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# High-dose HOOK effect in urinary DcR2 assay in patients with chronic kidney disease

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ABSTRACT

*Background:* Urinary DcR2 (uDcR2) is a biomarker for the early detection the tubulointerstitial injury (TII) in patients with chronic kidney disease (CKD), but the high-dose hook effect may lead to falsely low or even negative results when using an enzyme-linked immunosorbent assay (ELISA). This study aimed to investigate if the high-dose hook effect exists with ELISA testing, and to uncover a potential approach for reducing this effect.
*Methods:* 72 CKD patients were recruited and categorized into four groups based on TII scores. uDcR2 was measured in undiluted and serially diluted (two-, four-, eight- and 16-fold dilutions) urine using an ELISA kit. The results from the assay were normalized to urinary creatinine. We evaluated the correlation between uDcR2/cre levels at different dilutions and renal histological parameters. Receiver operating characteristic (ROC) curves were generated to examine the value of uDcR2/cre for predicting TII.
*Results:* uDcR2/cre levels in the undiluted urine were significantly higher in patients with CKD than those in the control. However, higher TII scores did not yield higher levels of uDcR2/cre in the undiluted urine. After serial dilution, uDcR2/cre levels were highest with the four-fold dilution. A positive correlation was found between uDcR2/cre levels at different dilutions and TII scores, with the highest correlation coefficient and the largest AUC being observed at the four-fold dilution.
*Conclusions:* The high-dose hook effect was apparent during ELISA testing of uDcR2 in CKD patients, yet dilution of the urine samples neutralized this effect. However, the use of a four-fold dilution of urine for uDcR2/cre testing may eliminate the high-dose hook effect and make it possible to effectively monitor the severity of TII in CKD patients.

## 1. Introduction

Decoy receptor 2 (DcR2), a 42 kDa receptor of the tumor necrosis factor (TNF)-related apoptosis inducing ligand, is a transmembrane protein found in senescent tumor cells [1]. We previously discovered that kidney expression of DcR2 is localized to renal tubular cells, and absent from the glomeruli [2, 3]. The ectodomain of DcR2 can be cleaved and detected in the urine, and urinary DcR2 (uDcR2) has been recognized as an important biomarker for the evaluation of tubulointerstitial injury (TII) and the progression of diabetic nephropathy [3]. The accurate assessment of uDcR2 levels is critical to successfully diagnose and treat patients with chronic kidney disease (CKD).

uDcR2 can be measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit with high sensitivity and specificity [3]. However, any ELISA may suffer from falsely low or negative results due to the high-dose hook effect [4]. The high-dose hook effect is a phenomenon in which a paradoxically lower signal is observed when higher concentrations of sample are tested. Since high uDcR2 concentrations are common in patients with severe TII, the high-dose hook effect may confound results from ELISA testing. In fact, we found that uDcR2 levels in undiluted urine remained the same, despite the severity of TII, suggesting the interference posed by the high-dose hook effect.

Many attempts have been made to overcome the limitation posed by the high-dose hook effect, including methods involving neural learning networks [5], reaction kinetics [6], and serial dilution [7]. Serial dilution has been deemed the simplest and most convenient means for avoiding the high-dose hook effect in the testing of many markers, including ferritin [7], human chorionic gonadotropin (hCG) [8, 9], and chromogranin A [10]. However, it is unclear if the high-dose hook effect is present in ELISA testing for uDcR2.

In this study, CKD patients with different TII scores were enrolled to investigate if ELISA testing of uDcR2 was affected by the high-dose hook effect. This was accomplished by measuring uDcR2 levels in both undiluted and diluted urine samples from CKD patients. In addition, we established the predictive value of uDcR2 levels for assessing TII at

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different folds of urine dilution.

## 2. Materials and methods

### 2.1. Subjects and ethical approval

A total of 72 patients with biopsy-proven CKD were recruited from the Department of Nephrology at the Daping Hospital of the Army Military Medical University (Yuzhong District, Chongqing, P. R. China). Exclusion criteria for this study included patients with a history of cancer and inflammatory diseases, along with patients receiving diuretics, Chinese herbal medicines, and nephrotoxic drugs. The causes of CKD in the 72 patients included IgA nephropathy ($n = 36$), diabetic nephropathy ($n = 27$), and focal segmental glomerulosclerosis ($n = 9$). The patients were categorized into four groups according to TII scores, as previously described [3, 11], with TII score 0 ($n = 18$), TII 1 (n = 18), TII 2 (n = 18), and TII 3 (n = 18). A TII score of 0 indicated the absence of tubulointerstitial injury, while scores TII 1 to TII 3 suggested increasing TII.

Healthy controls were selected from the Center for Health Examination. They were matched for age and gender with the CKD patients. Each healthy control had an estimated glomerular filtration rate (eGFR) > 90 mL/min/1.73 $m^2$ and an albumin-creatinine ratio (ACR) of < 30 mg/g creatinine, with no history of diabetes, renal disease, or cardiovascular disease. The study protocol was approved by the Ethical and Protocol Review Committee of the Army Medical University.

### 2.2. Collection of urine samples

Fresh midstream urine samples were collected in the early morning on the day of testing. Samples were immediately centrifuged (15 min × 1000 *g* at 4 °C), and the supernatant was stored at −80 °C. All urine samples were handled through the same practice.

### 2.3. Measurement of uDcR2 levels by ELISA

The uDcR2 levels were measured using a commercial ELISA kit (ab46017, Abcam, Cambridge, United Kingdom), with an optimal detection range of 312.5 pg/mL to 10,000 pg/mL. The lower limit of detection was 64 pg/mL. The urine samples were diluted with the diluent buffer, in accordance with manufacturer's protocol, before each sample was tested in triplicate. An automatic enzyme immunoassay reader (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance values. DcR2 assay results were normalized to the urinary creatinine (uDcR2/cre ratio) to account for the influence of urinary dilution and concentration.

### 2.4. High-dose hook effect

The serial dilution method was used to confirm the presence of the high-dose hook effect. All urine samples were diluted two-, four-, eight-, and 16-fold with the standard diluent buffer (Abcam). Changes in uDcR2/cre levels were studied using the diluted samples.

### 2.5. Pathological classification and histological scoring

Each biopsy specimen was processed and examined under light microscopy, immunofluorescence, and electron microscopy using standard procedures. Morphological changes were categorized based on the presence and degree of arteriolar hyalinosis, arteriosclerosis, glomerulosclerosis, and TII. Glomerulosclerosis scores ranged from 0 to 3, with a higher score indicating greater severity. The glomeruli in the biopsy specimens were calculated, as previously described [3, 12]. TII was scored as 0 for absent, 1 for < 25%, 2 for 25–50%, and 3 for > 50% of the total area. Arteriolar hyalinosis and arteriosclerosis were scored in accordance with the Renal Pathology Society guidelines [11]. All biopsy slides were randomly selected and independently scored by two blinded pathologists.

### 2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD) for normally distributed variables and median and interquartile range (IQR) for non-normally distributed variables. The one-way analysis of variance (ANOVA) was used to compare variables between more than two groups with normally distributed variables, and the Kruskal–Wallis test was used for non-parametric variables. The Pearson's correlation coefficient was used for normally distributed variables, while the Spearman's correlation coefficient was used for non-normally distributed variables. The predictive efficacy of uDcR2 levels at different dilutions for TII scores was evaluated using the receiver operating characteristics (ROC) analysis. *P*-values < .05 were considered significant, and all analyses were performed using SPSS 18.0 (SPSS Inc. Chicago, IL, USA).

## 3. Results

### 3.1. The uDcR2/cre levels in undiluted urine based on different TII scores in CKD patients

The demographic and clinical characteristics of the 72 enrolled patients are shown in Table 1. ACR and *N*-acetyl-β-D-glucosaminidase (NAG) levels were higher in the CKD group than the control group. The eGFR values in the CKD group were significantly lower than the values in the control group. The uDcR2/cre levels measured in the undiluted urine are shown in Table 1. While uDcR2/cre levels were significantly higher in the CKD group than those in the control, the values did not increase with higher TII scores.

### 3.2. uDcR2/cre levels at different dilutions in patients with CKD

To explore whether there was a high-dose hook effect, we measured uDcR2/cre levels in the urine at different dilutions (two-, four-, eight-, and 16-fold dilutions), as shown in Fig. 1. The uDcR2/cre levels increased with higher dilution strengths, and were highest at the four-fold dilution (Fig. 1A). Meanwhile, uDcR2/cre levels increased with higher TII scores when the urine was diluted at four-fold (Fig. 1B). These results verified the presence of the high-dose hook effect in the uDcR2 assay using undiluted urine samples, yet dilution of the urine samples could effectively eliminated this effect.

### 3.3. Correlations between uDcR2/cre levels at different dilutions and renal histological parameters

We next analyzed the correlations between the undiluted and diluted uDcR2/cre levels with the renal histological parameters in patients from the CKD group. The results showed a positive correlation between uDcR2/cre levels and TII scores, and the correlation coefficient was highest with four-fold dilution of the urine. Furthermore, the uDcR2/cre levels were positively correlated with glomerulosclerosis, arteriolar hyalinosis, and arteriosclerosis scores when the urine was four-fold diluted (Table 2).

### 3.4. ROC analysis of uDcR2/cre at different urine dilutions for assessing TII in CKD patients

We further studied if uDcR2/cre levels, obtained from different urine dilutions, could be used for predicting the severity of TII in CKD patients. The uDcR2/cre levels could effectively predict the severity of TII, with the highest AUC 0.923 (95% confidence interval [CI], 0.860–0.987, $P < .001$) at the four-fold dilution (Fig. 2). Table 3