Tohoku J. Exp. Med., 2020, **252**, 225-244

Urine Lactoferrin as a Potential Biomarker Reflecting the Degree of Malignancy in Urothelial Carcinoma of the Bladder

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Urothelial carcinoma of the bladder (UCB) is potentially life-threatening; therefore, we aimed to discover a novel urine biomarker for diagnosis and prognostication of UCB. This is a retrospective case-control study. Exploration of a new biomarker using urine from 20 UCB patients in the present study revealed that urinary level of lactoferrin (LF), a multifunctional glycoprotein released from neutrophils, was higher in 11 of 15 with invasive/high-grade UCB than 5 with non-invasive one, and 2 healthy adults. We therefore focused on LF and assessed the value of urine LF normalized by urine creatinine concentration (LF/Cr) using an enzyme-linked immunosorbent assay. Diagnostic performance of urine LF/Cr was examined using urine from 92 patients with primary (newly diagnosed) untreated UCB and 166 controls without UCB, including 62 patients with pyuria, and 104 subjects without pyuria consisting of 84 patients and 20 healthy adults. However, the diagnostic accuracies were accompanied by the risk of bias. In 92 primary UCB patients, both pyuria and tumor-infiltrating neutrophils (TINs) were independent predictors for urine LF/Cr. In contrast, TINs or urine LF/Cr were independent predictors for invasive histology, whereas pyuria was not. In terms of prognostication, urine LF/Cr and nodal metastasis were independent predictors of disease-specific survival in 22 patients with muscle-invasive bladder cancer, characterized by a high mortality rate, in the Cox proportional hazards model. In conclusion, urine LF/Cr linked to TINs was a predictor of both invasive histology and prognosis in UCB. Urine LF/Cr is a potential biomarker reflecting the degree of malignancy in UCB.

Keywords: degree of malignancy; lactoferrin; tumor-infiltrating neutrophils; urine biomarker; urothelial carcinoma of the bladder
Tohoku J. Exp. Med., 2020 November, **252** (3), 225-244.

Introduction

Urothelial carcinoma of the bladder (UCB) is the fourth most common cancer in men in developed countries (Jemal et al. 2011; Siegel et al. 2013). Upon detection of UCB, approximately 70-75% of the patients are diagnosed with non-muscle-invasive bladder cancer (NMIBC), while the remaining 25-30% of the patients are diagnosed with muscle-invasive bladder cancer (MIBC) (Burger et al. 2013; Witjes et al. 2014; Babjuk et al. 2017). The relationships between pathological T staging and NMIBC or MIBC are as follows: pTa, pTis, and pT1 are grouped as NMIBC, and pT2, pT3, and pT4 are classified as MIBC (The Japanese Urological Association et al. 2011; Grignon et al. 2016). Concurrently, invasive urothelial carcinoma is pathologically defined as invasion beyond the basement membrane (pT1 or more) (Grignon et al. 2016). The majority of NMIBC cases are managed by bladder preserving therapies such as transurethral resection of bladder tumor (TURBT) with or without intravesical instillation of Bacillus Calmette-Guérin (BCG). Bladder preserving therapy for NIMBC faces two major challenges, that is, recurrence and progression after TURBT (Sylvester et al. 2006). Recurrence indicates bladder tumor recurrence, and the

Received May 18, 2020; revised and accepted October 13, 2020. Published online November 6, 2020; doi: 10.1620/tjem.252.225.
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term “progression” is applied when NIMBC has progressed to muscle-invasive disease or one that is more advanced. Intravesical instillation of BCG has been widely used as the most effective treatment to prevent recurrence, whereas its ability to control progression remains controversial (Gontero et al. 2010). In contrast, the standard treatment of MIBC is radical cystectomy (Witjes et al. 2014; Babjuk et al. 2017). Despite this standard of care, the 5-year survival rate of MIBC patients who underwent radical cystectomy remains only 50% (Witjes et al. 2014). Nevertheless, patients with less muscle-invasive cancer are more likely to have a better overall survival (Hautmann et al. 2012). Since invasive UCB exhibits an aggressive behavior, early detection of invasive UCB may improve patient prognosis. Currently, the combination of cystoscopy and urinary cytology is the standard method for the detection and diagnosis of UCB, owing to the high sensitivity of cystoscopy and high specificity of cytology (Budman et al. 2008). However, cystoscopy may cause pain, bleeding, or infection, while urinary cytology has been shown to have low sensitivity and is highly dependent on a cytopathologist’s level of expertise (van Rhijn et al. 2005; Budman et al. 2008). Currently, there are no available urine biomarkers with high diagnostic accuracies to serve as substitutes for the combination of cystoscopy and cytology (Budman et al. 2008; Szarvas et al. 2018). Furthermore, urine biomarkers, which predict the prognosis of UCB patients, remain to be established for use in clinical practice (Szarvas et al. 2018), although CXCL1 and pyuria have been reported to be associated with recurrence and/or progression of NMIBC (Azuma et al. 2013; Nakashima et al. 2015). We hypothesized that there may exist a urinary biomarker for the diagnosis and prognostication of UCB patients; therefore, the purpose of this study was to identify such biomarkers. At the stage of exploration for a new biomarker, western blot analysis using urine from 20 patients with UCB revealed that level of urine lactoferrin (LF) was higher in 11 of 15 with invasive/high-grade UCB than five with non-invasive one, and two healthy adults. We therefore focused on LF and assessed the value of urine LF normalized by urine creatinine concentration (LF/Cr) using an enzyme-linked immunosorbent assay. LF is an iron-binding glycoprotein, which exists in exocrine secretions and in the secondary granules of neutrophils in humans, and has been known to have multifunctional properties such as antimicrobial, anti-inflammatory, anti-tumor activities, etc. (Kanwar et al. 2015). The endpoints of the present study were to investigate the possibility of urine LF/Cr as a diagnostic and prognostic marker. First, we examined the origin of LF, which revealed that UCB cells did not contribute to urine LF levels, although neutrophils are known to release LF during inflammation (Masson et al. 1969; Baggiolini et al. 1970). Second, we studied the relationship between level of urine LF/Cr and clinicopathological variables in UCB patients. We found that both pyuria and TINs were independent predictors of urine LF/Cr. In contrast, while TINs or urine LF/Cr were independent predictors for invasive histology, pyuria was not. Third, we investigated possibility of urine LF/Cr as a prognostic biomarker, and it was suggested that urine LF/Cr might be a predictor for the prognosis of MIBC patients. Lastly, we calculated the area under the receiver operating characteristic curve (AUC) of urine LF/Cr to distinguish patients with UCB or those with invasive UCB from the subjects without pyuria. However, the diagnostic accuracies of urine LF/Cr shown in the present study were accompanied by the risk of bias inherent to the case-control design. We also described the quality assessment of reporting according to the quality assessment items (Szarvas et al. 2018). To the best of our knowledge, this is the first report on the urine LF as a potential biomarker reflecting the degree of malignancy in UCB.

Materials and Methods

Patients and samples

Urine samples and tumor and non-tumor tissues were obtained from 144 patients presenting with UCB, who were admitted to the University of the Ryukyus Hospital and Naha City Hospital between January 2009 and June 2018. We failed to obtain samples from consecutive patients and therefore the cases were convenience series. Urine samples for the index test (LF/Cr) were collected 1 or 2 days before TURBT for the reference standard. Urine samples were also obtained by convenience sampling from 166 controls without UCB, which included 62 urologic patients with pyuria and 104 subjects without pyuria consisting of 84 urologic patients and 20 healthy adults including 7 healthy volunteers at the University of the Ryukyus Hospital between July 2009 and September 2013. Thus, most of the controls were hospital controls. Pyuria was defined as the presence of 10 or more leucocytes per high-power field (≥ 10/HPF) in the urinary sediment (Echols et al. 1999). Seven healthy volunteers were regarded as having no pyuria although they did not undergo examination of urinary sediments. All urine samples from UCB patients and control subjects without UCB were spot urine samples. After precipitation of the urinary sediment by centrifugation of urine at 2,000 × g, the supernatant was aliquoted and stored at –80°C until analysis. Six autopsy specimens of the urinary bladder were obtained from the Department of Pathology, University of the Ryukyus Hospital. Both Ethics Committees of the University of the Ryukyus and Naha City Hospital approved the present study, and informed consent was obtained from each patient and healthy volunteer. We have secured the records of the patients.

Urine samples used for exploration of a new biomarker

Urine samples from 20 patients with UCB consisting of 7 with primary (newly diagnosed) untreated tumors, 8 in state after TURBT for primary tumors, and 5 with recurrent tumors, were used for exploration of a new biomarker by western blot analysis. Out of 20 cases, 12 were invasive UCB, 3 were high-grade CIS, and 5 were non-invasive

UCB. We also performed western blot analysis using urine from 24 control subjects consisting of 12 with pyuria and 12 without pyuria.

Case definition and reference standard

Cases were defined by the reference standard, which was a pathological diagnosis based on TURBT specimens, independent of the index test. A single pathologist (N.K.), who was unaware of the values of urine LF/Cr, conducted the pathological evaluation of all patients (The Japanese Urological Association et al. 2011; Grignon et al. 2016). Histological grading (G1, G2 or G3) of UCB was judged according to the WHO 1973 classification (Mostofi et al. 1973). Thus, the European Organization for Research and Treatment of Cancer (EORTC) scores of recurrence and progression in NMIBC, which predict 1- and 5-year probabilities of both events after TURBT, were calculated (Sylvester et al. 2006). TINs were defined as positive when at least one part of 5 or more neutrophils per 200 × field of view were observed in the tumor or tumor stroma in a slide. TINs were evaluated on slides stained with hematoxylin-eosin.

Eligibility criteria

To investigate the possibility of urine LF/Cr as a diagnostic and prognostic marker, we selected patients based on the following criteria: postoperative ascertainment of pathological diagnosis as UCB based on the TURBT specimen, no history of UCB or upper urinary tract cancer, no other uncontrolled cancer, and no TURBT or chemotherapy before urine sampling. Out of 144 patients, 92 with primary untreated UCB (80 from the University of the Ryukyus Hospital and 12 Naha City Hospital) were included in this study (Fig. 1). As shown in the flow diagram, we avoided inappropriate exclusion.

The diagnosis of 166 control subjects were obtained from the medical records (see Table 3). All control subjects except seven healthy volunteers underwent ultrasound examination and were shown to have no UCB. All control patients were ascertained to have no history of cancer. However, we did not perform cystoscopy for these controls except patients with urinary stones, who underwent transurethral lithotripsy. All of 92 patients and all control subjects underwent the index test. There were no withdrawals in the UCB patients and control subjects.

Study design

This was a retrospective case-control study because medical records were reviewed after the reference standard and the index test was performed. The endpoints of this study were to examine whether urine LF/Cr was a diagnostic and prognostic marker. To this end, we analyzed the

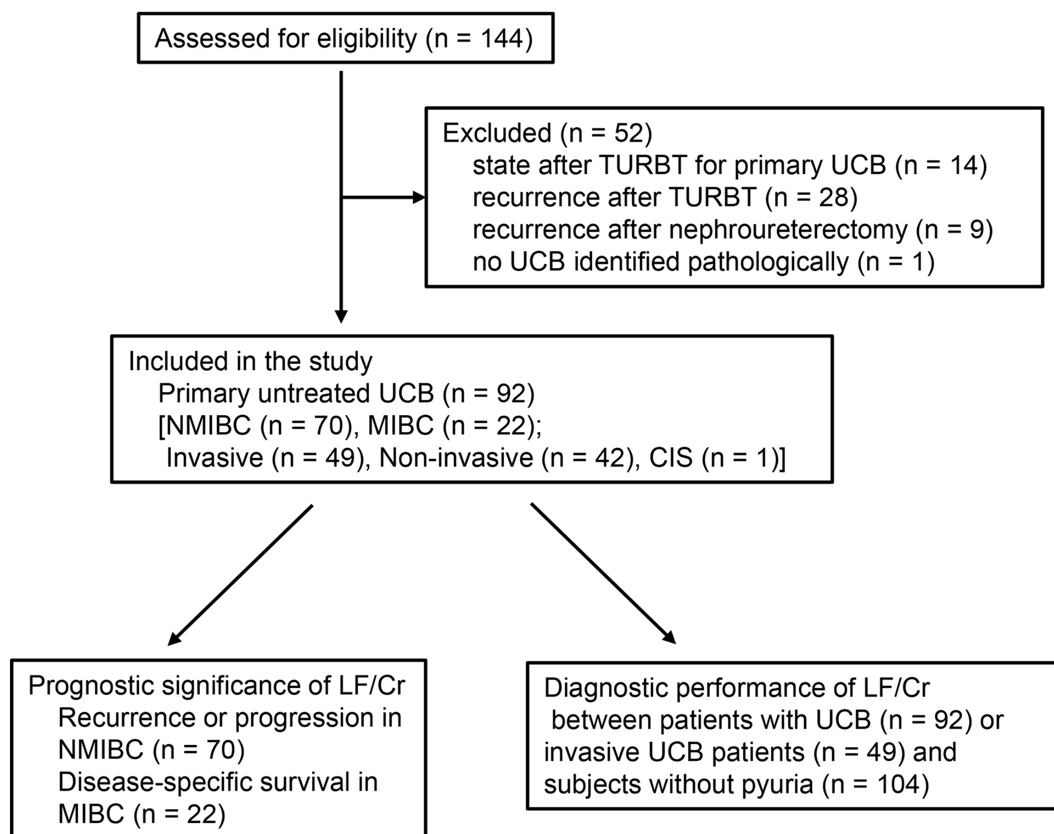

Fig. 1. Flow diagram of the UCB patients.
Participant flow diagram is shown.

explanatory variables for the level of urine LF/Cr and the variables predicting invasive histology. We also investigated whether the level of urine LF/Cr was predictors for recurrence or progression of NIMBC and prognosis of MIBC, and the diagnostic performance of urine LF/Cr. Preoperative variables examined were age, sex, body mass index (BMI), Brinkman index, urinary white blood cells (u-WBC), urinary red blood cells (u-RBC), urinary cytology, tumor number, maximum tumor diameter, and urine LF/Cr as a continuous or categorical variable. Postoperative variables included invasive or non-invasive histology, pathological T stage, and TINs. In survival analysis, stratification by stage was not used for the study on prognosis of MIBC patients because of the small number of patients. The end of the follow-up was June 2019. The median follow-up time of the patients with NMIBC and those with MIBC was 862 days (range: 62-3,459 days) and 700 days (range: 22-3,467 days), respectively.

Analysis of urine protein

Proteins from 15 μL aliquot of urine were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB) for the comparison between samples from patients with bladder cancer and healthy adults. The band with an approximate molecular mass of 80 kDa increased in cancer patients; this band was isolated with a razor blade and subjected to in gel digestion. The tryptic digest was analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Data were searched against NCBInr database and selected by Mascot Database Manager.

Urothelial cell lines

Human malignant urothelial cell lines and immortalized human normal urothelial cell lines were used in this study. Two human urothelial cancer cell lines T24 and 5637, both of which have invasive capacity (Pang et al. 2019), were purchased from RIKEN BioResource Center. The two immortalized human urothelial cell lines MC-SV-HUC T-2 and SV-HUC-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The identity of these cell lines was confirmed by short tandem repeat (STR) analysis (Takara Bio Inc, Shiga, Japan). 5637 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, NY) and T24 cells were cultured in Minimum Essential Medium (MEM; Gibco, Grand Island, NY). MC-SV-HUC T-2 cells were cultured in Ham's F-12 medium (Wako, Japan), while SV-HUC-1 cells were cultured in Ham's F-12K medium (Wako, Japan). Each medium was supplemented with or without 10% fetal bovine serum (Gibco) at 37°C in humidified air containing 5% CO_2.

Western blot analysis

Proteins from 15μL aliquot of urine, cell lysate or medium was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Mississauga, Ontario, Canada) using a semidry blotting system (Bio-Rad, Canada). Membranes were blocked with 1% bovine serum albumin (BSA; Sigma, USA) in phosphate-buffered saline (PBS) and Tween-20 (PBST, 137 mM sodium chloride [NaCl], 8.1 mM sodium phosphate [Na_2HPO_4], 2.68 mM potassium chloride [KCl], 1.47 mM monopotassium phosphate [KH_2PO_4] and 0.05%Tween-20) for 1hr at room temperature. Membranes were washed twice with PBST, and overnight incubated with anti-lactoferrin antibody (Abcam plc, Cambridge, UK) at 4°C. Blots were washed thrice with PBST, and probed for 1hr with horseradish peroxidase (HRP)-conjugated anti-mouse IgG + A + M (ZYMED, USA) at 1/2,000 dilution or anti-rabbit IgG (Santa Cruz Biotechnology, USA,) at 1/10,000 dilution. After washing thrice with PBST, the protein bands were visualized using an enhanced chemiluminescent detection system (Amersham, GE healthcare, Sweden).

Enzyme-linked immunosorbent assay (ELISA)

To detect and quantify the concentration of urine LF, LF ELISA assay was conducted using human LF ELISA kit (Assaypro LCC, St. Charles, MO) according to the manufacturer's instructions by one of the authors (A.S.), who was unaware of the clinicopathological data. The absorbance for the LF ELISA was measured at 450 nm, with 595 nm as a reference wavelength, using an iMark Plate Reader (Bio-Rad Laboratories, Inc. Hercules, CA). The concentration of urine creatinine was measured by creatinine (urinary) colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The concentration of urine LF measured by ELISA was normalized with that of urine creatinine, to obtain the value of LF/Cr (ng/mL/gram creatinine [ng/mL/g Cr]). All tests were performed in duplicates.

To detect LF in the culture media of the cell lines by ELISA, malignant urothelial cell lines (5637 and T24) and immortalized normal urothelial cell line (SV-HUC-1 cells) were maintained in their respective complete medium. Cells were seeded at a density of 2×10^5 (T24 cells) or 4×10^5 cells (5637 and SV-HUC-1 cells) onto a 10-cm tissue culture dish in the complete medium. After 24 h, the cells were washed twice with PBS, and grown in Opti-MEM® I reduced-serum medium (Life Technologies Japan, Tokyo, Japan). Three days later, the conditioned media were collected by centrifugation and the concentration of LF in the conditioned media was measured using human LF ELISA kit (Assaypro LLC, St. Charles, MO) according to the manufacturer's instructions. This experiment was carried out in duplicates.

Immunohistochemical staining of tissue sections

Tissue specimens were surgically obtained and fixed in 10% formalin for 24 h at room temperature, followed by paraffin embedment at 56°C. From each tissue block, 4-μm

thick sections were cut, mounted on MAS-coated slide glasses (Matsunami Glass Ind., Tokyo, Japan), and de-paraffinized in xylen. The sections were rehydrated in graded ethanol solutions. For antigen retrieval of tissue sections, the slides were incubated with Proteinase K (DAKO) for 5 min at room temperature in a moist chamber. Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) for 10 min at room temperature and the slides were washed thrice with PBS. The sections were blocked with 5% BSA in PBS and incubated with primary antibody (rabbit polyclonal anti-human LF; Abcam, UK) at 4°C overnight. The slides were washed thrice with PBS and probed with a secondary antibody (EnVison/HRP; DAKO, Japan) according to the instructions supplied by the manufacturers. Visualization was performed with 3′, 3′-diaminobenzidine substrate solution. The slides were counterstained with hematoxylin 3G (Sakura-finetek, Tokyo) for 45 s at room temperature. LF immunoreactivity was classified into 2 categories: positive, ≥ 10% of tumor cells were immunostained; negative, < 10% of tumor cells were immunostained.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (Quiagen, Germany) according to the manufacturer's instructions. The expression of LF mRNA was first analyzed by RT-PCR. Briefly, 4 μg of total RNA sample was used to synthesize cDNA using Cloned AMV first-stranded cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. The forward and reverse primers were synthesized by Greiner, Japan. The sequences of the forward (F) and reverse (R) primers were as follows: LF F, 5′-GAGAGATACTACGGCTACAC-3′; and R, 5′-CTGGGCCATCTTCTTCGGTT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F, 5′-CCATGGAGAAGGCTGGGG-3′; and R, 5′-CAAAGTTGTCATGGATGACC-3′. PCR was performed for 3 min at 95°C, followed by 30 cycles using the following profile: 95°C, 15 s; 55°C, 15 s; 72°C, 30 s; and a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on 2% agarose gel and the bands were visualized by ethidium bromide staining. The expression level of LF was compared with that of the housekeeping gene GAPDH.

Real-time quantitative PCR (RT-qPCR)

The quantification of lactoferrin was performed by real-time PCR, using Syber GreenI chemistry (Roche, Germany). Assays were run with the Light Cycler instrument. Amplifications were carried out in 20 µl of reaction volume, mixing 5 µl of target cDNA and 15µl of the master mix containing forward and reverse primers. The PCR conditions used in the Light Cycler were as the following profile: 95ºC, 10 s; 60ºC,10 s; 72ºC, 10 s per cycle. Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer on the PCR products. β-actin was used as an internal reference. RT-qPCR on LF and β-actin cDNAs for every cell line could be performed in triplicate except T 24 cells. Threshold cycle (Ct) value for each LF and β-actin cDNA was obtained, respectively, and ΔCt was calculated as the difference between LF Ct and β-actin Ct values. Then, using SV-HUC-1 as a calibrator sample, ΔΔCt as the difference between target Ct and calibrator Ct was calculated. Relative level of PCR product to a calibrator sample was determined using the formula $2^{-\Delta\Delta Ct}$.

Cell proliferation assay

Cell proliferation assay was conducted on malignant urothelial cell lines and immortalized normal urothelial cells as previously described, with some modifications (Shaheduzzaman et al. 2007). Cells in the basal medium were added to each well of the 96-well plates at a density of 1×10^3 and incubated for 24 h. Cells were grown with various concentrations (0, 3.1, 6.3, 12.5, 25, and 50 μg/mL) of LF (Sigma-Aldrich Co., St. Louis, MO) in Opti-MEM® I reduced-serum medium (Life Technologies Japan, Tokyo, Japan). The plates were incubated for 24, 48, 72 and 96 h and the cell viability evaluated using cell counting reagent SF (Nacalai tesque, Inc., Kyoto, Japan), following the manufacturer's instructions. All samples were analyzed in triplicates. Statistical analysis was performed by a Mann-Whitney *U*-test.

Statistical analysis

Statistical analyses were conducted using JMP Pro 15.0 of the SAS Institute (SAS Institute Inc., Cary, NC). The association between the value of urine LF/Cr and each categorical or continuous variable was conducted by non-parametric Wilcoxon/Kruskal-Wallis test or regression analysis. The association between two categorical variables was analyzed using the chi-square test. Both multiple regression and multivariate logistic regression analysis models were applied to examine the explanatory variables for the level of urine LF/Cr as a dependent variable. In these analyses, the level of urine LF/Cr was handled as both a continuous and categorical variable. Urine LF/Cr as the categorical variable was dichotomized by the cut-off value, which was set at the median of UCB patients (293.5 ng/mL/g Cr). Multivariate logistic regression analysis models were used to estimate the explanatory variables for the invasive histology of UCB as a dependent variable. In this situation, urine LF/Cr was used as an explanatory variable and handled as both a continuous and categorical variable. The cut-off value of urine LF/Cr, which gave the maximum Youden index (sensitivity – [1 – specificity]) in discriminating invasive from non-invasive histology based on the receiver operating characteristic (ROC) curve, was determined to be 203.3 ng/mL/g Cr (Youden 1950). At this value, the sensitivity and specificity of the cut-off value were 0.74 and of 0.67, respectively. To calculate the diagnostic accuracy of LF/Cr, the area under the ROC curve

(AUC) was utilized. The cut-off value was determined to provide the maximum Youden index based on the ROC curve. For survival analysis, the cut-off of LF/Cr was set at a median value in both NMIBC and MIBC patients. Kaplan-Meier survival curves and log-rank tests were applied for survival analysis. The Cox proportional hazards model was applied to examine the independent predictors of the disease-specific survival of MIBC patients. Histological grading (G1, G2, or G3) based on WHO 1973 classification was not included as a categorical variable. Instead, invasive or non-invasive histology was used according to WHO Classification of Tumors of the Urinary Systems and male Genital Organs, 4th edition published in 2016 (Grignon et al. 2016). When the relationships between clinicopathological parameters and invasive or non-invasive histology were analyzed, one case of CIS was included in the non-invasive histology group. There were no indeterminate or missing data on the index test (LF/Cr) and the reference standard (Table 1). However, urinary cytology data were not obtained in 14 out of 92 patients. Therefore, both models of multivariate analysis, including and excluding urinary cytology, were made.

Quality assessment of reporting

We described the quality assessment of reporting in the present study according to the Newcastle-Ottawa Scale (NOS) (Wells et al. 2014), Quality Assessment of Studies of Diagnostic Accuracy (QUADAS / QUADAS-2) (Whiting et al. 2004, 2011), Standards for Reporting of Diagnostic Accuracy (STARD) (Cohen et al. 2016), and REporting recommendations for tumor MARKer prognostic studies (REMARK) criteria (Sauerbrei et al. 2018). The diagnostic accuracy in the present study was based on a case-control design, which harbors the risk of bias in selection of cases and controls, comparability, and ascertainment of exposure (Wells et al. 2014). Therefore, comparability of age and sex between cases and controls were statistically analyzed by non-parametric Wilcoxon/Kruskal Wallis test and the chi-square test, respectively.

Results

Characteristics of patients with primary untreated UCB and diagnosis of control subjects without UCB

The clinicopathological backgrounds of the 92 patients with primary untreated UCB are shown in Table 1. No adverse events were recorded after TURBT for the reference standard. Adverse events did not occur when performing the index test because measurements were extracorporeal. There were no uninterpretable, indeterminate, or missing results of pathological diagnosis. The proportion of NMIBC (pT1 or less) was 70 out of 92 (76.0%), confirming previous statistical analyses (approximately 70-75%) (Burger et al. 2013; Witjes et al. 2014; Babjuk et al. 2017), despite the convenience sampling with a potential risk of selection bias. Forty-two cases were non-invasive urothelial carcinoma (pTa), one case was carcinoma *in situ* (CIS)

Table 1. Clinicopathological background of patients with primary UCB.

Parameters	No. / median	% / range
Patients	92	100.0
Male	73	79.0
Female	19	21.0
Age	71	35-94
BMI	24.1	15.3-39.6
Brinkman Index	378	0-4,000
Not examined	6	
Charlson Comorbidity Index		
Low: 0	53	57.6
Medium: 1-2	37	40.2
High: 3	2	2.2
Histology		
Invasive	49	53.2
Non-invasive	42	45.7
CIS	1	1.1
Grade		
G1	15	16.3
G2	33	35.9
G3	44	47.8
pT		
pTa	42	45.7
pTis	1	1.1
pT1	27	29.3
≥ pT2	22	23.9
N		
N0	85	92.4
N1-2	7	7.6
M		
M0	89	96.7
M1	3	3.3
Maximum tumor size		
< 3cm	58	63.0
≥ 3cm	34	37.0
Tumor number		
1	48	52.2
2-7	26	28.2
≥ 8	18	19.6
Urinary cytology		
Negative	33	35.9
Suspicious	21	22.8
Positive	24	26.1
Not examined	14	15.2
Urinary WBC sediments		
WBC < 10/HPF	53	57.6
WBC ≥ 10/HPF	39	42.4
Urinary RBC sediments		
RBC < 5/HPF	36	39.1
RBC ≥ 5/HPF	56	60.9
Tumor-infiltrating neutrophils		
Negative	60	65.2
Positive	31	33.7
Not available	1	1.1

BMI, body mass index; pT, pathological T stage; N, regional lymph node metastasis; M, distant metastasis.

Table 2. Treatments for the UCB patients.

Treatments received	No.	%
TURBT	92	100.0
2nd TURBT	24	26.1
Upstage to MIBC	4	4.3
NMIBC (n = 70)		
Intravesical BCG instillation		
Induction therapy	28	40.0
Maintenance therapy	17	24.3
Intravesical pirarubicin instillation	2	2.9
EBRT	1	1.4
Chemotherapy for nodal metastasis	1	1.4
MIBC (n = 22)		
Radical cystectomy	9	40.9
Neoadjuvant chemotherapy	1	
Adjuvant chemotherarpy	2	
Bladder preservation intended	8	36.4
EBRT	2	
Chemotherapy	3	
Intravesical BCG	2	
TURBT only	4	
EBRT for palliative care	2	9.1
Chemotherapy for metastasis	2	9.1
No treatment because of advanced age	1	4.5

NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; BCG, Bacillus Calmette-Guérin; EBRT, external beam radiation therapy.

(pTis), and 49 were invasive urothelial carcinoma (pT1 and pT2 or more). Twenty-seven of 49 invasive urothelial carcinomas were NMIBC (pT1). Ninety of 92 patients had a Charlson Comorbidity Index 0 or 1-2 (Charlson et al. 1987). Twenty-eight (40%) and 17 (24.3%) of 70 patients with NMIBC underwent intravesical instillation of BCG as induction and maintenance therapy, respectively (Table 2). Nine and eight out of 22 MIBC patients underwent radical cystectomy and intended bladder preservation, respectively. The diagnosis of 166 control subjects without UCB were obtained from the medical records (Table 3). Data on age and sex of the three volunteers were missing.

Urine LF levels

At the stage of exploration for a new biomarker, we used urine samples from patients with primary tumors and those with recurrent ones. Coomassie Brilliant Blue (CBB) staining analysis identified a more intense band of approximately 80 kDa that was stronger in urine samples from patients with UCB as compared with that in samples from healthy adults (Fig. 2A). Protein analysis of the 80-kDa band revealed two major glycoproteins: transferrin and LF. Western blot analysis using urine from 20 patients with UCB showed that urinary levels of LF were higher in 11 out of 15 with invasive/high-grade (G3) UCB as compared to 5 with non-invasive ones, and 2 healthy adults (Fig. 2B). Non-cancer subjects with pyuria also had higher levels of urine LF as compared to those without pyuria (Fig. 2C). However, the immunoreactivity level of urine transferrin was not higher in the patients with UCB than that in healthy adults (data not shown).

Urine LF/Cr levels in patients with primary untreated UCB and control subjects without UCB

Urine LF/Cr levels were measured by ELISA. Median urine LF/Cr with inter-quartile range (IQR) was higher in the order of 62 control subjects with pyuria [1,543.1 (455.9-4,253.8) ng/mL/g Cr], 92 patients with primary untreated UCB [293.5 (76.9-2,622.1) ng/mL/g Cr] and 104 control subjects without pyuria [53.9 (25.8-139.8) ng/mL/g Cr] (Fig. 2C) ($p < 0.0001$), among the three groups. LF/Cr levels were significantly different between groups (Fig. 2D). There were no uninterpretable, indeterminate, or missing results of urine LF/Cr.

Immunostaining results of LF in urothelial cancer and normal urothelial tissues

Immunohistochemical staining of LF was based on a case-control design. To detect the source of urine LF in the patients with UCB, immunohistochemical staining of LF could be conducted in 84 out of 92 surgically obtained UCB tissue samples, and in 6 autopsy-derived non-tumor urothelial tissue samples. LF-positive staining was observed in 8 out of 11 cases of non-tumor urothelia (72.7%), which included 5 non-tumor portions of 84 UCB tissues and 6 from autopsy-derived (Fig. 3A, B). In contrast, only 2 out of 84 cases evaluated (2.3%) were positive for LF staining of cancer cells (data not shown). Tumor-infiltrating neutrophils (TINs) were observed in 31 out of 91 cases (34%) evaluated (Fig. 3C and Table 1). An example of LF immunostaining of TINs is shown in Fig. 3D.

Relationships between urine LF/Cr levels and clinicopathological variables in patients with UCB

We evaluated the relationships between urine LF/Cr levels and clinicopathological variables in 92 patients with UCB. In univariate analysis, invasive histology, pathological T stage, pyuria, TINs, hematuria, and maximum tumor diameter were significantly associated with the level of urine LF/Cr in either case where urine LF/Cr was handled as a continuous or categorical variable (Fig. 4A-F, Tables 4, 5). Through stepwise multiple regression and stepwise multivariate logistic regression analysis models, both pyuria and TINs were independent predictors of urine LF/Cr, and pyuria was a stronger predictor (Tables 4 and 5). In terms of malignant potential, urinary cytology, TINs, and hematuria were independently associated with invasive histology (Table 6). When urinary cytology was excluded because cytology data were shown to be lacking in 14 cases, TINs

Table 3. Backgrounds and diagnosis of 166 control subjects.

	No. or range		WBC ≥ 10/HPF (%)	Both WBC ≥ 10/HPF and symptomatic
Age (years)	median 58 (12-96)		62 (39.0)	
Missing (healthy volunteer)	3			
Sex				
Male	87		35 (40.2)	
Female	76		27 (35.5)	
Missing (healthy volunteer)	3			
Urinary stone	58		46 (79.3)	14 (30.4)
Adrenal tumor	22			
Primary aldosteronism		10	0	0
Pheochromocytoma		3	0	0
Myelolipoma		3	0	0
Cushing syndrome		3	0	0
Adenoma		2	0	0
Cyst		1	1	0
Pelvic organ prolapse	20		4	0
Healty adults	13			
Donar of renal transplantation		13	0	0
Healthy volunteer		7	0*	
Male infertility	11		0	0
Varicocele	6		0	0
Benign prostatic hyperplasia	5		3	0
Stress urinary incontinense	6		1	1
Ureteral stenosis	2		2	1
Acute cystitis	1		1	1
Others	15			
Renal bleeding		1	1	0
Renal pelvic-ureteral junction stenosis		1	0	0
Renal arteriovenous fistula		1	0	0
Chronic renal dysfunction		1	0	0
Retroperitoneal ganglioneuroma		1	0	0
Vesico-ureteral reflux		1	0	0
Uretero-rectal fistula		1	0	0
Atrophic bladder		1	1	1
Interstitial cystits		1	1	1
Polypoid cystits		1	0	0
Pyourachus		1	0	0
Uretheral stenosis		1	0	0
Scrot-adenomatoid tumor		1	0	0
Phimoses		1	1	0
Penile foreign object		1	0	0
	166		62	19 (30.6%)

*Healthy volunteers were regarded as having no pyuria.

remained independently associated with invasive histology, whereas pyuria was not an independent predictor in the multivariate analysis models (Table 6). Furthermore, we examined the relationship between preoperative variables and invasive histology. Instead of TINs as a postoperative variable, urine LF/Cr was incorporated as a preoperative variable and handled as a continuous or categorical variable dichotomized by the cut-off value (203.3 ng/mL/g Cr). Although urine LF/Cr was not an independent predictor in the multivariate models including cytology, urine LF/Cr (whether as a continuous or categorical variable) was independently associated with invasive histology in a multivari-

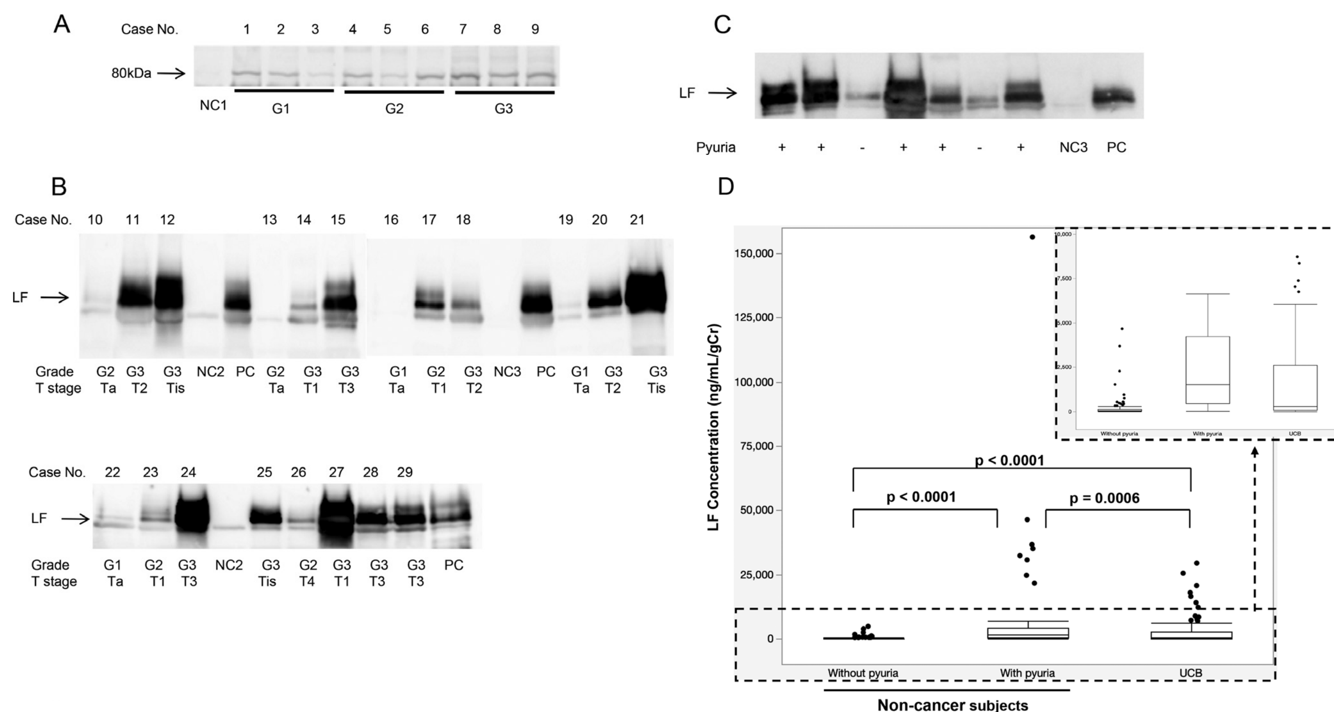

Fig. 2. Comparison of urine LF level between patients with UCB and non-cancer subjects.
(A) Examples of CBB staining of urine samples from UCB compared with that from a healthy adult designated as negative control (NC). Case No. 2, 3, 4, and 5: primary tumor without prior TURBT. Case No.1, 6, 7, 8, and 9: recurrent tumor. G, grade. (B) Western blot analysis of urine LF in 20 patients with UCB. Case No. 10, 14, 15 17, 19, 20, and 27: a primary untreated tumor. Case No. 11, 12, 16, 21, 23, 24, 28, and 29: state after TURBT for a primary tumor. Case No. 13, 18, 22, 25, and 26: a recurrent tumor. (C) Examples of western blot analysis of urine from control subjects with or without pyuria. NC1, NC2, and NC3: healthy adults; PC (positive control): a case of a recurrent tumor with stage T3. The same amount of urine (15 µL) from each case was used for CBB and western blotting. (D) Levels of urine LF/Cr measured by ELISA in 92 patients with primary untreated UCB and 166 non-cancer subjects, which included 62 subjects with pyuria and 104 without pyuria. Each median value of urine LF/Cr with IQR was shown as the box plot. In each box plot, the boundary of the box closest to zero indicates the 25th percentile (first quartile), a black line within the box is the median (second quartile), and the upper boundary of the box shows the 75th percentile (third quartile). T bar means the upper whisker (maximum) and inverted T bar the lower whisker (minimum). Outliers were plotted as circle dots. Scale of the dotted rectangle was expanded in the upper right corner.

ate model excluding cytology, whereas pyuria was not an independent predictor in any multivariate model (Tables 7, 8 and 9). Multivariate analysis showed that urine LF/Cr was a stronger predictor of invasive histology than pyuria.

Relationships between urine LF/Cr levels and recurrence, progression and prognosis of UCB

The correlation coefficient between urine LF/Cr and the EORTC scores in NMIBC were initially calculated. The computed values of urine LF/Cr were weakly related to both recurrence ($r = 0.29$, $p = 0.0153$) and progression scores ($r = 0.33$, $p = 0.0053$). In the Kaplan-Meier analysis, the median values of urine LF/Cr, which were 179.9 ng/mL/g Cr for NMIBC and 2,901.5 ng/mL/g Cr for MIBC, were set at the cut-off values. Urine LF/Cr levels were not significantly correlated with recurrence-free survival (log-rank test, $p = 0.6724$), whereas urine LF/Cr levels showed a nearly significant correlation with progression-free survival in 70 NMIBC patients (log-rank test, $p = 0.0688$) during a median follow-up period of 862 days (range: 62-3,459 days) (Fig. 5A, B). In contrast, urine LF/Cr levels were significantly correlated with disease-specific survival (DSS) in 22 MIBC patients (log-rank test, $p = 0.0169$) during a median follow-up period of 700 days (range: 22-3,467 days) (Fig. 5C). During the follow-up period, 0 or 3 events of progression occurred in NMIBC patients having less or more than the median LF/Cr values, while 2 or 6 events of disease-specific death occurred in MIBC patients having less or more than the median LF/Cr values. The 5-year DSS of all MIBC patients was 50.5%, that for 11 MIBC patients with less than the median LF/Cr was 74.1%, whereas the 4-year DSS of 11 MIBC patients with more than the median LF/Cr was 20.1% (Fig. 5C). In the Cox proportional hazards model, urine LF/Cr and nodal metastasis were significant predictors of DSS in 22 MIBC patients (Table 10).

Diagnostic performance of urine LF/Cr

The AUC of urine LF/Cr, utilized to differentiate 92 patients with UCB from 104 non-cancer subjects without pyuria, was 0.76 (95% CI: 0.68-0.82) (Fig. 6A). Additionally, the AUC of urine LF/Cr to distinguish 49

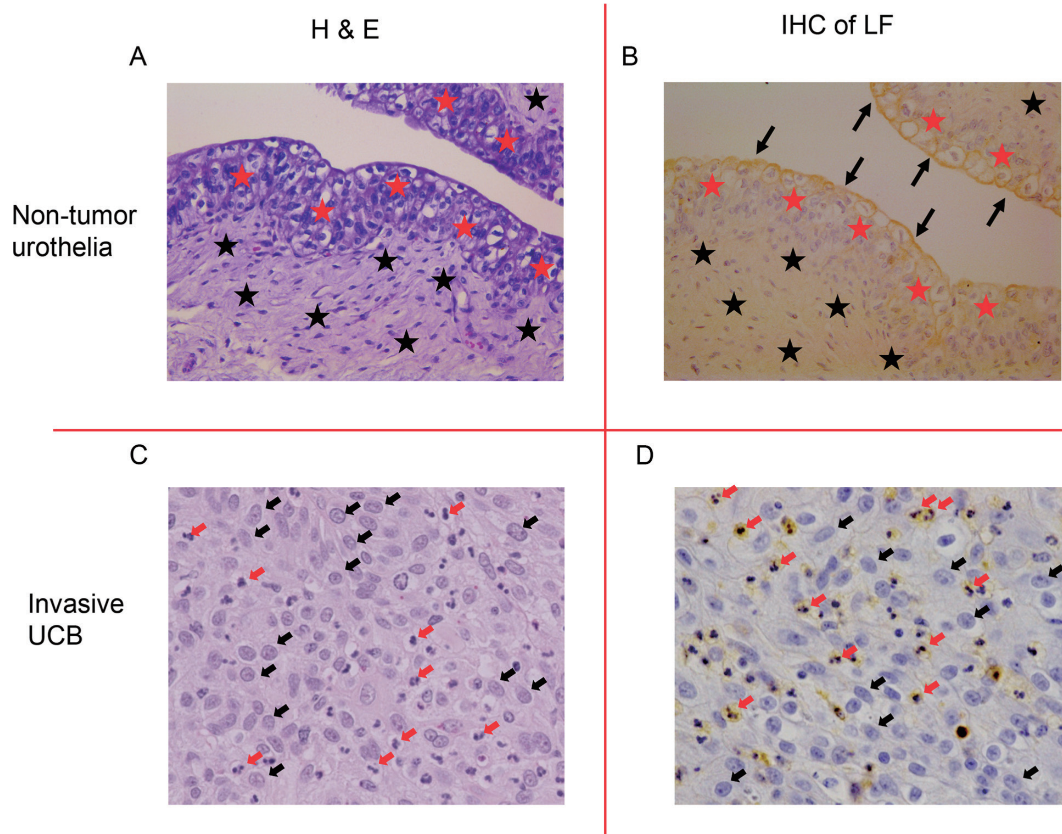

Fig. 3. Representative cases of LF immunostaining in UCB and non-tumor urothelial tissues.
Left column (A, C): Hematoxylin and eosin (H&E) staining. Right column (B, D): Immunohistochemical (IHC) staining of LF. (A) and (B): an example of non-tumor urothelia, which were positive for LF as indicated by black arrows. The urothelial and stromal areas of the bladder were designated by red and black stars, respectively. Each panel, × 200. (C) and (D): an example of tumor-infiltrating neutrophils in invasive UCB. Neutrophils were positive for LF, whereas cancer cells were negative for LF (D). Neutrophils are multinucleated. Some of the neutrophils were indicated by red arrows. Cancer cells have large nuclei. Some of them were indicated by black arrows. Each panel, × 400.

patients with invasive UCB from 104 subjects without pyuria was 0.82 (95% CI: 0.73-0.89) (Fig. 6B). The maximum Youden index was obtained when the cut-off level of urine LF/Cr was set at 629 ng/mL/g Cr, providing a specificity of 0.94 and a sensitivity of 0.63 for urine LF to detect invasive UCB. Subjects with a urinary tract infection (UTI) were excluded from the calculation of specificity and sensitivity. However, the proportion of symptomatic patients with pyuria was 30.6% at the time of urine sampling, as shown in Table 3. Urine LF/Cr was not useful for differentiating between UCB patients and benign controls, which included both subjects with and without pyuria (AUC = 0.40 [95% CI: 0.33-0.47]).

Expression of LF mRNA in urothelial cell lines and LF concentration in culture media of urothelial cell lines

To explain the low rate of LF immunostaining in UCB tissues, we measured the expression level of LF mRNA in malignant urothelial cell lines (5637 and T24) and two immortalized normal urothelial cell lines (SV-HUC-1 and MC-SV-HUC T-2) by real-time quantitative polymerase chain reaction (RT-qPCR). The relative expression levels of LF mRNA were virtually undetectable in 5637 and T24 cells, both of which have invasive capacity (Pang et al. 2019), whereas they were detected in immortalized normal urothelial cells (Fig. 7).

Next, we measured the LF concentrations in culture media of urothelial cell lines after 3 days of culture, showing that there were no meaningful levels of LF, irrespective of lactoferrin mRNA expression levels. In fact, the LF concentrations in 5637, T24 and SV-HUC-1 cells were 59.17, 60.52 and 59.05 pg/mL, respectively, and these values were similar to the background level in the control group (only culture media without cell lines) (58.93 pg/mL). These results indicate that LF is not secreted from these urothelial cell lines.

Effects of exogenous LF on cell proliferation

To determine additional reasons for the low rate of LF immunostaining in UCB tissues, we examined whether addition of exogenous LF in culture media suppresses the proliferation of urothelial cell lines. LF exhibited the similar growth inhibitory effects against all urothelial cell lines within the range of 3.1 μg/mL to 50 μg/mL (Fig. 8). The

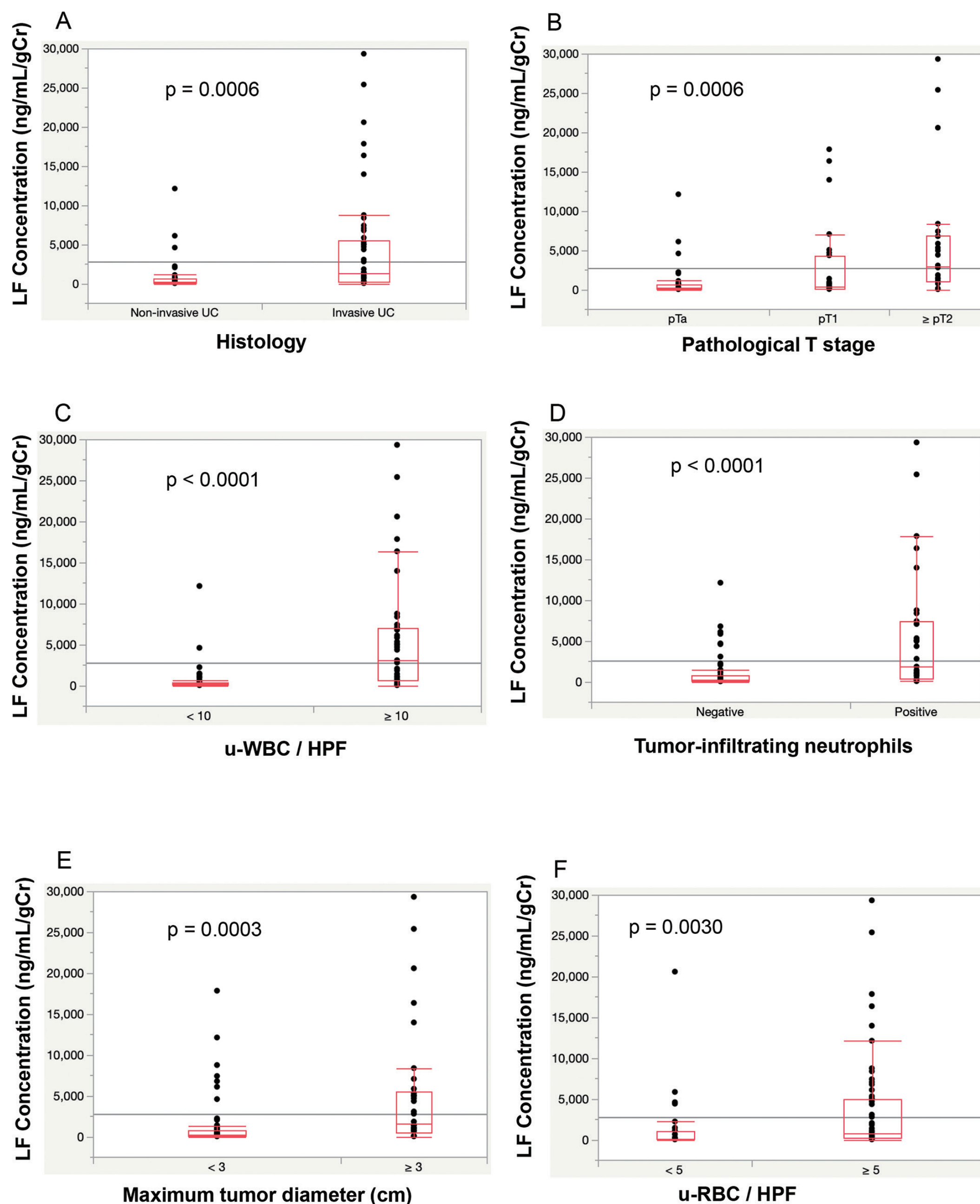

Fig. 4. Relationships between the value of urine LF/Cr and clinicopathological variables in patients with primary UCB.
The relationships between levels of urine LF/Cr and (A) histology (non-invasive [n = 42] or invasive [n = 49]), (B) pathological T stage (pTa [n = 42], pT1 [n = 27] or ≥ pT2 [n = 22]), (C) level of pyuria (u-WBC < 10/HPF [n = 53] or ≥ 10/HPF [n = 39]), (D) tumor-infiltrating neutrophils (negative [n = 60] or positive [n = 31]), (E) maximum tumor diameter (< 3cm [n = 58] or ≥ 3cm [n = 34]), and (F) level of u-RBC (u-RBC < 5/HPF [n = 36] or ≥ 5/HPF [n = 56]) were shown, respectively. Data of CIS was not shown in panels A and B because of only one case. Each box plot with IQR is shown in red. Explanation of box plot is the same as in Fig.2.
UC, urothelial carcinoma.

concentrations (3.1 μg/mL to 50 μg/mL) we used were almost consistent with 10 μg/mL of LF used in vitro studies (Xiao, et al. 2004; Arcella et al. 2015), while the maximized effect of LF on tumor size was obtained by daily oral intake of 60 mg LF/kg weight (60 μg LF/g weight) in vivo study (Wolf et al. 2007), and the other in vivo experiment adopted daily oral intake of 65 mg/kg LF (65 μg LF/g weight) (Arcella et al. 2015). Considering the potential absorption efficiency from gastrointestinal tract and stability of LF in blood, both of which remained to be clarified (Wolf et al. 2007; Arcella et al. 2015), the concentrations of LF we adopted in vitro study might not be much different from the oral dose used in vivo studies. In T24 cells, higher concentration of LF (25 μg/mL and 50 μg/mL) showed a lower growth inhibitory effect as compared to lower concentrations of LF, although the mechanism remains to be deter-

Table 4. Multiple regression analysis models of the explanatory variables predicting level of urine lactoferrin as a continuous variable.

Variables	Univariate	Multivariate model 1		Multivariate model 2 (Stepwise)	
	p value	F value	p value	F value	p value
Age	0.0911				
Sex	0.4519				
Body mass index	0.6062				
Invasive vs. Non-invasive histology	0.0009	0.03	0.8732		
T stage (≥ pT2 vs. pT1 vs. pTa)	0.0003	1.01	0.3700		
u-cytology (positive vs. suspicious vs. negative)	0.2193				
Tumor-infiltrating neutrophils (positive vs. negative)	< 0.0001	6.90	0.0103	8.30	0.0050
u-WBC (≥ 10/HPF vs. < 10)	< 0.0001	3.03	0.0855	11.53	0.0010
u-RBC (≥ 5/HPF vs. < 5)	0.0030	0.30	0.5848		
Tumor number (≥ 8 vs. 2-7 vs. 1)	0.1544				
Maximum tumor diameter (≥ 3cm vs. < 3cm)	0.0023	0.89	0.3434		
Brinkman index	0.3078				

Table 5. Multivariate logistic regression analysis models of the explanatory variables predicting level of urine lactoferrin as a categorical variable.

Variables	Univariate	Multivariate 1			Multivariate 2 (Stepwise)		
	p value	OR (95% CI)	$\chi 2$	p value	OR (95% CI)	$\chi 2$	p value
Age	0.6768						
Sex	0.7967						
Body mass index	0.9093						
Invasive vs. Non-invasive histology	0.0003	29,151,478 (0-ND)	0.00	0.9931			
T stage (≥ pT2 vs. pT1 vs. pTa)	0.0002	90,596,264 (0-ND) ≥ pT2 / pTa	2.81	0.2447			
u-cytology (positive vs. suspicious vs. negative)	0.0742						
Tumor-infiltrating neutrophils (positive vs. negative)	< 0.0001	5.15 (1.39-19.09)	6.00	0.0143	3.85 (1.27-12.35)	5.72	0.0168
u-WBC (≥ 10/HPF vs. < 10)	< 0.0001	5.36 (1.45-19.87)	6.31	0.0120	9.20 (3.31-28.19)	19.02	< 0.0001
u-RBC (≥ 5/HPF vs. < 5)	0.0025	1.18 (0.30-4.74)	0.07	0.7963			
Tumor number (≥ 8 vs. 2-7 vs. 1)	0.4510						
Maximum tumor diameter (≥ 3cm vs. < 3cm)	< 0.0001	3.08 (0.88-10.89)	3.08	0.0790			
Brinkman index	0.9781						

OR, odds ratio; CI, confidence interval; $\chi 2$, Chi-square; ND, not determined.

mined. The difference in proliferation between each cell line with and without exogenous LF was significant, following 96 h of treatment for the range of LF concentration tested ($p < 0.05$, Fig. 8). Except the inverse relationship between the higher concentration of LF and growth inhibitory effect on T24 cells, concentration dependency of LF on cell proliferation was not observed.

Quality assessment of reporting

We described the quality assessment of reporting in the present study according to the five assessment tools described in Materials and Methods. The diagnostic accuracy of the present study was based on a case-control design. As one of the risk of bias, comparability between cases and controls were analyzed. Age was significantly lower ($p < 0.0001$) and the female-to-male ratio was significantly higher in control subjects ($p < 0.0001$) than in UCB patients (statistically analyzed from data in Tables 1 and 3). Thus, the comparability of age and sex between cases and controls were not obtained.

Discussion

Chemoattractants secreted from cancer cells exert chemotaxis-induced effects on motile inflammatory cells, such as tumor-associated macrophages and neutrophils (Coussens and Werb 2002). These proinflammatory cells localize to the tumor site where mitogenic factors are secreted, creating a favorable environment for cancer cell

Table 6. Multivariate logistic regression analysis models of the preoperative and postoperative variables predicting invasive histology.

Variables	Univariate	Multivariate model 1			Multivariate model 2(cytology excluded)		
	p value	OR (95% CI)	$\chi 2$	p value	OR (95% CI)	$\chi 2$	p value
Age	0.1601						
Sex	0.6488						
Body mass index	0.7036						
Tumor-infiltrating neutrophils (positive vs. negative)	< 0.0001	5.40 (1.26-28.46)	5.23	0.0223	4.51 (1.38-16.61)	6.28	0.0122
u-WBC (≥ 10/HPF vs. < 10)	< 0.0001	1.21 (0.28-5.63)	0.06	0.7992	1.50 (0.43-5.18)	0.41	0.5207
u-RBC (≥ 5/HPF vs. < 5)	< 0.0001	5.01 (1.27-22.23)	5.33	0.0210	8.03 (2.49-29.34)	12.58	0.0004
Urinary cytology (positive vs. negative)	< 0.0001	13.18 (2.72-85.76)	11.31	0.0035			
Tumor number (≥ 8 vs. 2-7 vs. 1)	0.0643						
Maximum tumor diameter (≥ 3cm vs. < 3cm)	0.0005	3.78 (0.95-16.48)	3.55	0.0596	3.97 (1.17-14.85)	4.90	0.0269
Brinkman index	0.1648						

OR, odds ratio; CI, confidence interval; $\chi 2$, Chi-square.

Table 7. Multivariate logistic regression analysis models of the preoperative variables predicting invasive histology when cytology was included.

Variables	Univariate	Multivariate model 1			Multivariate model 2		
	p value	OR (95% CI)	$\chi 2$	p value	OR (95% CI)	$\chi 2$	p value
Age	0.1601						
Sex	0.6488						
Body mass index	0.7036						
Urine LF							
as a continuous variable	0.0101	22.31* (0.04-12,925.66)	1.23	0.2675			
as a categorical variable	< 0.0001				2.13 (0.58-7.88)	1.27	0.2579
u-WBC (≥ 10/HPF vs. < 10)	< 0.0001	1.46 (0.33-6.51)	0.24	0.6210	1.51 (0.34-6.62)	0.30	0.5850
u-RBC (≥ 5/HPF vs. < 5)	< 0.0001	4.65 (1.22-17.69)	5.29	0.0241	4.94 (1.29-18.90)	5.44	0.0197
Urinary cytology (positive vs. negative)	< 0.0001	10.70 (2.21-54.74)	10.29	0.0058	9.27 (1.90-45.31)	8.08	0.0175
Tumor number (≥ 8 vs. 2-7 vs. 1)	0.0643						
Maximum tumor diameter (≥ 3cm vs. < 3cm)	0.0005	2.90 (0.74-11.37)	2.36	0.1247	2.87 (0.73-11.36)	2.27	0.1316
Brinkman index	0.1648						

OR, odds ratio; CI, confidence interval; $\chi 2$, Chi-square.
*Range OR.

survival, proliferation, and metastasis (Coussens and Werb 2002). In recent years, inflammation has been reported to play a crucial role in the malignancy of cancer cells, and therefore it is no surprise that inflammation has also been demonstrated to play a role in urothelial carcinoma (Colotta et al. 2009). For example, a leucocyte chemoattractant known as chemokine (C-X-C motif) ligand 1 (CXCL1) is overexpressed in highly invasive bladder cancer cell lines (Nakashima et al. 2015). Urine CXCL1 was shown to be a predictor of tumor recurrence as well as a marker for tumor detection (Nakashima et al. 2015). The malignant potential of NMIBC was reflected on urine leucocytes chemotaxis in response to chemokines (Azuma et al. 2013). Higher-grade urothelial bladder cancer was shown to be associated with the presence of pyuria, which was an independent predictor of recurrence and progression in NMIBC (Azuma et al. 2013). In addition, persistent leukocytosis in blood has been known to be associated with a poor prognosis in patients with UCB (Izard et al. 2015). Neutrophil-to-lymphocyte ratio (NLR), which reflects inflammation, has been utilized as a prognostic marker for urothelial carcinoma (Marchioni et al. 2016).

As previous studies have shown the relationship between inflammation and the poor prognosis, it is possible that LF as a substitute of neutrophils reflects the malignant potential of UCB because neutrophils are known to release LF during inflammation (Masson et al. 1969; Baggiolini et al. 1970). In the present study, both pyuria and TINs were independent predictors for urine LF/Cr levels in UCB patients, where pyuria was a stronger predictor. In contrast, while TINs were one of the independent predictors for invasive histology, pyuria was not. Thus, it was thought that the attraction of neutrophils to the tumor site has the relevance to invasive potential, or neutrophils preferentially migrate

Table 8. Multivariate logistic regression analysis models of the preoperative variables predicting invasive histology when cytology was excluded.

Variables	Multivariate model 3 (cytology excluded)			Multivariate model 4 (cytology excluded)		
	OR (95% CI)	$\chi2$	p value	OR (95% CI)	$\chi2$	p value
Age						
Sex						
Body mass index						
Urine LF						
as a continuous variable	44.32* (0.19-10,346.02)	2.71	0.0999			
as a categorical variable				2.85 (0.96-8.68)	3.54	0.0597
u-WBC (≥ 10/HPF vs. < 10)	1.52 (0.43-5.36)	0.42	0.5174	1.64 (0.48-5.65)	0.63	0.4270
u-RBC (≥ 5/HPF vs. < 5)	7.66 (2.38-24.64)	13.08	0.0003	7.35 (2.38-25.32)	12.39	0.0004
Urinary cytology (positive vs. suspicious vs. negative)						
Tumor number (≥ 8 vs. 2-7 vs. 1)						
Maximum tumor diameter (≥ 3cm vs. < 3cm)	3.43 (1.00-11.78)	4.02	0.0450	3.36 (1.00-12.25)	3.85	0.0497
Brinkman index						

OR, odds ratio; CI, confidence interval; $\chi2$, Chi-square.
*Range OR.

Table 9. Multivariate logistic regression analysis models of the preoperative variables predicting invasive histology when cytology was excluded and stepwise analysis was applied.

Variables	Multivariate model 5 (u-WBC & cytology excluded)			Multivariate model 6 (urine LF & cytology excluded)			Multivariate model 7 (u-WBC & cytology excluded)		
	OR (95% CI)	$\chi2$	p value	OR (95% CI)	$\chi2$	p value	OR (95% CI)	$\chi2$	p value
Age									
Sex									
Body mass index									
Urine LF									
as a continuous variable	83.30* (1.15-76,769.08)	4.15	0.0416						
as a categorical variable							3.21 (1.12-9.21)	4.78	0.0298
u-WBC (≥ 10/HPF vs. < 10)				2.25 (0.70-7.45)	1.87	0.1720			
u-RBC (≥ 5/HPF vs. < 5)	8.76 (3.07-28.40)	17.59	< 0.0001	7.58 (2.51-25.65)	13.32	0.0003	8.63 (2.86-25.98)	14.71	< 0.0001
Urinary cytology (positive vs. suspicious vs. negative)									
Tumor number (≥ 8 vs. 2-7 vs. 1)									
Maximum tumor diameter (≥ 3cm vs. < 3cm)	3.94 (1.28-13.64)	5.76	0.0163	3.81 (1.15-13.75)	5.43	0.0282	3.98 (1.25-12.71)	5.43	0.0156
Brinkman index									

OR, odds ratio; CI, confidence interval; $\chi2$, Chi-square.
*Range OR.

into invasive UCB. Instead of TINs as a postoperative variable, urine LF as a preoperative variable was also an independent predictor of invasive histology depending on the multivariate model, that is, in a multivariate model excluding urinary cytology. However, pyuria was not a predictor of invasive histology in any multivariate analysis model. Thus, the present study showed that TINs or urine LF/Cr were more stronger predictors of invasive potential than pyuria. In terms of prognostication, urine LF/Cr predicted the DSS of MIBC patients and the likelihood of progression of NMIBC to muscle-invasive disease or one that is more advanced based on the Kaplan-Meier survival curve. Furthermore, urine LF/Cr and nodal metastasis were independent predictors of DSS in MIBC patients in the Cox

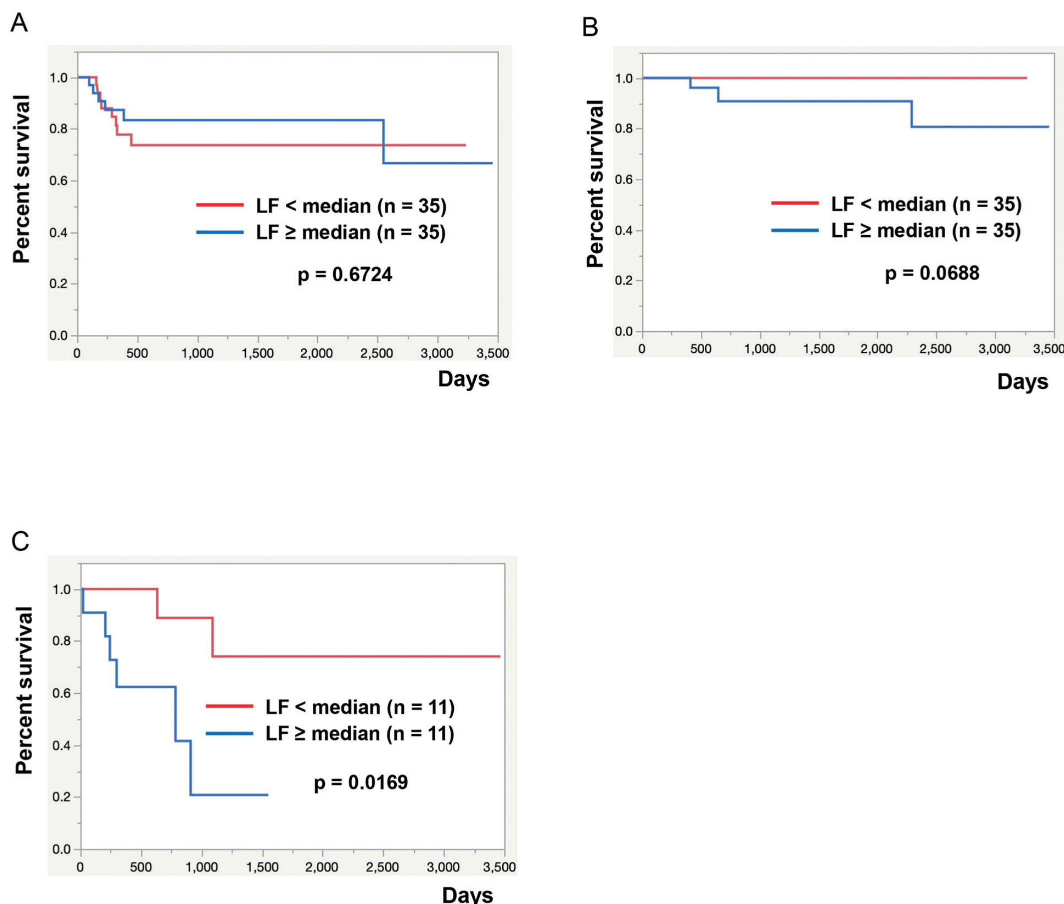

Fig. 5. The Kaplan-Meier survival curves according to the level of urine LF/Cr, the cut-off of which was set at a median value in both NMIBC and MIBC patients.
(A) Recurrence-free and (B) progression-free survival of 70 NMIBC patients (p = 0.6724, and p = 0.0688, respectively). The number of less or more than the cut-off value of urine LF/Cr were 35, respectively. (C) Disease-specific survival of 22 MIBC patients (p = 0.0169). The number of less or more than the cut-off value of urine LF/Cr were 11, respectively.

proportional hazards model, although the number of patients was small (n = 22). In a systematic review and meta-analysis, high levels of intratumoral neutrophils were reported to be associated with lower survival rates (Shen et al. 2014). A previous study on the relationship between intratumoral neutrophils and poor prognosis of urothelial carcinoma was conducted in upper urinary tract tumors (Al'-Shukri et al. 2004). Thus, urine LF/Cr linked to TINs (intratumoral neutrophils) was thought to be a potential biomarker reflecting the degree of malignancy in UCB, as shown by the poor prognosis of MIBC. Considering that level of urine LF/Cr can be measured quantitatively before treatment, it may be useful in determining the treatment strategy of UCB.

To evaluate the possibility of LF/Cr as a detection biomarker for UCB, we examined the AUC of urine LF/Cr to differentiate patients with UCB from non-cancer subjects without pyuria. The AUC of urine LF/Cr was determined to be 0.82 (95% CI: 0.73-0.89) for the differentiation of patients with invasive UCB from non-cancer subjects without pyuria. This AUC is equivalent to a very good diagnostic accuracy (Šimundić 2009). The maximum Youden index was obtained when the cut-off level of LF/Cr was set at 629 ng/mL/g Cr, providing a specificity of 0.94 and a sensitivity of 0.63 for the detection of invasive UCB. Thus, urine LF/Cr had a high specificity for detecting invasive UCB unless the subjects had a UTI. However, the diagnostic accuracy of LF/Cr in the present study was based on a case-control study, which has a potential risk of bias. Convenience sampling and lack of comparability between cases and controls may have affected the AUC. Therefore, the diagnostic accuracy shown in the present study was regarded just as a reference. Furthermore, UTI did not necessarily accompany symptoms at the time of urine sampling. Thus, there remained another problem with the differentiation between UCB patients and asymptomatic subjects based on urine LF/Cr alone. Early diagnosis of invasive UCB is clinically necessary, due to the potentially life-threatening nature of invasive cancer. Nevertheless, a patient with MIBC will have a better prognosis if UCB is found at a less invasive stage (Hautmann et al. 2012). Future studies should focus on evaluating the combination of additional methods with the application of urine LF/Cr based on a screening population, but not on a case-control

Table 10. The Cox proportional hazards model of the explanatory variables for predicting the disease-specific survival of MIBC patients.

Variables	Univariate			Multivariate		
	RR (95% CI)	$\chi 2$	p value	RR (95% CI)	$\chi 2$	p value
Age	1.68 (0.09-41.01)	0.12	0.7290			
Sex (M vs. F)	0.89 (0.18-4.48)	0.02	0.8935			
BMI	2.26 (0.17-23.51)	0.43	0.5120			
Brinkman index	1.94 (0.12-27.50)	0.24	0.6261			
Charlson comobidity index (medium vs. low)	0.47 (0.09-2.34)	0.95	0.3310			
u-WBC (≥ 10/HPF vs. < 10)	3.27 (0.40-26.80)	1.61	0.2046			
u-RBC (≥ 5/HPF vs. < 5)	0.47 (0.11-2.01)	0.95	0.3304			
Tumor-infiltrating neutrophils (positive vs. negative)	1.50 (0.33-6.76)	0.29	0.5926			
Urinary cytology (positive vs. negative)	0.13 (0.01-1.18)	3.24	0.1981			
LF value (≥ median in MIBC vs. < median)	6.14 (1.17-32.21)	5.50	0.0190	7.86 (1.29-48.00)	6.09	0.0136
pT (≥ pT3 vs. pT2)	1.17 (0.23-5.93)	0.03	0.8523			
N (1-2 vs. 0)	8.13 (1.53-60.44)	5.98	0.0145	11.66 (1.76-128.59)	6.57	0.0104
M (1 vs. 0)	7.62 (0.98-47.28)	3.78	0.0519			
Tumor number (≥ 8 vs. 1)	0.94 (0.17-5.20)	0.18	0.9152			
Maximum tumor diameter (≥ 3cm vs. < 3cm)	3.60 (0.44-29.45)	1.92	0.1656			

RR, risk ratio; CI, confidence interval; $\chi 2$, Chi-square.

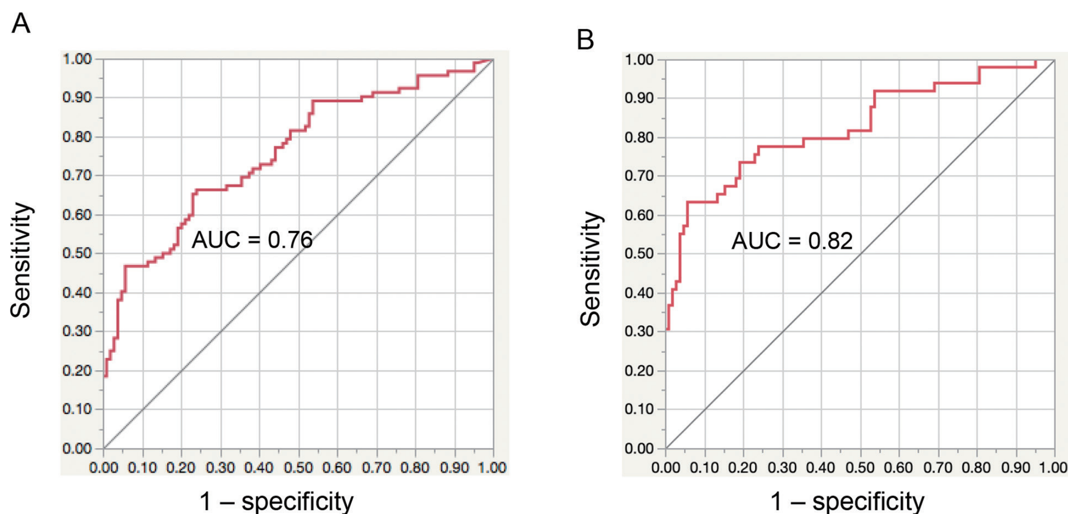

Fig. 6. Diagnostic performance of urine LF/Cr.
AUCs of urine LF/Cr to differentiate 92 patients with UCB from 104 subjects without pyuria (A) and to differentiate 49 patients with invasive UCB from 104 subjects without pyuria (B) were 0.76 and 0.82, respectively.

design, for the detection of invasive UCB before progression to MIBC or at an earlier stage of MIBC.

LF displays tumor suppressive activity in various cancer cell types (Xiao et al. 2004; Wolf et al. 2007; Arcella et al. 2015). The chemopreventive effect of LF has been shown in mutagen-induced rat bladder carcinogenesis (Masuda et al. 2000). In the present study, the levels of LF mRNA were hardly detected in 5637 and T24 cells, both of which have invasive capacity (Pang et al. 2019). We also observed that exogenous LF suppressed the proliferation of these two malignant urothelial cell lines. It was interesting that concentration dependency of LF was not observed within the range of 3.1 μg/mL to 50 μg/mL, whereas the higher concentrations of LF (25 μg/mL and 50 μg/mL) showed a lower growth inhibitory effect in T24 cells. It was assumed that LF receptors involved in suppression of cell proliferation may be saturated and activated with a low concentration of LF. In contrast, it was suggested that a lower growth inhibitory effect with the higher concentration of LF may have the relevance to the malignant potential of UCB linked to the level of urine LF. The remaining issue is whether a higher concentration of LF shows an inhibitory

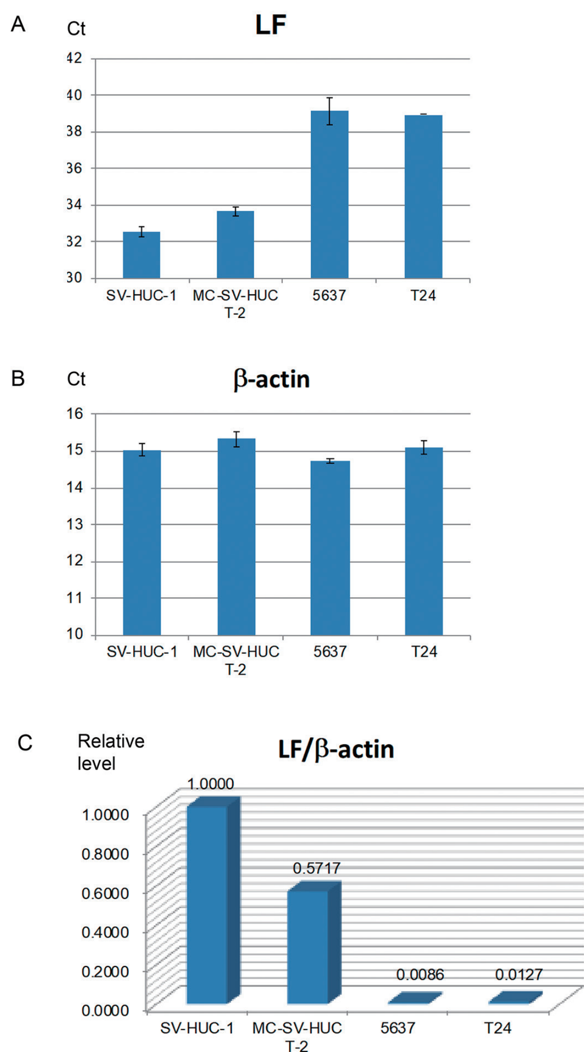

Fig. 7. Expression levels of LF mRNA in urothelial cell lines by RT-qPCR.
Ct values of (A) LF and (B) β-actin mRNA are shown on the vertical axis. Each error bar is standard deviation. Ct, threshold cycle. (C) Relative levels of LF/β-actin mRNA using SV-HUC-1 as a calibrator are shown. They were virtually undetectable in 5637 and T24 cells as compared to SV-HUC-1 and MC-SV-HUC T-2 cells. The relative level of LF/β-actin mRNA was designated as a numerical value on top of each bar.

or stimulatory effect on proliferation of cancer cells. Collectively, the results of the present study suggest that the production of endogenous LF, which may have the potential to suppress proliferation, is disadvantageous to the survival of cancer cells. Therefore, endogenous LF may be suppressed in cancer cells as shown by the low rate of LF immunostaining in UCB cases and down-regulation of mRNA in the two malignant urothelial cell lines, although the suppressive effect on proliferation by exogenous LF is not a direct proof of down-regulation of endogenous LF production. Nonetheless, an increase in the number of neutrophils known to secrete LF was observed in invasive UCB. Recent studies have shown that neutrophils, the most abundant leucocyte in humans, have a dual role in cancer development, displaying pro-tumor and anti-tumor actions (Tecchio and Cassatella 2016; Mishalian et al. 2017). Neutrophils have been shown to enhance cancer cell growth, angiogenesis, and recruit regulatory T cells (Tregs), whereas they exert direct tumor cytotoxicity and induce the proliferation of CD4+ and CD8+ T cells (Tecchio and Cassatella 2016; Mishalian et al. 2017). It was assumed that the pro-tumor function of neutrophils was the main action, leading to the suppression of anti-tumor LF activity in invasive UCB. Based on our results, the present study suggests the possibility that a molecule such as LF secreted from the cells other than cancer cells has the potential as a biomarker and the duality that even a molecule with anti-tumor activity could reflect the degree of malignancy.

This study had several limitations. First, the number of the newly diagnosed cases without prior TURBT was small because a considerable number of the patients had undergone TURBT before referral to our institute. In addition, we did not intend to analyze the sample size. Second, we failed to obtain urine samples from consecutive patients with UCB, and the cases were collected over a long period. Although our patients were representative of the cases, convenience sampling may have caused the potential risk of bias. Third, diagnostic accuracy was based on a case-control study, which harbors the potential risk of bias. In the present study, comparability such as age and sex between cases and controls was not obtained. Thus, the diagnostic accuracy should be regarded as a reference. Since urine LF/Cr was not useful for differentiating between UCB patients and benign subjects regardless of UTI, a hurdle remains for the generalizability of urine LF/Cr measurement to detect UCB. Fourth, urine LF/Cr as a possible prognostic predictor for MIBC patients was based on a small number of patients. Lastly, urinary cytology data, which were lacking in 14 out of 92 UCB cases, may have led to statistical uncertainty.

In conclusion, both pyuria and TINs were independent predictors of urine LF/Cr. In contrast, TINs or urine LF/Cr as a postoperative or preoperative variable were independent predictors of invasive histology, whereas pyuria was not. In terms of prognostication, urine LF/Cr and nodal metastasis were independent predictors of the disease-specific survival in patients with MIBC in the Cox proportional hazards model. Thus, urine LF/Cr may be a potential biomarker reflecting the degree of malignancy in UCB. Considering that level of urine LF/Cr can be measured quantitatively before treatment, it may be useful in determining the treatment strategy of UCB. However, since the number of MIBC patients in this study was small, a prospective study including a large number of MIBC patients is necessary to confirm our findings. As to the diagnostic accuracy of urine LF/Cr, further studies are necessary

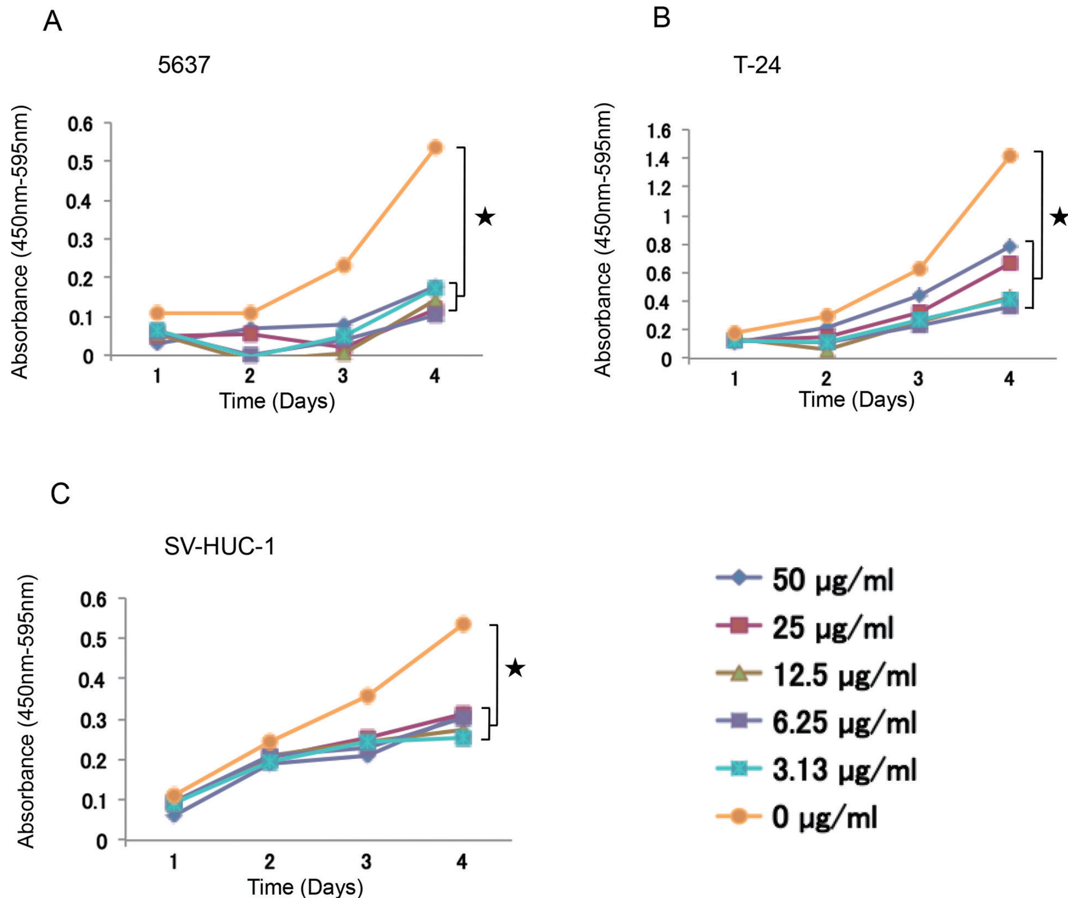

Fig. 8. Effects of exogenous LF on cell proliferation.
Cell proliferation curves of (A) 5637, (B) T24 and (C) SV-HUC-1 cells with treatment of LF were shown. Proliferation of the urothelial cell lines tested was suppressed by addition of exogenous LF within the concentration range used in this assay. It is of note that a higher concentration of LF rather exhibited a lower growth inhibitory effect on T 24 cells as compared to lower concentrations.
★$p < 0.05$.

because of the potential risk of bias inherent to a case-control design in the present study.

Acknowledgments

This study was supported by JSPS KAKENHI Grant Numbers 21592051 and 23592341 (Grant in Aids for Scientific Research C). We are grateful to the participating patients and their families. We thank Drs. Noriko Machida and Ayako Kaneyuki for technical assistance, and Dr. Mitsuyoshi Tamaki for survey of medical records. We would like to thank Editage (https://www.editage.com) for English language editing.

Conflict of Interest

The authors declare no conflict of interest.

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