male patients with UTI ($p<0.001$) than those with BC.

The study protocol was approved by the Human Ethics Review Committee of Nagasaki University Hospital (No. 15102604–2; registered as UMIN000020152). All experiments complied with the principles embodied within the Declaration of Helsinki, and patients provided written informed consent to participate in all aspects of the study.

IC-antigens identification by nano-LC-MS/MS. Immune complexes (ICs) were purified by magnetic beads with immobilized Protein A (PureProteome®, Millipore, Darmstadt, Germany). Protein A-beads (40 μ l) were incubated with 50 μ l of each patient’s urine diluted with 50 μ l PBS (9.0 mmol/l Na_2HPO_4 , 2.9 mmol/l NaH_2PO_4 , 137 mmol/l NaCl) for 30 min with gentle mixing, then the liquid was removed. The beads with bound ICs were washed three times with 500 μ l PBS. The beads were resuspended in 100 μ l of 10 mM dithiothreitol and incubated at 56°C for 45 min. Next, 100 μ l of 55 mM iodoacetamide was added to the mixtures and incubated at room temperature in the dark for 30 min. Ammonium hydrogen carbonate (100 μ l of 50 mM) and 100 μ l of ultrapure water were added, followed by trypsin in 0.05% acetic acid to achieve a final concentration of 5 mg of trypsin/l, then the mixture was incubated overnight at 37°C. We then added 12 μ l of 10% trifluoroacetic acid and mixed the beads for 2 min to stop the digestion. This aliquot (about 400 μ l) was vacuum-reduced to approximately 80 μ l and stored at 4°C for subsequent analysis by nano-LC-MS/MS.

The tryptic digests (peptides) (1 μ l) were injected into an MS/MS instrument (LTQ XL, Thermo Fisher Scientific, Waltham, MA, USA) equipped with custom nano-LC system consisting of a Shimadzu LC pump (Kyoto, Japan) with an LC flow splitter (Dionex) and an HCT PAL autosampler (CTC Analytics, Zwingen, Switzerland). MS/MS data were extracted using Proteome Discoverer 1.3.1.339 (Thermo

Table II. Correlation with clinicopathological features and urinary tract recurrence.

IC-antigens	Clinicopathological features			Invasive potential		Recurrence in NMIBC
	Grade	T stage	Meta	Muscle	pTa/T1	
Serum albumin	0.819	0.909	0.105	0.435	0.585	0.001
α 1 antitrypsin	0.286	0.982	0.949	0.734	0.806	0.637
Fibrinogen γ chain	0.006	0.017	0.946	0.096	0.017	0.133
Hemoglobin subunit α	0.003	0.044	0.087	0.007	0.969	0.073
Hemoglobin subunit β	0.010	0.096	0.087	0.007	0.580	0.408
Complement C3	0.124	0.211	0.460	0.055	0.227	0.058
Ceruloplasmin	0.009	0.024	0.419	0.012	0.032	0.001
Fibrinogen β chain	0.106	0.089	0.751	0.027	0.110	0.071

Meta: Metastasis; NMIBC: non-muscle invasive bladder cancer. All data are *p*-values. Bold values indicate statistical significance.

Fisher Scientific). Spectra were searched against sub-databases from the public nonredundant protein database of UniProt Knowledgebase (human, 2015.01.29 download).

Statistical analyses. Student's *t*-test and Chi-square test were used to compare continuous and categorical variables, respectively. Scheffé's method was used for multiple comparisons of the data. Survival analyses were assessed using Kaplan-Meier curves and the log-rank test and were expressed using hazard ratios (HRs), 95% confidence intervals (CIs), and *p*-values. All statistical analyses were two-sided and performed using StatView for Windows (version 5.0; Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Detection of BC-related IC-antigens. We detected 328 IC-antigens from urine samples based on researching of a database including 20,165 proteins. Next, we excluded IC-antigens whose positive rates in BC were under 30% or those whose rates in normal controls were over 50%. Finally, eight IC-antigens were selected as candidates for BC-related factors. A flow chart and selected IC-antigens as BC-related factors are shown in Figure 1 and Table I respectively. The positive rates of these eight IC-antigens in BC were significantly higher ($p < 0.050$) than those in normal controls.

As shown in Table I, IC-serum albumin and IC- α 1 antitrypsin were most frequently detected (52.6%), followed by IC-fibrinogen γ chain (47.4%), IC-hemoglobin subunit α (46.4%), and IC-hemoglobin subunit β (46.4%). On the other hand, these factors also had a trend showing relatively high positive rates in normal controls. In short, the positive rates of IC-serum albumin and IC- α 1 antitrypsin in normal controls were 23.9 and 17.4%, respectively, and the rate of IC-hemoglobin subunit β was over 10% in normal controls (15.2%). On the other hand, regrading positive rates in UTI, there was no remarkable difference among all IC-antigens. Indeed, all positive UTI rates were under 20% (Table I). In contrast, the positive rates of IC-antigens in the US were

relatively higher than those in other non-tumoral conditions. In particular, the rates of IC-serum albumin, IC- α 1 antitrypsin, and IC-fibrinogen γ chain were over 50.0% in US patients. Finally, IC-ceruloplasmin was the only factor in which the positive rate in US was under 30%.

Correlation with clinicopathological features. Although IC-serum albumin and IC- α 1 antitrypsin were detected most frequently in urine samples of BC, they were not significantly associated with all the pathological parameters (Table II). On the other hand, the IC-fibrinogen γ chain, IC-hemoglobin subunit α , and IC-ceruloplasmin were significantly associated with grade and T stage, and IC-hemoglobin subunit β was associated with grade, but not with T stage (Table II). Similar to IC-serum albumin and IC- α 1 antitrypsin, none of the other factors showed a significant correlation with metastasis. When we analyzed the invasive potential for muscle (Ta and 1 *versus* T2-4) and lamina propria (Ta *versus* T1), IC-hemoglobin subunit α , IC-hemoglobin subunit β , IC-ceruloplasmin, and IC-fibrinogen β chain were significantly associated with muscle invasion, and IC-fibrinogen γ chain and IC-ceruloplasmin were associated with lamina propria invasion (Table II). Thus, only IC-ceruloplasmin was associated with both muscle invasion and lamina propria invasion. Based on these findings, detailed data on the relationships between these five IC-antigens and pathological features are shown in Tables III and IV.

Correlation with urinary tract recurrence. The relationship between our eight factors and urinary tract recurrence was investigated in 67 NMIBC patient IC-serum albumin and IC-ceruloplasmin levels were identified as significant predictors of urinary tract recurrence ($p = 0.001$; Table II). Hemoglobin subunit α , IC-complement C3, and IC-fibrinogen β chain had a trend to be significantly associated with urinary tract recurrence; however, the difference was not significant

Table III. Detailed data on relationships between IC-antigens and clinicopathological features.

Clinico-pathological features; N (%)	Fibrinogen γ chain		Hemoglobin subunit α		Hemoglobin subunit β		Ceruloplasmin		Fibrinogen β chain	
	Negative N=51	Positive N=46	Negative N=52	Positive N=45	Negative N=52	Positive N=45	Negative N=60	Positive N=37	Negative N=61	Positive N=36
Grade										
Low	26 (51.0)	11 (23.9)	27 (51.9)	10 (22.2)	26 (50.0)	11 (24.4)	29 (48.3)	8 (21.6)	27 (44.3)	10 (27.8)
High	25 (49.0)	35 (76.1)	25 (41.7)	35 (77.8)	26 (50.0)	34 (75.6)	31 (51.7)	29 (78.4)	34 (55.7)	26 (72.2)
<i>p</i> -Value	0.006		0.003		0.010		0.009		0.106	
T stage										
Ta	28 (54.9)	12 (26.1)	25 (48.1)	15 (33.3)	24 (46.2)	16 (35.6)	32 (53.3)	8 (21.6)	31 (50.8)	9 (25.0)
T1	11 (21.6)	16 (34.8)	17 (32.7)	10 (22.2)	18 (34.6)	9 (20.0)	15 (25.0)	12 (32.4)	16 (26.2)	11 (30.6)
T2	4 (7.8)	8 (17.4)	4 (7.7)	8 (17.8)	4 (7.7)	8 (17.8)	6 (10.0)	6 (16.2)	6 (9.8)	6 (16.7)
T3	3 (5.9)	8 (17.4)	2 (3.8)	9 (20.0)	3 (5.8)	8 (17.8)	5 (8.3)	6 (16.2)	4 (6.6)	7 (19.4)
T4	5 (9.8)	2 (4.3)	4 (7.7)	3 (6.7)	3 (5.8)	4 (8.9)	2 (3.3)	5 (13.5)	4 (6.6)	3 (8.3)
<i>p</i> -Value	0.017		0.044		0.096		0.024		0.089	
Metastasis										
Absence	44 (85.1)	40 (85.1)	47 (90.4)	35 (77.8)	47 (90.4)	35 (77.8)	54 (87.1)	30 (81.1)	54 (85.7)	30 (83.3)
Presence	8 (15.4)	7 (14.9)	5 (9.6)	10 (22.2)	5 (9.6)	10 (22.2)	8 (12.9)	7 (18.9)	9 (14.3)	6 (16.7)
<i>p</i> -Value	0.946		0.087		0.087		0.419		0.751	

Bold values indicate statistical significance.

Table IV. Detailed data on relationships between IC-antigens and invasive potential.

Invasive status N (%)	Fibrinogen γ chain		Hemoglobin subunit α		Hemoglobin subunit β		Ceruloplasmin		Fibrinogen β chain	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Muscle	N=51	N=46	N=52	N=45	N=52	N=45	N=60	N=37	N=61	N=36
Absence; Ta/1	39 (76.5)	28 (60.9)	42 (80.8)	25 (55.6)	42 (80.8)	25 (55.6)	47 (78.3)	20 (54.1)	47 (77.0)	20 (55.6)
Presence; T2-4	12 (23.5)	18 (39.1)	10 (19.2)	20 (44.4)	10 (19.2)	20 (44.4)	13 (21.7)	17 (45.9)	14 (23.0)	16 (44.4)
<i>p</i> -Value	0.097		0.007		0.007		0.012		0.027	
Lamina propria	N=39	N=28	N=42	N=25	N=40	N=27	n=47	n=20	n=47	n=20
Absence; pTa	28 (71.8)	12 (42.9)	25 (59.5)	15 (60.0)	24 (57.1)	16 (64.0)	32 (68.1)	8 (40.0)	21 (66.0)	9 (45.0)
Presence; pT1	11 (28.2)	16 (57.1)	17 (40.5)	10 (40.0)	18 (42.9)	9 (36.0)	15 (31.9)	12 (60.0)	16 (34.0)	11 (55.0)
<i>p</i> -Value	0.017		0.969		0.580		0.032		0.110	

Bold values indicate statistical significance.

($p=0.073$, 0.058 , 0.071 , respectively). The corresponding Kaplan-Meier survival curves for IC-serum albumin and IC-ceruloplasmin are shown in Figure 2A and B.

Discussion

This is the first study on immune complexome analysis in not only urine samples, but also BC patients. We previously identified novel disease-specific factors in various pathological conditions, such as autoimmune diseases, infection, and transplant rejection, by using immune complexome analysis (10, 13-15). In addition to these non-tumoral disorders, immune complexome analysis successfully identified disease-specific IC antigens (gelsolin and inter-alpha-trypsin inhibitor heavy chains) in patients

with lung cancer (16). Furthermore, the usefulness of immune complexome analysis to identify disease-specific factors and biomarkers has been confirmed by other investigators in patients with Bechet's disease and those with pancreatic cancer (17,18). First, the original method of immune complexome analysis was designed to detect ICs in blood samples (10). However, in the present study, this analysis was modified to evaluate the ICs in other fluid samples. In fact, we identified disease-specific IC-antigen from cerebrospinal fluid in patients with central nervous system autoimmune diseases and from follicular fluid in infertile patients (19, 20).

One of most important results in the present study is that IC-ceruloplasmin was the most useful BC-related urine marker because its positive rate in BC was higher than that in all non-

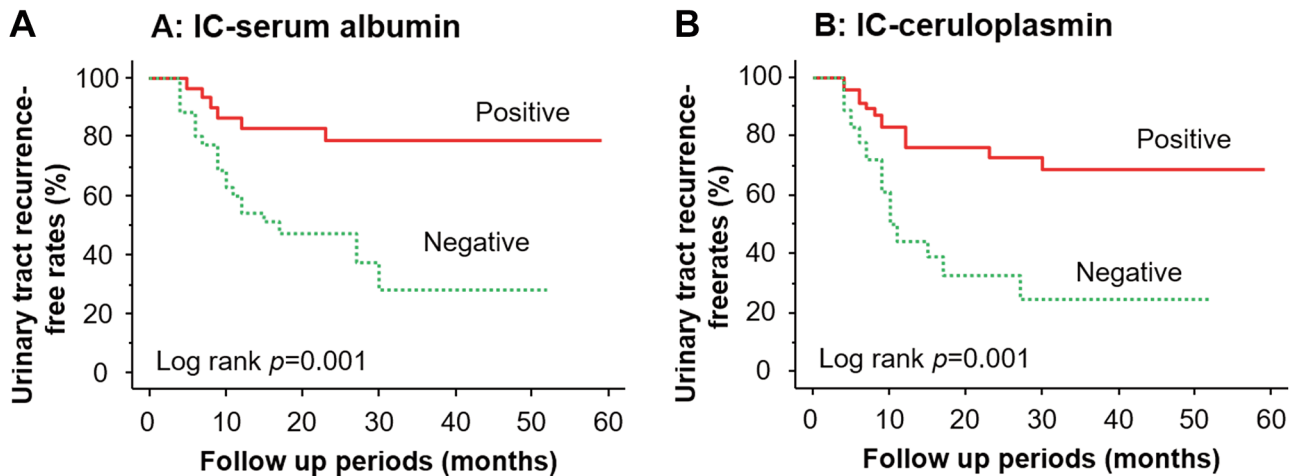


Figure 2. Kaplan-Meier survival curves for recurrence-free survival rates of immune complex-serum albumin (A) and immune complex-ceruloplasmin (B).

tumoral conditions and was also significantly associated with grade, T stage, invasive potential, and urinary tract recurrence. Ceruloplasmin is a glycoprotein with a molecular weight of 132 kDa, and it is recognized as a multicopper oxidase with functions including copper transport, ferroxidase activity, and superoxide dismutase activity (21, 22). The most representative ceruloplasmin-related disease is Wilson disease, which is caused by a low level of serum ceruloplasmin, and a high level of ceruloplasmin leads to various diseases, such as inflammatory and neurodegenerative diseases (23-25). On the other hand, many investigators support the opinion that ceruloplasmin is closely associated with carcinogenesis, malignant behavior, and prognosis in various types of malignancies (21, 26, 27). In addition, serum ceruloplasmin levels in cancer patients were increased compared to those in normal controls, and it was positively correlated with malignant aggressiveness in various malignancies, including Hodgkin lymphoma, cervical cancer, and lung cancer (28-30). Thus, there is a general agreement that ceruloplasmin plays important roles in tumorigenesis, cancer cell progression, and prognosis in many types of malignancies. On the other hand, in BC patients, increased serum ceruloplasmin levels were reported by several investigators; however, there was a finding that the sensitivity of serum ceruloplasmin levels in urinary tract urothelial cancers was lower than that in renal cell carcinoma (31, 32). Nevertheless, regarding ceruloplasmin in the urine samples, BC patients were reported to have significantly higher ($p<0.05$) levels than those in controls (patients with hernia) (33). In addition, the same study showed that urine ceruloplasmin levels were significantly associated with stage and grade in patients with BC (33). These results were similar to our results; however, it was difficult to verify them because their data were obtained using multiplexed

liquid chromatography-multiple reaction monitoring/mass spectrometry. Furthermore, there were no detailed data on relationship between urine ceruloplasmin levels and pathological features, including pTa and pT1, because the main purpose is to discover biomarkers for BC diagnosis. On the other hand, we emphasize the new information in the present study; for example, the relationship between detection of IC-ceruloplasmin and pT stage, invasion into muscle or lamina propria, and outcome, is important to understand the clinical significance of ceruloplasmin and to discuss the observation and treatment strategies in BC patients.

In addition to IC-ceruloplasmin, our results also showed that IC-serum albumin, IC-fibrinogen γ chain, fibrinogen β chain, IC-hemoglobin subunit α , IC-hemoglobin subunit β were associated with any malignant aggressiveness in patients with BC. In a previous study on the detection of newly diagnostic urine marker of urothelial neoplasm of the bladder, two-dimensional gel electrophoresis and image analysis demonstrated that albumin was increased in urothelial neoplasm samples compared to normal controls (34). That study also showed that such changes in urine albumin were not dependent on tumor grade (34). These findings are similar to our results obtained by the immune complexome. However, there was no information on the relationships between urine albumin and T stage, metastasis, and prognosis because of the small number of patients ($N=12$). Other investigators have studied the clinical significance of albuminuria in various types of cancers, including BC (35). They found that albuminuria was associated with the incidence of BC and lung cancer. In addition, they suggested that proteinuria was recognized as a non-specific marker of these cancers because it reflected vascular permeability caused by cancer-related cytokines

(35). Unfortunately, this study also had no detailed information on the pathological significance and prognostic roles of proteinuria in BC patients.

Although there is no study on fibrinogen β chain in urine samples of BC patients, several studies have shown that fibrinogen β chain precursor in urine was reported to be one of most useful biomarkers for diagnosis and prediction of tumor stage in BC patients (36, 37). Thus, there is a possibility that urine fibrinogen β chain is associated with any malignant aggressiveness and prognosis in these patients. We suggest detailed study on pathological significance of urine fibrinogen β chain in patients with BC. On the other hand, in lung cancer patients, there was a report that urine level of fibrinogen γ chain was elevated compared to normal controls (38). Unfortunately, there is little information about the pathological significance of urine fibrinogen γ chain in BC patients; however, higher frequency of fibrinogen γ chain dysregulation in blood samples compared to healthy controls was shown by analysis using dithiothreitol-based protein equalization technology in blood samples of BC patients (39). This study did not mention the detailed pathological significance of such dysregulation of the fibrinogen γ chain in BC patients. However, their results might support our finding that positive rates of the IC-fibrinogen γ chain in BC were remarkably higher than those in normal controls.

On the other hand, there are few reports on the pathological significance and prognostic roles of hemoglobin subunit α and β . Actually, this is the first report showing that hemoglobin subunit α - and hemoglobin subunit β -related parameters were identified as potential urine markers and associated with malignant potential, including grade and muscle invasion in BC patients. Hemoglobin subunits α and β are members of the globin family, and they are well-known to play important roles in the binding and transport of O_2 in erythrocytes (40). Regarding malignant cells, there was a report that hemoglobin subunits α and β were potential serum biomarkers of the diagnosis and prognosis in patients with ovarian cancer (41). Similar findings were reported by other investigators in lung cancer patients (42). However, these studies were performed in 35 ovarian cancer patients and 30 lung cancer patients, and the detailed mechanisms of these findings are not clear. On the other hand, in *in vivo* and *in vitro* studies, several investigators have shown that hemoglobin subunit β played crucial roles in malignant aggressiveness and prognosis in breast cancer and neuroblastoma (43, 44). In these reports, they speculated that modulation of tumor growth, apoptosis, and invasion-related factors were associated with such pathological roles (43, 44). However, there are many unknown issues about the pathological significance of hemoglobin subunit α and β in malignant cells including BC.

The major limitation is that this study had a relatively small number of patients with BC, UTI, US, and normal

controls. In addition, patient background, such as age and gender, was not unified in our study population. The next limitation is that urine levels of BC-related factors were not evaluated using versatile methods, such as enzyme-linked immune-sorbent assay or radioimmunoassay. In short, our immune complexome analysis can detect the IC form of an antigen, but not a simple protein. However, this study was performed as a preliminary study to clarify whether our original methods could detect BC-related factors in urine samples. In addition, this study is useful to narrow down the clinically significant BC-related factors among numerous candidates. Furthermore, our method can detect antigen-antibody complexes which form as a result of immune response under pathological conditions. On the other hand, various factors are reported as disease-specific marker in patients with BC (45, 46). Therefore, further studies on the urine levels of our significant BC-related IC-antigens are necessary, and the results obtained by such future studies and findings showed by other studies may be important to discuss the diagnostic tools and observation strategies in BC.

Conclusion

By using our original method that detected ICs in fluid samples, our study showed that six IC-antigens (serum albumin, fibrinogen γ chain, hemoglobin subunit α , hemoglobin subunit β , ceruloplasmin, and fibrinogen β chain), in urine, were associated with any of the clinicopathological features and outcomes in patients with BC. In particular, IC-ceruloplasmin was recognized as the most useful urine biomarker for malignant aggressiveness, except for metastasis.

Conflicts of Interest

The Authors have no conflicts of interest regarding this study.

Authors' Contributions

NA performed the experiments and contributed to the sample collection and writing of the manuscript. Yasuyoshi Miyata and Kaname Ohyama conceived and designed the experiments, performed data analysis, and contributed to the writing of the manuscript. KA performed the experiments and analyzed the data. YS, KM, TM, Kojiro Ohba, and Yasushi Mochizuki contributed to the sample and clinical data collection and to the writing of the manuscript. HS designed the experiments and acted as supervisor.

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