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Urinary albumin and transferrin as early diagnostic markers of chronic kidney disease

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ABSTRACT. Feline renal diseases are increasingly noted in veterinary practice. It is important to diagnose and identify the pathological basis of renal dysfunction accurately at an early stage, but there are only a few reports on this area in clinical veterinary medicine. We investigated the efficacy of measurement of urinary albumin (u-Alb) and urinary transferrin (u-Tf) for early diagnosis using 5-μl urine samples collected noninvasively by catheterization from normal (IRIS stage I) cats and cats with stage I chronic kidney disease (CKD). The u-Alb levels in normal and stage I CKD cats were 6.0 ± 4.5 and 11.2 ± 8.4 mg/d*l*, respectively, and the u-Tf levels were 0.09 ± 0.42 and 0.52 ± 0.79 mg/d*l*, respectively. Based on ROC curve analysis, the sensitivity and specificity of u-Alb and u-Tf were higher than those of the currently used biomarker, the plasma creatinine level. The sensitivity of u-Alb was higher than that of u-Tf, whereas the specificity of u-Tf was higher than that of u-Alb. The validity of the threshold albumin level (20 mg/d*l*) was confirmed by measurements using SDS-PAGE. Since leakage of u-Tf in urine precedes leakage of u-Alb, inclusion of u-Tf in biochemistry tests may be appropriate for IRIS staging as a diagnostic marker of early diagnosis of renal disorder in cats.

KEY WORDS: albumin, chronic kidney disease, feline, transferrin, urine

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Feline renal diseases are increasingly noted in veterinary practice [16]. It is important to diagnose and identify the pathological basis of renal dysfunction accurately at an early stage. Early diagnosis and initiation of treatment of renal dysfunction delay progression of the disease, which leads to prolongation of the survival time and improvement of QOL [7]. But, there are only a few reports on this area in clinical veterinary medicine [18]. In veterinary practice, diseases are classified based on the plasma creatinine level (p-Cre) following the disease classification criteria established by the International Renal Interest Society (IRIS) [17]. Urinary protein and urinary albumin (u-Alb) tests are performed, but urinary protein positivity may be found regardless of the presence or absence of renal disease. Moreover, the u-Alb test uses the method for human u-Alb measurement, and thus, the sensitivity is low, although there is homology between the human and feline proteins. For these reasons, a method with high sensitivity and specificity for early-stage evaluation of feline renal dysfunction is required. Transferrin is an iron-transporting protein with a molecular weight similar to that of albumin, but the isoelectric point is different [6]. Therefore, the kinetics of transferrin differ from those of albumin, and thus, abnormal renal function may be evaluated at an early stage by the urinary transferrin (u-Tf).

We have previously shown that u-Alb on micro two-dimensional polyacrylamide gel electrophoresis (M2D-PAGE) is useful as a diagnostic marker of early stage feline chronic kidney disease (CKD) and that the u-Alb level in M2D-PAGE analysis does not exceed 20 mg/d*l* in normal cats.

In this study, we investigated the efficacy of measurement of u-Alb and u-Tf for early diagnosis using 5-μl urine samples collected noninvasively by catheterization from normal (IRIS stage I) cats and cats with stage I CKD [9].

MATERIALS AND METHODS

Animals: Urine samples collected from 31 cats with nephropathy (stage I CKD) brought to Maeda Veterinary Hospital between July 28, 2007 and July 10, 2008 (American Shorthair: 3, Chinchilla: 1, Mix: 27; 15 males and 16 females aged 3–14 years) were used in the study. Urine samples col-

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lected from 92 cats in IRIS stage I aged ≤2 years (Scottish Fold: 1, American Shorthair: 1, Mix: 90; 42 males and 50 females aged 4 months to 2 years) were used as normal controls. Regarding the diagnostic criteria, cats <2 years old clinically diagnosed as normal based on serum biochemistry test results were defined as normal cats, and cats aged ≥6 years showing clinical signs of CKD in blood chemistry were defined as those with stage I CKD. Criteria for normal urinalysis were USG >1.030, UPC <0.4 and a negative bacteriologic urine culture [1]. Diagnosis of CKD was made prior to inclusion in the study, based on clinical and laboratory (i.e., renal azotemia and USG <1.030) findings. Cats were classified into 4 stages according to the IRIS guidelines after stabilization [1], with p-Cre of <1.6, 1.6–2.8 mg/d*l* and 2.9–5.0 mg/d*l* in stages I, II and III, respectively. Urine was collected by catheterization. Samples were centrifuged at 1,190 *g* for 10 min (Kubota, Tokyo, Japan) and then stored at −80°C for further use. All owners gave signed informed consent to participation of their animal in the study. Urine samples collected from 31 cats with nephropathy (stage I CKD) brought to Maeda Veterinary Hospital.

SDS-PAGE analysis: Urine samples were dissolved in PAGE sample buffer (pH 6.8; 50 mM Tris-HCl, 50 mM dithiothreitol, 0.5% SDS and 10% glycerol), and the resulting solution was analyzed using SDS-PAGE (e-PAGEL, 10% acrylamide and 14 wells; Atto Corp., Tokyo, Japan). The gel was stained with Coomassie brilliant blue (CBB) (PhastGel Blue R; GE Healthcare, Little Chalfont, U.K.). The intensities of the albumin and transferrin bands were quantified using CS Analyzer ver. 3.0 (Atto Corp.) and used as indices of the level of protein expression. Bovine serum albumin and human transferrin (both from Sigma-Aldrich, St. Louis, MO, U.S.A.) were used as reference proteins.

In-gel digestion of proteins: The gel was cut into small pieces, destained in 50% Acetonitril/50 mM NH_4HCO_3 and washed with deionized water. The gel pieces were dehydrated in 100% Acetonitril for 15 min and dried in a SpeedVac Evaporator (Wakenyaku, Kyoto, Japan) for 45 min. The gel pieces were rehydrated in 10–30 μl of 25 mM Tris-HCl/20% Acetonitril containing 25 ng/*l* trypsin (Trypsin sequence grade, Roche, Basel, Swiss) for 45 min. After removal of unabsorbed solution, the gel pieces were incubated in 10–20 μl of 50 mM Tris-HCl/20% Acetonitril for 20 hr at 37°C. The solution containing digested fragments of proteins was transferred to a new tube, and peptide fragments remaining in the gel were extracted in 5% formic acid/50% ACN for 20 min at room temperature [15].

Protein identification: In-gel digested peptides were injected into a 0.3 × 5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan) and a 0.1 × 50 mm analytical Monolith column (AMR, Tokyo, Japan) attached to a HPLC system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). The flow rate of the mobile phase was 1 μl/min. The solvent composition of the mobile phase was programmed to change in 35-min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–50% B for 20 min, 50 to 95% B for 1 min, 95% B for 3 min, 95 to 5% B for 1 min and 5% B for 10 min. Purified peptides were introduced from HPLC to an LTQ-XL ion trap mass spectrometer (Thermo Scientific, Waltham, CA, U.S.A.) via an attached Pico Tip (New Objective, Woburn, MA, U.S.A.). MS and MS/MS peptide spectra were measured in a data-dependent manner based on the manufacturer's operating specifications. The Mascot search engine (Matrix Science, London, U.K.) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the International Protein Index database (European Bioinformatics Institute, Cambridge, U.K.) using the MASCOT engine. The minimum criterion for the probability-based MASCOT/MOWSE score was set at 5% as the significant threshold level.

Other procedures: Creatinine was measured by an enzymatic method with creatinine deiminase using a Fuji DRI-CHEM Slide CRE-PIII kit (Fuji Film Medical, Tokyo, Japan). BUN was measured using a N-Assay BUN-L Nittobo D-Type kit (Nittobo Medical Co., Ltd., Tokyo, Japan). The specific gravity was measured using a pocket refractometer for urine specific gravity PAL-09S (Atago, Tokyo, Japan). Numerical data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics 19 software (SPSS Inc., Chicago, IL, U.S.A.) with $P<0.05$ considered to be significant.

Histopathological examination: The u-Tf level was >1.5 mg/d*l* in one each of the normal and stage I CKD cats, and these animals were examined histopathologically. Renal biopsy was performed with a Tru-Cut needle (16G, 9 cm) [19]. Fixed tissues embedded in paraffin were sectioned and stained with hematoxylin and eosin (H&E) after appropriate standard treatments. [14]. The biopsy specimens were reviewed by experienced pathologists.

RESULTS

SDS-PAGE analysis: The molecular weight of transferrin was estimated to be 77 kDa based on the band position of the reference transferrin. A band was present at a position corresponding to 77 kDa in the electrophoretic profile of urinary protein and was identified as transferrin (score: 602). The molecular weight of albumin was estimated to be 67 kDa based on the band position of the reference albumin. A band was also present at a position corresponding to 67 kDa in the electrophoretic profile of urinary protein and was identified as albumin (score: 814). Favorable protein band separation was obtained with 4-hr electrophoresis on a 10% polyacrylamide gel at a constant current of 5 mA, and the transferrin and albumin bands were clearly visible. Thus, analysis was performed under these conditions (Fig. 1). In normal cats, the frequencies of u-Alb and u-Tf detection on SDS-PAGE were 94.5% and 5.4%, respectively.

The cutoff values of u-Alb and u-Tf were set at the means+2SD of those in the normal cats. The cutoff values of u-Alb and u-Tf were 15.0 and 0.93 mg/d*l*, respectively. The CKD-positive rates in the cats with stage I CKD were 29.0 and 33.3% for u-Alb and u-Tf, respectively.

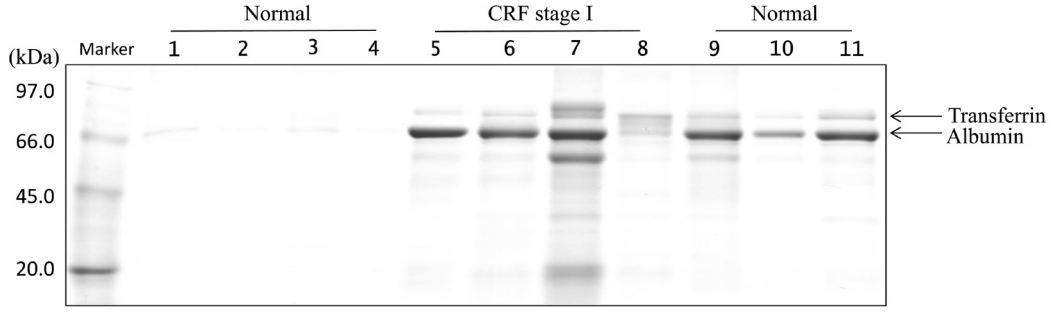

Fig. 1. SDS-PAGE profiles of urine in normal and stage I CKD cats. Lanes 1–4 and 9–11 are serum samples from normal cats, and lanes 5–8 are serum samples from cats with stage I CKD.

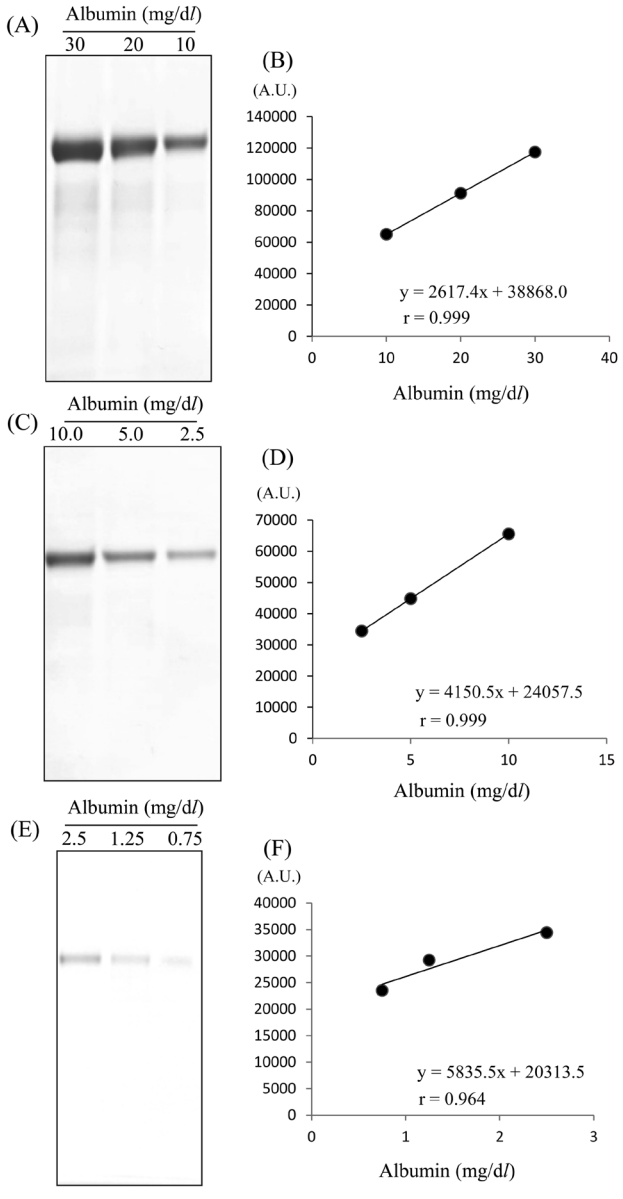

Fig. 2. Reference SDS-PAGE profiles (A, C, E) and calibration curves (B, D, F) for high (A, B), intermediate (C, D) and low (E, F) concentration ranges of albumin.

Calibration curves were prepared using the reference albumin and transferrin, respectively. The equations of the calibration curves for reference albumin were y=2,617.4x+38,868.0 (r=0.999) at high concentration (10–30 mg/d*l*); y=4,150.5x+24,057.5 (r=0.999) at intermediate concentration (2.5–10 mg/d*l*); and y=5,835.5x+20,313.5 (r=0.963) at low concentration (0.75–2.5 mg/d*l*) (Fig. 2A–2C). The equation of the calibration curve for reference transferrin was y=6,964.7x+2,894.8 (r=0.999) in a range of 0.75–3.0 mg/d*l* (Fig. 3).

Quantitative analysis: The u-Alb levels in normal (n=92) and stage I CKD (n=31) cats were 6.0 ± 4.5 and 11.2 ± 8.4 mg/d*l*, respectively, and the u-Tf levels were 0.09 ± 0.42 and 0.52 ± 0.79 mg/d*l*, respectively, with significant differences for both proteins (both $P<0.001$) (Fig. 4). The u-Alb level did not exceed 20.0 mg/d*l* in any of the 92 normal cats (Fig. 4). The correlation coefficients between u-Alb and creatinine levels were r=–0.087 in normal cats and r=–0.380 in CKD cats, indicating no correlation (Fig. 5). The correlation coefficients between u-Tf and creatinine levels were r=–0.160 in normal cats and r=–0.022 in CKD cats, again with no correlation (Fig. 5). In ROC curves, the AUCs of albumin, transferrin and creatinine were 0.719, 0.651 and 0.577, respectively, with significant differences between albumin and creatinine ($P<0.01$) and between transferrin and creatinine ($P<0.05$) (Fig. 6A). The AUC of albumin+transferrin was 0.798, with significant differences between albumin and albumin+transferrin ($P<0.05$) (Fig. 6B). ROC analysis was performed to evaluate cats with stage I CKD, with normal cats as controls.

Histopathological examination: The u-Tf level was >1.5 mg/d*l* in one each of the normal and stage I CKD cats, and these animals were examined histopathologically. The normal cat showed mild mesangial cell outgrowth and an increase in matrix in glomeruli; and mild chronic interstitial nephritis, renal tubular atrophy and interstitium fibrosis (Fig. 7A). In the stage I CKD cat, there was thickening of the glomerular capsule and widening of the mesangial region, and atrophy of the capillary vascular lumen, showing moderate chronic interstitial nephritis, renal tubular atrophy and interstitial fibrosis (Fig. 7B).

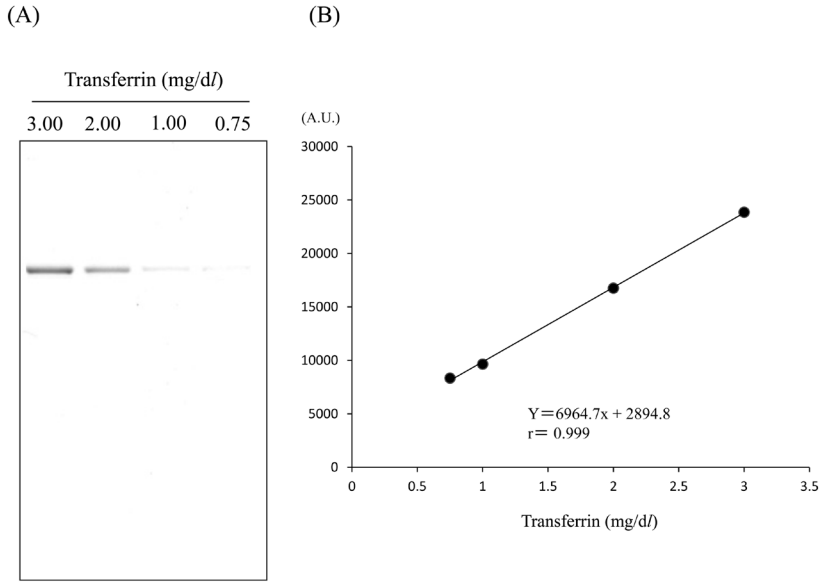

Fig. 3. Reference SDS-PAGE profile (A) and calibration curve (B) for transferrin.

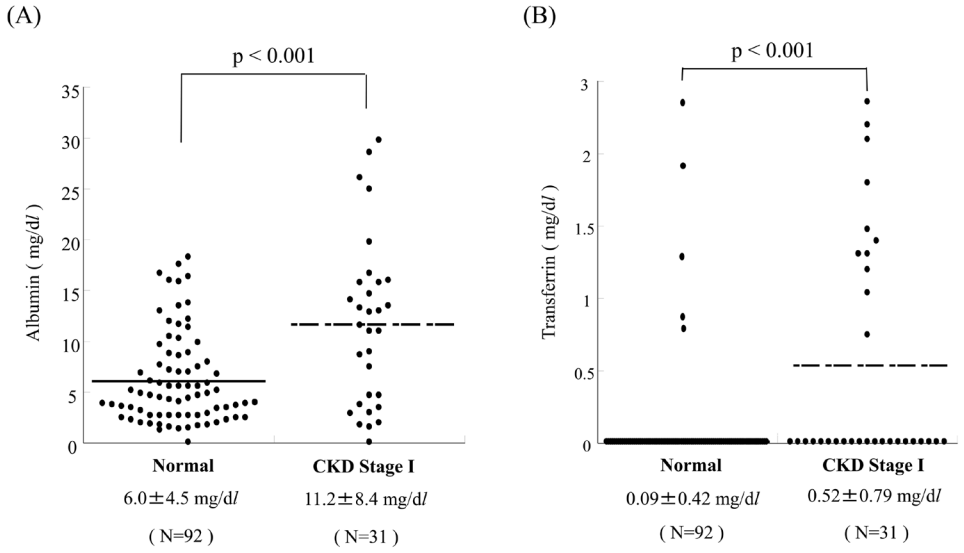

Fig. 4. Urinary albumin (A) and urinary transferrin (B) in normal and stage I CKD cats.

DISCUSSION

Feline renal diseases are increasingly seen in veterinary practice, and a test is needed for early and accurate diagnosis and identification of the pathology of renal dysfunction. In veterinary practice for small animals, the total urinary protein and u-Alb tests are performed. However, the total urinary protein test may not reflect the stage of pathological progression of feline renal disease and high urinary protein levels are often found in normal cats [10–12], while urinary protein-negative cats may have renal disease. Thus, an early diagnosis based on the results of these tests may not be useful for establishing a therapeutic strategy. Also, the human Alb method is used for urinary Alb in veterinary practice for small animals, and thus, the sensitivity is low, despite the homology between feline and human Alb. Therefore, a method with high sensitivity is needed for early stage evaluation of feline renal dysfunction, but only a few related studies have been performed in clinical veterinary medicine. Diseases in veterinary practice are classified based on the p-Cre level following the IRIS disease classification criteria [4], but creatinine levels are influenced by factors, such as muscle mass, gender, age and diet [3].

We measured the u-Alb level in 0.25 μl of feline urine us-

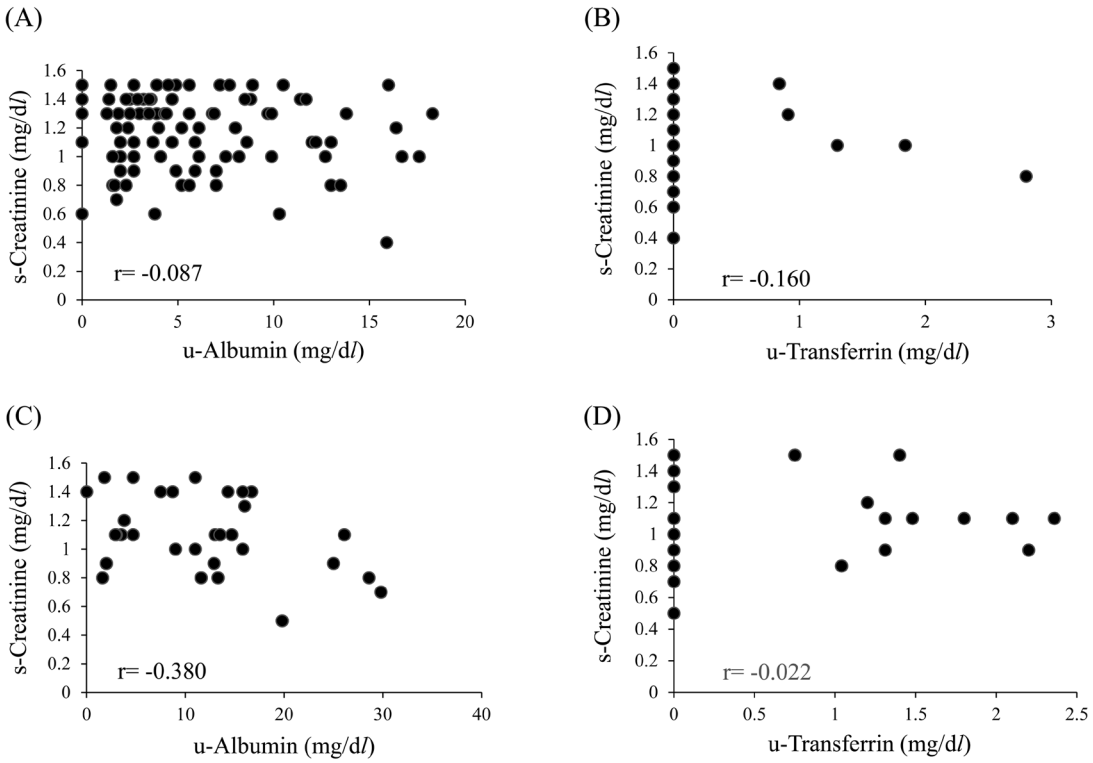

Fig. 5. Correlations of s-creatinine with urinary albumin (A) and urinary transferrin (B) in normal cats (n=92); and with urinary albumin (C) and urinary transferrin (D) in stage I CKD cats (n=31).

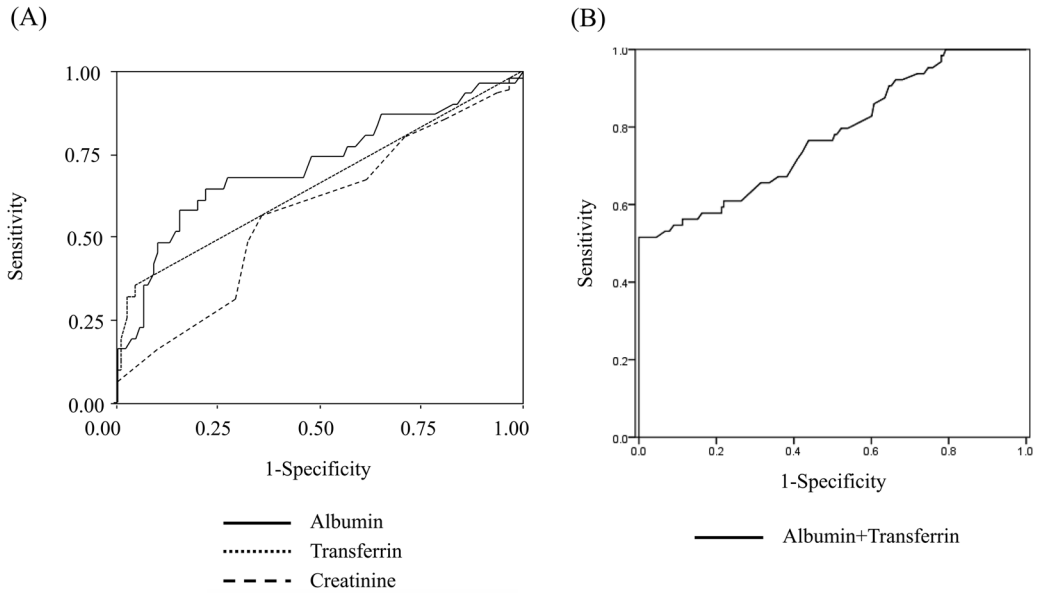

Fig. 6. ROC curves of urinary albumin, urinary transferrin and serum creatinine (A) and ROC curves of urinary albumin+ urinary transferrin (B).

ing M2D-PAGE and confirmed the usefulness of u-Alb as a diagnostic marker of early stage CKD. However, glomerular lesions were observed in some cases with no increase in u-Alb excretion. Thus, another marker is needed for accurate diagnosis of these lesions. There was no change in the p-Cre or u-Alb level in some cases of mild chronic interstitial

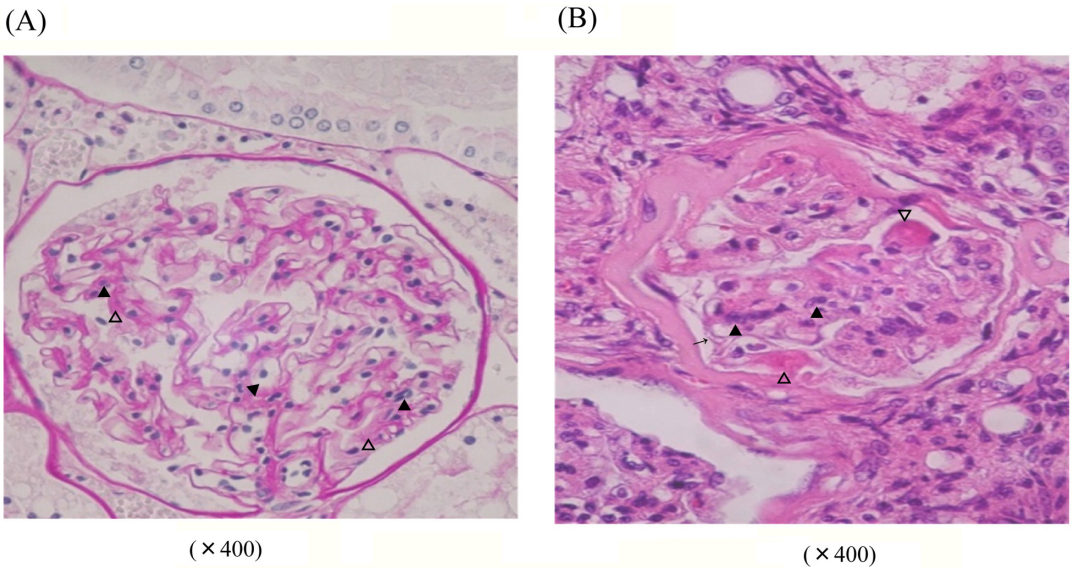

Fig. 7. PAS staining of renal tissue in urinary transferrin-positive normal and stage I CKD cats. (A) Renal tissue of a normal 6-year-old female cat with contraception (BUN: 22.0 mg/d*l*, specific gravity of urine: >1.030, serum creatinine: 1.3 mg/d*l*, urinary albumin: 14.1 mg/d*l* and urinary transferrin: 1.8 mg/d*l*). Mild mesangium cell outgrowth (▲) and increased matrix (Δ) were noted in the glomeruli. Mild to moderate chronic nephritis was also observed. (B) Renal tissue of a stage I CKD 9-year-old female cat with contraception (BUN: 38.2 mg/d*l*, urine specific gravity of urine: 1.015, serum creatinine: 1.5 mg/d*l*, urinary albumin: 11.2 mg/d*l* and urinary transferrin: 1.5 mg/d*l*). Mesangium cell outgrowth (▲), expansion of the matrix region (Δ), hypertrophy of the basement membrane (→) and collapse were noted. Mild to moderate chronic nephritis, atrophy of the renal tubule and interstitial fibrosis were also observed.

nephritis with outgrowth of glomerular mesangium cells and basement membrane hypertrophy [5]. It is difficult to confirm the transferrin spot on M2D-PAGE, because only a small volume of sample can be applied. In contrast, the transferrin band can be identified and measured on SDS-PAGE, because a large volume of sample (and many samples) can be applied. Thus, SDS-PAGE is applicable for clinical testing in veterinary practice. In this study, we measured u-Alb and u-Tf, which are excreted in urine in humans with renal diseases, in IRIS classification-based normal cats and stage I CKD cats using 5 μl of urine that was noninvasively collected by catheterization, and investigated the utility of SDS-PAGE analysis of these samples for diagnosis of early CKD. Establishment of suitable electrophoretic conditions for quantitative determination of u-Alb and u-Tf bands permitted measurements of u-Alb and u-Tf up to 0.75 mg/d*l*. SDS-PAGE measurements showed that the u-Alb and u-Tf in 5 μl of urine in stage I CKD cats were about 2 times and 5 times higher, respectively, than those in normal cats. The u-Alb level did not exceed 20 mg/d*l* in any of the 92 normal cats. Correlations between u-Alb and p-Cre levels in normal cats ($r=-0.087$) or stage I CKD cats ($r=-0.38$) were not significant.

In normal cats, the frequencies of u-Alb and u-Tf detection on SDS-PAGE were 94.5% and 5.4%, respectively, indicating that detection of u-Tf is rare in normal cats and thus may be indicative of CKD. However, there was no significant correlation between u-Tf and p-Cre levels in normal cats ($r=-0.16$) or stage I CKD cats ($r=-0.022$). u-Tf leakage was detected in 5.4% of normal cats, despite the u-Alb and p-Cre levels being within the normal ranges. This result supports previous findings of a significant correlation between the level of u-Tf leakage and stage of glomerular lesions [4, 8, 13], and the u-Tf level may be abnormal despite a normal u-Alb level in some humans with diabetic neuropathy [2]. Normal and stage I CKD cats with a u-Tf level ≥1.5 mg/d*l* were definitely diagnosed with chronic interstitial nephritis by histopathological examination, consistent with the findings on SDS-PAGE.

Based on ROC curve analysis, the sensitivity and specificity of u-Alb and u-Tf were higher than those of the currently used biomarker, p-Cre. The sensitivity of u-Alb was higher than that of u-Tf, whereas the specificity of u-Tf was higher than that of u-Alb. The validity of the threshold Alb level (20 mg/d*l*) was confirmed by measurements using SDS-PAGE. Since leakage of u-Tf in urine precedes leakage of u-Alb, inclusion of u-Tf in serum biochemistry tests for IRIS staging as a diagnostic marker of early stage renal failure may be useful for early diagnosis of renal disorder in cats.

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