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Urinary carboxylesterase 5A fragment as an early diagnostic marker of cat chronic kidney disease

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SUMMARY

Chronic kidney disease (CKD) is a common disorder and cause of death in cats. In the classification proposed by the International Renal Interest Society (IRIS), stage I and II CKD are difficult to diagnose accurately using markers, in comparison with normal controls. We recently described a simple and highly reproducible two-step method for identifying potential disease-marker candidates among low-abundance urine proteins. Urine samples were taken from 56 normal control cats as the control group and from 56 cats with CKD (stage I). A carboxylesterase 5A fragment and filaggrin-2 fragment were identified as two proteins with higher levels in normal control cats. The performance of the ELISA of urine carboxylesterase 5A fragment was satisfactory in terms of recovery (97.2–102.4%) and within-run (1.3–3.6%) and between-day (1.5–4.1%) reproducibility. Urine carboxylesterase 5A fragment levels were significantly greater in normal cats (3.4 ± 0.6 mg/dL) than in CKD (stage I) (1.9 ± 0.5 mg/dL) ($p < 0.001$). A carboxylesterase 5A fragment may be useful as a complementary marker to P-Cre and BUN for detection of CKD (stage I).

Key words: cat, chronic kidney disease, urine, carboxylesterase 5A fragment, ELISA

INTRODUCTION

Chronic kidney disease (CKD) is a common disorder and cause of death in cats. The prevalence of renal diseases in cats ranges from 1.6 to 20%^{1,2)} and CKD occurs in cats of all ages, but mortality due to CKD commonly increases with age. In human and veterinary medicine, CKD is diagnosed by detection of chronic decreases in glomerular filtration rate (GFR) or the presence of chronic renal damage³⁾. GFR can be evaluated indirectly from concentrations of plasma and urine markers, which are dependent on the amount eliminated by the kidneys. CKD in cats is usually diagnosed based on data obtained from past history, physical examination, measurements of plasma/serum urea and creatinine, measurement of urinary specific gravity (USG), and quantitative determination of proteinuria. In particular, renal failure is defined based on plasma creatinine (P-Cre) > 1.6 mg/dL, urine specific gravity (USG) < 1.035 , and a urinary protein to creatinine ratio (UPC) > 0.2 ⁴⁾. Blood urea

nitrogen (BUN) and P-Cre concentrations are widely used as endogenous markers to evaluate renal function in dogs and cats because the measurements are simple, rapid and inexpensive⁵⁾.

Early detection of stage I and II CKD is important because the incidence of CKD in cats tends to increase with age and complete cure is difficult after development of CKD. However, in the classification proposed by IRIS, stage I and II CKD are difficult to diagnose accurately compared to normal controls using these markers. The IRIS classification is currently used clinically for estimation of renal function. However, laboratory data for creatinine can be affected by non-renal factors such as sex, age, and muscle mass; and abnormal values may not be seen in mildly decreased renal function. This is known as the so-called “blind area”⁶⁾. Changes in BUN are also affected by numerous factors such as protein intake and are not specific to renal function⁷⁾.

Serum contains thousands of proteins and peptides at large dynamic concentrations, and this provides a technical

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challenge in plasma proteome analysis. Indeed, 22 abundant proteins, including albumin, immunoglobulins, and transferrin, constitute up to 99% of the protein content of plasma^{8,9}. Depletion of these abundant proteins and further fractionation of samples is necessary in proteomic studies searching for low-abundance serum proteins or peptides. We recently described a simple and highly reproducible two-step method for identifying potential disease-marker candidates among low-abundance urine proteins, using extraction of glycoprotein followed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this study, we used this method of two-step proteome analysis to gain more insight into changes of urine proteins in cats with CKD. The results showed that a carboxylesterase 5A (CES5A) fragment was decreased in urine in cats with CKD (stage I).

MATERIALS AND METHODS

Animals

Urine samples collected from 56 cats with CKD (stage) (mix: 56; all males of age 2–14 years) and 56 normal controls (mix: 56; all males of age 2–14 years) brought to Maeda Veterinary Hospital between December 14, 2013 and June 30, 2016 were used in the study. The test-set consisted of urine samples collected from 6 cats with CKD (stage) and 6 normal controls. For the validation study, urine samples were obtained from 50 cats with CKD (stage I) and 50 normal controls.

Criteria for normal urinalysis were USG > 1.035, UPC ≤ 0.2, urine albumin/urine creatinine < 10 mg/g and a negative bacteriologic urine culture³.

Diagnosis of CKD was made prior to inclusion in the study, based on clinical and laboratory findings of renal azotemia, USG < 1.030 and urine albumin/urine creatinine > 30 mg/g. Cats were classified into 4 stages according to IRIS guidelines after stabilization³, with P-Cre < 1.6 mg/dL indicating stage I. Urine was collected by catheterization. Samples were centrifuged at 1,190 g for 10 min and then stored at –80°C for further use. All owners gave signed informed consent to participation of their animal in the study.

Isolation of glycoproteins

Glycoproteins from urine of normal control and CKD cats were isolated using magnetic glyco-capturing beads (Bruker Daltonik GmbH, Germany) with lectin wheat germ agglutinin (WGA)-functionalized particles for specific capture. Briefly, 20 µL of urine was incubated with 20 µL of magnetic particles in the provided binding buffer. After washing, bound proteins were eluted under acidic conditions and dried in a vacuum centrifuge¹⁰.

SDS-PAGE analysis

In the second step of the analysis, each sample was subjected to SDS-PAGE. Lyophilized 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 solution was analyzed using SDS-PAGE (Perfect NT Gel W; DRC Co. Ltd., Tokyo, Japan, 10 to 20% acrylamide, 20 wells). The gel was stained with 2D-Silver Stain II (Cosmo Bio Co. Ltd., Tokyo, Japan). TotalLab TL120 software v. 2006 (Shimadzu Co. Ltd., Tokyo, Japan) was used to quantify the intensity of each protein band, and the intensity was used as an index of the level of protein expression¹¹.

In-gel digestion of proteins

The gel was cut into small pieces, destained in 50% CH₃CN/50 mM NH₄HCO₃, and washed with deionized water. The gel pieces were dehydrated in 100% ACN for 15 min and then dried in a SpeedVac Evaporator (Wakenyaku, Kyoto, Japan) for 45 min. The pieces were rehydrated in 10–30 µL of 25 mM Tris-HCl/20% CH₃CN containing 25 ng/L trypsin (Trypsin sequence grade; Roche Diagnostics GmbH, Mannheim, Germany) for 45 min. After removal of the unabsorbed solution, the pieces were incubated in 10–20 µL of 50 mM Tris-HCl/20% CH₃CN for 20 h at 37°C. The solution containing digested fragments of proteins was transferred to a new tube, and the peptide fragments remaining in the gel were extracted in 5% formic acid/50% CH₃CN for 20 min at room temperature¹¹.

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 Monolith analytical 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 CH₃CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH₃CN and 0.1% v/v HCOOH): 5–50% B for 20 min, 50–95% B for 1 min, 95% B for 3 min, 95–5% B for 1 min, and 5% B for 10 min. Purified peptides from HPLC were introduced into an LTQ-XL ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) via an attached Pico Tip (New Objective, Woburn, MA, USA). MS and MS/MS peptide spectra were measured in a data-dependent manner. The MASCOT search engine (Matrix Science, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the NCBI database using the MASCOT engine. The minimum significant threshold level for the probability-based MASCOT/MOWSE score was set at 5%¹².

ELISA assay

Immunogens for the development of anti- carboxy-lesterase 5A fragment antibody

The recombinant CES5A fragment were created by FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) (Fig. 1).

Synthetic peptides of 20 amino acid (Toray research center, Tokyo, Japan) corresponding to the sequences of the N-terminal peptide (CES5A fragment N peptide) and synthetic peptides of 20 amino acid (Toray research center) corresponding to the sequences of the C-terminal peptide (CES5A fragment C peptide) of CES5A fragment, coupled to keyhole limpet hemocyanin (KLH) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). The peptide-keyhole limpet hemocyanin conjugates were dissolved in distilled water and used as antigens for the preparation of monoclonal antibodies.

Immunization and establishment of hybridoma cell lines

The CES5A fragment N peptide and C peptide (50 mg at 1 mg/mL in PBS buffer) were used for the immunization of BALB/c mice. Hybridoma cell lines were prepared as described¹⁵⁾. The hybridoma cell lines, CES5A fragment N-01 and C-02, were established and antibody isotypes were determined using Mouse Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Oxford, UK) following the manufacturer's instructions. To obtain pure monoclonal antibodies on a large scale, BALB/c mice were initially stimulated with 1.0 mL pristine (Sigma Aldrich Japan) and then inoculated 2 week later. Monoclonal antibodies were purified as described¹⁵⁾.

Western blot analysis of anti- CES5A fragment antibodies

To examine the specificity of the antibodies, recombinant CES5A fragment and urine sample of cats with CKD (stage I) were separated by SDS-PAGE using a 10–20% gradient gel (DRC, Tokyo, Japan) in the absence of β -mercaptoethanol, and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). To minimize nonspecific binding, the membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan). After the membrane was washed three times with PBST (PBS buffer including 0.05% Tween-20), the membranes were incubated with anti- CES5A fragment N peptide antibody or anti- CES5A fragment C peptide antibody as primary antibodies for 1 h at room temperature. The membrane was washed

again three times with PBST and then incubated with a secondary Rabbit anti-mouse immunoglobulins/HRP (DAKO Japan, Tokyo, Japan) for 1 h at room temperature. The reactive antibodies were visualized by staining with Pierce Western Blotting substrate (Thermo Fisher Scientific, MA, USA).

Immobilization of antibodies to a polystyrene microtiter plate

The anti- CES5A fragment C peptide antibody dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific) at 0.5 mg/well and incubated for 1 day at 4°C. The plate was washed three times with PBS containing 0.05% Tween-20. The microtiter plate was coated with 20% NOF102 containing 10% sucrose for 1 day at 4°C.

ELISA assay conditions

Synthetic CES5A fragment was diluted with PBS buffer for calibration. After washing the microtiter plate with PBS buffer containing 0.05% Tween-20, 100 μ L aliquots of 10-diluted urine samples were added in duplicate to wells. The plates were incubated at room temperature for 1 h and then washed three times. Anti-CES5A fragment N antibody conjugated to HRP in PBS containing 0.05% Tween-20 (100 μ L) was added to each well and the plate was incubated at room temperature for 30 min. The plate was washed three times, and then 100 μ L of TMB solution (Wako Pure Chemical Industries, Tokyo, Japan) was added. After incubation at room temperature for 10 min, 100 μ L of stop solution was added and the absorbance at 450 nm was measured.

Other procedures

BUN, P-Cre and urine Creatinine were measured by an enzymatic method using an automatic analyzer (7180 Automatic Analyzer; Hitachi High-Technologies Corporation, Tokyo, Japan). Numerical data are presented as the mean $\pm$ standard deviation (SD). Significance was evaluated using IBM SPSS Statistics 18 software (SPSS Inc., Chicago, IL, USA), with p values <0.05 considered significant.

RESULTS

Two-step proteome analysis

Six urine samples from normal control and CKD (stage I) cats were subjected to two-step urine proteome analysis. A representative 2D-Silver Stain II-stained SDS-PAGE gel is shown in Fig. 2A. After converting the intensity of each band to a numerical value using TotalLab TL120 software v.2006, the urine samples showed a significant difference in the expression level of a 39.0 kDa band between normal control and CKD (stage I) cats ($p=0.012$) (Fig. 2B). The expression level of a 39.0 kDa band corrected with urine creatinine level between normal control and CKD (stage I) cats ($p=0.002$) (Fig. 2C). The levels of 6 bands in controls

DN1 EFGGDPKRSV TIFGESAGAI SVSSILSP1
ANGLFHKAIM ESGVAILPLL MRPPGDERKK DLOVLARICG CHASDSAALL QOLRAKPSSE
LMDISKKLTF SIPVIDDFFF PDEPVALLTQ KAFNSVPSII GVNNECAFL LSTEFSEILG
GSNRSLLALYL VHTFLNIPTQ YLHLVADHYF YNKHSPVEIR DSFLDLLGDV LFWPGVVTA
RYHRDAGAPV YFEFQHPPO CLNDTRPAFV KADHSDEIRF VFSGAFLKGD IVMFEGATEE
EK

Fig. 1. Amino acid sequence of the recombinant CES5A fragment.

were significantly decreased in CKD (stage I) cats.

Identification of proteins

The five bands with significant changes in CKD (stage I) cats were digested by trypsin and the products were subjected to tandem mass spectrometry for identification (Table 1). The two proteins that had higher levels in control cats were identified as carboxylesterase 5A and filaggrin-2.

Western blot analysis

Anti-CES5A fragment N peptide antibody (Fig. 3A) and anti-CES5A fragment C peptide antibody (Fig. 3B) recognized recombinant CES5A fragment and urine sample of normal control, respectively.

ELISA assay

Range, dilution analysis and detection limit

A standard curve was drawn based on the colorimetric

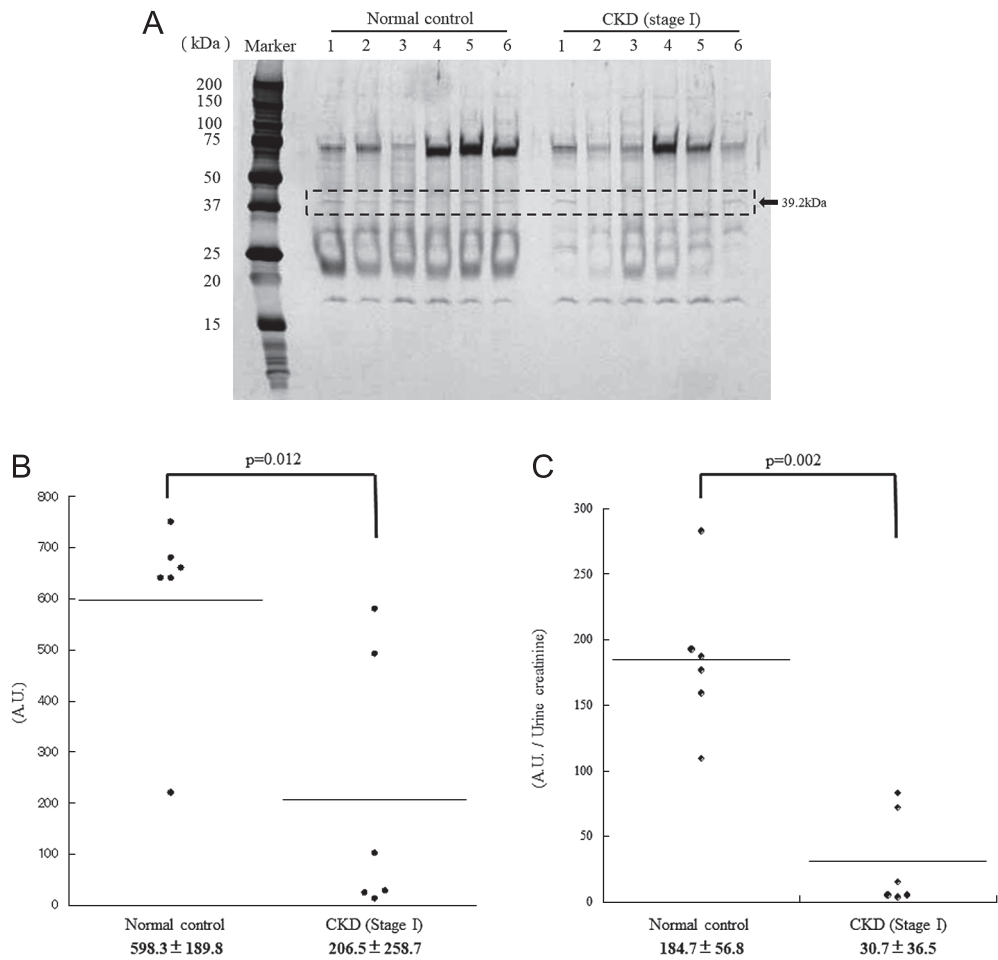


Fig. 2. Representative SDS-PAGE gel and comparison of band intensities by densitometry.

Glycoproteins were isolated from urine samples (20 μ L each), subjected to 10 to 20% SDS-PAGE, and visualized using silver staining. (A) An image showing the band corresponding to the 39.2 kDa carboxylesterase 5A fragment. (B) The intensity of the 39.2 kDa band differed significantly between samples from normal control cats (n=6) and CKD (stage I) cats (n=6). (C) The intensity of the 39.2 kDa band corrected with urine creatinine level differed significantly between samples from normal control cats (n=6) and CKD (stage I) cats (n=6).

Table 1. Urine proteins detected at lower levels in CKD (stage I) cats compared to normal controls in a two-step proteome analysis

No.	Database Accession No.	ID	Molecular weight	Score	Number of matching peptides	Sequence coverage
1	gi57163725	Carboxylesterase 5A	60467	138	4	6%
2	gi57163725	Carboxylesterase 5A	60467	123	3	5%
3	gi57163725	Carboxylesterase 5A	60467	105	3	5%
4	gi586996825	Filaggrin-2	191739	125	4	2%
5	gi586996825	Filaggrin-2	191739	112	4	2%

intensity of diluted synthetic CES5A fragment to establish the relationship between the intensity and the CES5A fragment concentration (Fig. 4). The working range of the assay was 0–10 mg/dL and the assay gave linear results from 0 to 10 mg/dL ($y=0.0201x+0.0009$, $r^2=0.9999$, $p<0.0001$). The detection limit was estimated by assaying the zero concentration eight times, and defined as the CES5A fragment “zero” concentration+3SD. The limit was found to be 0.22 mg/dL.

Within-run and between-run reproducibility

The precision of the assay was determined using two concentrations (0.6 and 3.2 mg/dL). Within-assay CVs were determined with eight replicates of each sample. Between-assay CVs were determined based on assays performed on 5 different days (two replicates of each sample per day). The within-run CV was 1.3–3.6% and the between-run CV was 1.5–4.1%.

Interference

Interference was assessed in samples containing 10 mg/dL of CES5A fragment. Potential interference materials

were added to urines at various concentrations. There was no substantial interference from hemoglobin (up to 5000 mg/L), free bilirubin (up to 207 mg/L), ditauobilirubin (up to 204 mg/L), chyle (up to 1400 formazine turbidity units even equal 1176 mg/L as triglyceride), ascorbic acid (up to 500 mg/L) and RF (up to 500 U/L).

Recovery test

To evaluate recovery in the ELISA, two concentrations (0.5 and 5.0 mg/dL) of synthetic CES5A fragment were added to pooled urine (3.2 mg/dL). The percentage recovery ranged from 97.2 to 102.4%.

Carboxylesterase 5A fragment levels are increased in the urine of cats with CKD

We measured urine CES5A fragment levels among stage I CKD cats ($n=56$) and age-matched normal cats ($n=56$). As shown in Fig. 5, the urine CES5A fragment levels in the normal cats (3.4 ± 0.6 mg/dL) were significantly greater than in stage I CKD cats (1.9 ± 0.5 mg/dL) ($P<0.001$; Mann–Whitney U-test).

Receiver operator characteristic (ROC) curves were conducted to evaluate the value of urine CES5A fragment, serum creatinine, and serum BUN levels for distinguishing stage I CKD cats from normal cats. For distinguishing between stage I CKD and normal cats, the respective area under the ROC curve (AUC) was 0.900 for urine CES5A fragment, 0.612 for serum creatinine, 0.492 for serum BUN. These results suggest that urine CES5A fragment expression is potentially a better diagnostic stage I CKD biomarker than serum creatinine and BUN expressions (Fig. 6).

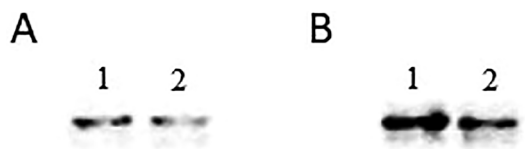


Fig. 3. Western blotting analysis.

Immunoreactive bands were observed at CES5A fragment when recombinant and urine CES5A fragment were incubated with anti-CES5A fragment N antibody (A) or anti-CES5A fragment C antibody (B).

Lane1: recombinant CES5A fragment.

Lane2: urine.

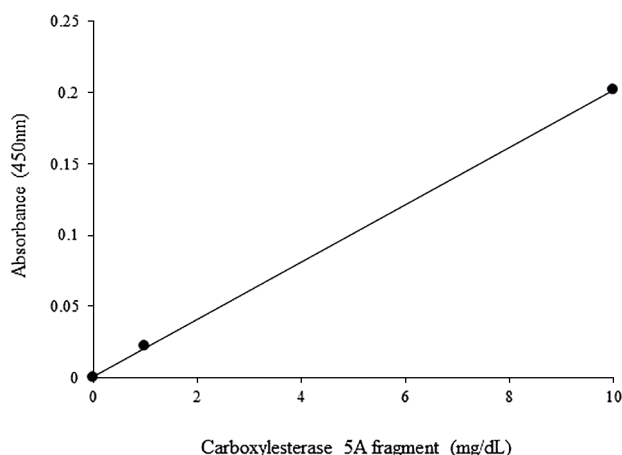


Fig. 4. Standard curves for CES5A fragment in the ELISA.

The relationship between colorimetric intensity and CES5A fragment concentration in the range of 0–10 mg/dL. Three concentrations of CES5A fragment were determined by ELISA.

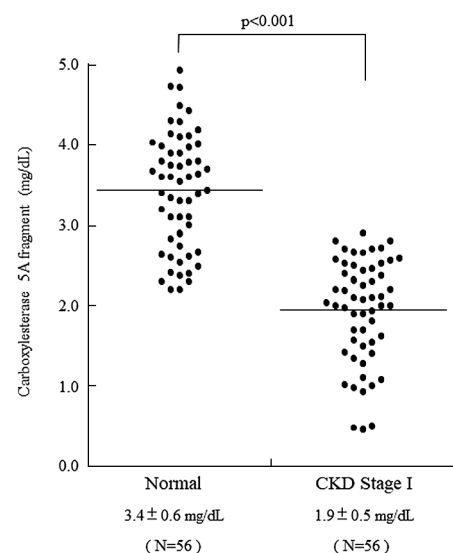


Fig. 5. Urine CES5A fragment levels in normal cats and CKD (stage I) cats.

The levels are significantly lower in CKD (stage I) cats (1.9 ± 0.5 mg/dL) compared with the normal cats (3.4 ± 0.6 mg/dL). Urine CES5A fragment levels in normal cats were significantly higher than in CKD (stage I) cats ($P<0.001$; Mann–Whitney U-test).

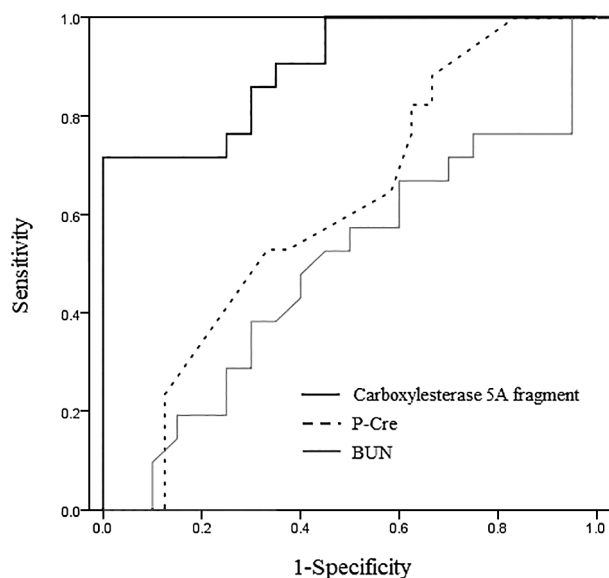


Fig. 6. Urine CES5A fragment excels P-Cre and BUN in the early detection of stage I CKD.

The ROC analyses were performed for the serum levels of urine CES5A fragment, P-Cre and BUN between stage I CKD and normal cats. The respective AUC was 0.900 for urine CES5A fragment, 0.612 for P-Cre, 0.492 for BUN.

Discussion

Recent advances in proteomic technology have provided promising ways to identify biomarkers in clinical veterinary medicine. Application of gel-based and gel-free methods has also facilitated discovery of potential clinical biomarkers, although there is a long and uncertain path from marker discovery to clinical utility. In this study, we used a two-step proteomics analysis to search for biomarkers for CKD (stage I) in cats, with the finding that the urine level of a 39.2 kDa CES5A fragment was significantly greater in controls than in CKD (stage I) cats.

Carboxylesterase 5A is also referred to as cauxin. The carboxylesterase 5A gene was isolated from a feline kidney cDNA library based on the amino acid sequence of a partial protein fragment¹³. Carboxylesterase 5A is present in urine of adult male cats at a high level of about 1 mg/mL¹³. Carboxylesterase 5A excretion is greater in males than in females¹³, is decreased by castration¹⁴, and increases with growth¹⁴. Carboxylesterase 5A is involved in production of a pheromone precursor, felinine, in urine¹⁵, and reduction of carboxylesterase 5A expression has been found in chronic interstitial nephritis in feline renal impairment¹⁶.

In this study, we have constructed that the performance of the ELISA was satisfactory in terms of recovery (97.2–102.4%) and within-run (1.3–3.6%) and between-day (1.5–4.1%) reproducibility. To the end, we have described that urine CES5A fragment level is a potential urine biomarker to distinguish CKD (stage I) cats from normal cats.

Advanced techniques for proteomics analysis have re-

sulted in identification of many new biomarkers, but the most of those are not insufficient for clinical application. The procedures for developing biomarkers include comparison of disease and control groups by semiquantitative analysis (discovery phase) and selection of marker candidates (validation phase), followed by ELISA validation of markers found by proteome analysis.

Proteomics using advanced analytical techniques gives a comprehensive analysis of protein levels in vivo and relationships with disease. Biomarker candidates can be detected by serum/plasma proteome analysis, leading to acquisition of disease-specific diagnostic information^{17, 18}. However, few of these markers are currently practically useful for diagnosis and tests. We detected a 5.9 kDa α C-chain near the C-terminal fragment (FIC5.9) as a marker for drinking behavior¹⁹. For FIC5.9-specific detection, antibodies against the N-terminal and C-terminal domains of FIC5.9 were produced and a sandwich ELISA was constructed²⁰. A multicenter clinical trial showed that detection of FIC5.9 was effective for assessment of early fibrogenesis in chronic hepatic dysfunction²¹.

The urine levels of CES5A fragment in normal cats and CKD (stage I) cats were 3.4 ± 0.6 mg/dL and 1.9 ± 0.5 mg/dL, respectively. The CES5A fragment level was significantly decreased in CKD (stage I) cats compared with normal cats ($p < 0.001$). These results suggest that CES5A fragment is a new marker for CKD that is complementary to conventional markers. The simple ELISA developed in this study is useful for establishing the diagnostic significance of urine CES5A fragment in CKD (stage I) cats.

CONCLUSION

Our results suggest that the 39.2 kDa carboxylesterase 5A fragment may be used as a marker complementary to P-Cre and BUN for detection of CKD (stage I) in cats. We note that the present study is limited by the number of samples, and evaluation of more samples from multiple facilities is required in a future study.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ABBREVIATIONS

BUN, blood urea nitrogen; CES5A, carboxylesterase 5A; CKD, chronic kidney disease; GFR, glomerular filtration rate; IRIS, International Renal Interest Society; P-Cre,

plasma creatinine; UPC, urinary protein to creatinine ratio; USG, urine specific gravity

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