

## Microproteinuria for detecting calcineurin inhibitor-related nephrotoxicity after liver transplantation

Jing Li, Bin Liu, Lu-Nan Yan, Lan-Lan Wang, Wan Y Lau, Bo Li, Wen-Tao Wang, Ming-Qing Xu, Jia-Yin Yang, Fu-Gui Li

Jing Li, Department of Anesthesiology and Critical Care Medicine, West-China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Bin Liu, Lu-Nan Yan, Bo Li, Wen-Tao Wang, Ming-Qing Xu, Jia-Yin Yang, Fu-Gui Li, Division of Liver Transplantation, West-China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Lan-Lan Wang, Department of Clinical Immunological Laboratory, West-China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Wan Y Lau, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong SAR, China

**Author contributions:** Li J and Liu B took care of the patients, designed the study, collected and analyzed the data, and wrote the manuscript; Yan LN designed the study, collected and analyzed the data, and wrote the manuscript; Wang LL collected and analyzed the data, coordinated the work group and contributed to the discussion; Lau WY contributed to the discussion; Li B, Wang WT, Xu MQ, Yang JY, Li FG took care of the patients, collected and analyzed the data.

**Correspondence to:** Lu-Nan Yan, Division of Liver Transplantation, West China Hospital, Sichuan University, Chengdu 610041, China. [surgeryliubin@163.com](mailto:surgeryliubin@163.com)

Telephone: +86-28-85422867 Fax: +86-28-85423724

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### Abstract

**AIM:** To investigate whether microproteinuria could be used as an early and sensitive indicator to detect calcineurin inhibitor (CNI)-related nephrotoxicity after liver transplantation.

**METHODS:** All liver transplant recipients with normal serum creatinine (SCr) and detectable microproteinuria at baseline were included in this study. The renal function was monitored by the blood clearance of  $^{99m}\text{Tc}$ -diethylenetriaminepentaacetic acid every 6 mo. Microproteinuria, SCr and blood urea nitrogen (BUN) were measured at entry and at subsequent follow-up visits. The patients were divided into different groups according to the mean values of glomerular filtration rate (GFR) at the follow-up time points: Group 1, GFR decreased from baseline by 0%-10%; Group 2, GFR decreased from baseline by 11%-20%; Group 3, GFR

decreased from baseline by 21%-40%; Group 4, GFR decreased from baseline by > 40% and/or SCr was increasing.

**RESULTS:** A total of 143 patients were enrolled into this study (23 females and 120 males). The mean follow-up was 32 mo (range 16-36 mo). Downward trends in renal function over time were observed in the study groups. SCr and BUN increased significantly only in Group 4 patients ( $P < 0.001$ ).  $\beta_2$ -microglobulin ( $\beta_2\text{m}$ ) and  $\alpha_1$ -microglobulin ( $\alpha_1\text{m}$ ) significantly increased with the subtle change of renal function in recipients who were exposed to CNI-based immunosuppression regimens. The reductions in GFR were closely correlated with elevated  $\alpha_1\text{m}$  ( $r^2 = -0.728$ ,  $P < 0.001$ ) and  $\beta_2\text{m}$  ( $r^2 = -0.787$ ,  $P < 0.001$ ).

**CONCLUSION:**  $\beta_2\text{m}$  and  $\alpha_1\text{m}$  could be useful as early and sensitive indicators of CNI-induced nephrotoxicity.

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**Key words:** Microproteinuria; Liver transplantation; Calcineurin inhibitors; Nephrotoxicity

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### INTRODUCTION

Calcineurin inhibitors (CNIs) have improved survival significantly after liver transplantation, but nephrotoxicity is an adverse effects common to both cyclosporine and tacrolimus<sup>[1-3]</sup>. Deterioration of renal function with CNI therapy has been widely reported in liver transplant

recipients<sup>[4-6]</sup>. A recent analysis of 36 849 liver transplant recipients performed in the United States between 1990 and 2000 revealed an 18.1% incidence of chronic renal failure after 5 years, with CNI dose and duration of exposure correlated with subsequent renal damage<sup>[7]</sup>.

The monitoring of transplant patients, however, is still dependent on somewhat old methodologies: serum creatinine (SCr) levels, total urine output, blood urea nitrogen (BUN), or calculated glomerular filtration rate (GFR). However, these tests are particularly problematic as they do not have sufficient specificity, sensitivity, or accuracy to allow appropriate and timely interventions<sup>[8]</sup>. Additionally, CNIs are characterized by a narrow therapeutic index, so renal damage is often irreversible when these indices become abnormal, or the indices can still fluctuate in the normal range while renal insufficiency is obvious. Given these limitations, more and more transplant specialists are looking to emerging fields, such as proteomics and metabolism, to improve the current situation. Microproteinuria, as a hallmark of reflecting early changes in the glomeruli and proximal tubular function, can be used as an accurate predictor to monitor early changes in renal function<sup>[9,10]</sup>.

The aim of this prospective study was to find out whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients. It was approved by the Ethics Committee at the West China Hospital, Sichuan University.

## MATERIALS AND METHODS

From April 2005 to December 2008, 423 adult patients underwent liver transplantation in our hospital. CNI-based immunosuppression regimens comprising a CNI (cyclosporine A or tacrolimus) + azathioprine + prednisone were delivered to recipients after transplantation. All patients who received a CNI-based regimen for immunosuppression after liver transplantation were potential candidates for this study. Recipients receiving CNI therapy without interruption after liver transplantation, with normal SCr at baseline and detectable microproteinuria in fresh urine were all included in this study. If death occurred within 3 mo posttransplantation or renal dysfunction was caused by non-CNI drugs (such as antibiotics or antivirals), or by other means during follow-up period, the recipients were excluded from the study.

Follow-up protocols were performed once every month for the first 6 mo after liver transplantation, once every 2 mo during months 7-12, and once every 6 mo after 1 year. At each time point, the patient contributed a urine sample which was correlated with the measurements from blood samples. Midstream fresh urine samples were collected and not centrifuged. Measurements of microproteinuria including  $\alpha$ 1-microglobulin ( $\alpha$ 1m),  $\beta$ 2-microglobulin ( $\beta$ 2m), immunoglobulin, microalbumin and transferrin were performed immediately with a Dade Behring array nephelometry system (Dade Behring Inc, USA). SCr (picric acid method) was measured by a

Modula clinical chemistry analyzer (Roche Diagnostics, Roche, Switzerland). GFR was measured every 6 mo by the Gates method (PHILIPS Helix SPX-6D SPECT, Holland) which analyzed the blood clearance of <sup>99m</sup>Tc-diethylenetriaminepentaacetic acid (DTPA) (5 mCi, from the China Institute of Atomic Energy, radiochemical purity 95%). As consent for a renal biopsy was difficult to obtain, this was performed only when clinically indicated, especially in patients with increasing SCr (an indication of progressive deterioration in renal function).

The actual GFR is considered to be the best overall index of renal function in health and disease<sup>[11]</sup>. Therefore, we chose the actual GFR as the criteria for renal function in this study. The patients were divided into 4 groups according to the mean values of GFR at every 6-mo follow-up: Group 1, GFR declined from baseline by 0%-10%; Group 2, GFR declined from baseline by 11%-20%; Group 3, GFR declined from baseline by 21%-40%; Group 4, GFR declined from baseline by > 40% and/or SCr was increasing.

The normal concentrations of individual proteins present in the urine are illustrated by maximum values<sup>[12,13]</sup>, and are significantly different between different laboratories<sup>[12-14]</sup>. The normal reference values of microproteinuria provided by Dade Behring, Inc. are: microalbumin < 30 mg/L,  $\alpha$ 1m < 12.0 mg/L,  $\beta$ 2m < 0.20 mg/L, immunoglobulin < 9.6 mg/L, and transferrin < 1.9 mg/L. According to the values determined in 500 Chinese healthy individuals in our laboratory, the normal values used in this study for microalbumin were < 19 mg/L,  $\alpha$ 1m < 12.5 mg/L,  $\beta$ 2m < 0.22 mg/L, immunoglobulin < 20.0 mg/L, and transferrin < 2.2 mg/L.

Statistical analysis was performed by SPSS 13.0 (SPSS Inc., Chicago, IL). Differences in microproteinuria, serum creatinine and BUN were tested with the one way ANOVA test for multiple comparisons. Data were expressed as mean  $\pm$  SD, or median (range). The Kruskal-Wallis rank sum test (individual comparisons were done by the Wilcoxon rank sum test) and correlation analyses were used in this study. A *P*-value less than 0.05 indicated statistical significance.

## RESULTS

A total of 143 liver transplant patients were recruited into this study. Of these, 16 withdrew during the follow-up period. The reasons for withdrawal were infection in 3 patients, acute rejection in 2, use of non-CNI drugs in 4, uncontrolled hypertension in 3, abnormal liver function in 2, and 2 patients died. There were 23 females and 120 male, aged 21-68 years. The grafts and recipients were blood group identical in 127 cases and compatible in 16 cases. The mean follow-up was 32 mo (range 16-36 mo). The primary disease of the 143 recipients included diffuse ischemic intrahepatic biliary stenosis in 8, Caroli disease in 7, Budd-Chiari syndrome with liver cirrhosis in 4, liver cirrhosis after hepatitis B in 56, postoperative liver failure after right lobe hepatectomy caused by hepatic trauma in 4, Wilson disease in 11,  $\alpha$ 1-antitrypsin deficiency in 2, echinococcus disease of the liver in

Table 1 Demographic data of patients at the point of entry into this study (mean  $\pm$  SD)

Variable ( <i>n</i> = 143)	Group 1 ( <i>n</i> = 102)	Group 2 ( <i>n</i> = 35)	Group 3 ( <i>n</i> = 6)	Group 4 ( <i>n</i> = 0)
Age (yr), median(range)	48.6 (28-66)	49.2 (33-67)	48.5 (21-68)	0
Body mass index (BMI) (kg/m <sup>2</sup> )	22.0 $\pm$ 2.8	20.5 $\pm$ 2.4	21.8 $\pm$ 2.9	0
Pre-transplant MELD score	17.8 $\pm$ 7.6	16.2 $\pm$ 8.4	15.7 $\pm$ 9.7	0
BUN (mmol/L)	5.6 $\pm$ 2.3	6.0 $\pm$ 2.5	5.9 $\pm$ 2.8	0
SCr ( $\mu$ mol/L)	54.7 $\pm$ 15.8	60.5 $\pm$ 20.1	68.7 $\pm$ 17.4	0
GFR (mL/min per 1.73 m <sup>2</sup> )	105.4 $\pm$ 20.5	99.6 $\pm$ 15.2	103.3 $\pm$ 23.1	0
Dosage (mg/kg per day)				
Cyclosporine A	4.0 $\pm$ 1.1	4.6 $\pm$ 1.5	5.1 $\pm$ 1.2	0
Tacrolimus	0.055 $\pm$ 0.03	0.061 $\pm$ 0.07	0.057 $\pm$ 0.05 <sup>a</sup>	0
Trough levels (ng/mL)				
Cyclosporine A	212.4 $\pm$ 45.8	243.7 $\pm$ 56.2	251.3 $\pm$ 61.6	0
Tacrolimus	6.3 $\pm$ 0.6	6.7 $\pm$ 0.7	7.5 $\pm$ 0.9 <sup>a</sup>	0
Duration of previous treatment (mo)				
Cyclosporine A	5.8 $\pm$ 3.4	6.7 $\pm$ 4.6	6.9 $\pm$ 3.9	0
Tacrolimus	5.3 $\pm$ 2.8	6.2 $\pm$ 3.5	6.7 $\pm$ 2.5	0
Time post-transplant	7.5 $\pm$ 2.2	8.8 $\pm$ 2.6	8.2 $\pm$ 3.1	0
Microproteinuria (mg/L)				
$\beta$ 2m	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.5 $\pm$ 0.3 <sup>a</sup>	0
$\alpha$ 1m	14.2 $\pm$ 10.6	18.5 $\pm$ 17.3	24.8 $\pm$ 20.1 <sup>a</sup>	0
Microalbumin	28.6 $\pm$ 19.3	37.7 $\pm$ 28.1	40.1 $\pm$ 30.5	0
Immunoglobulin	27.4 $\pm$ 8.3	25.1 $\pm$ 11.4	29.3 $\pm$ 15.6	0
Transferrin	2.8 $\pm$ 0.7	2.4 $\pm$ 1.6	3.1 $\pm$ 1.4 <sup>a</sup>	0

<sup>a</sup>*P* < 0.05, *vs* group 1. Group 1: GFR declined from baseline by 0%-10%; Group 2: GFR declined from baseline by 11%-20%; Group 3: GFR declined from baseline by 21%-40%; Group 4: GFR declined from baseline by > 40% and/or SCr was increasing.

Table 2 Mean values of every follow-up time point in the study groups (mean  $\pm$  SD)

Variable ( <i>n</i> = 143)	Group 1 ( <i>n</i> = 73)	Group 2 ( <i>n</i> = 40)	Group 3 ( <i>n</i> = 30)	Group 4 ( <i>n</i> = 5)
GFR (mL/min per 1.73 m <sup>2</sup> )	97.4 $\pm$ 12.7	85.6 $\pm$ 17.9	62.3 $\pm$ 20.5	49.6 $\pm$ 20.2
The declining percentage of GFR from baseline (%)	7.3 $\pm$ 2.6	16.7 $\pm$ 10.1 <sup>a,c</sup>	32.5 $\pm$ 12.9 <sup>a,c</sup>	52.4 $\pm$ 20.8 <sup>a,c</sup>
SCr ( $\mu$ mol/L)	76.3 $\pm$ 16.2	83.7 $\pm$ 15.4	90.3 $\pm$ 19.8	173.7 $\pm$ 28.5 <sup>a,c</sup>
BUN (mmol/L)	5.8 $\pm$ 1.7	6.3 $\pm$ 1.2	6.9 $\pm$ 1.5	11.2 $\pm$ 2.6 <sup>a,c</sup>
Dosage (mg/kg per day)				
Cyclosporine A	2.5 $\pm$ 0.8	3.0 $\pm$ 1.0	3.3 $\pm$ 0.9	3.8 $\pm$ 1.1 <sup>a</sup>
Tacrolimus	0.049 $\pm$ 0.03	0.052 $\pm$ 0.07	0.054 $\pm$ 0.05	0.058 $\pm$ 0.03 <sup>a</sup>
Trough levels				
Cyclosporine A (ng/mL)	145.2 $\pm$ 30.5	154.8 $\pm$ 25.6	165.3 $\pm$ 39.4	183.2 $\pm$ 31.2 <sup>a</sup>
Tacrolimus (ng/mL)	5.1 $\pm$ 0.8	5.5 $\pm$ 1.2	6.2 $\pm$ 1.0 <sup>a</sup>	6.8 $\pm$ 1.3 <sup>a,c</sup>
Diabetes (%)	9.2	10.5	9.3	11.1
Hypertension (%)	38.5	34.2	37.2	33.3
Follow-up time, mean (range, mo)	35.6 (23-36)	33.7 (24-36)	30.4 (19-36)	27.8 (16-36)
Microproteinuria				
$\beta$ 2m (mg/L)	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2 <sup>a</sup>	1.0 $\pm$ 1.3 <sup>a,c</sup>	4.2 $\pm$ 2.5 <sup>a,c</sup>
$\alpha$ 1m (mg/L)	22.6 $\pm$ 21.1	38.9 $\pm$ 25.4 <sup>a</sup>	42.3 $\pm$ 35.9 <sup>a,c</sup>	65.1 $\pm$ 30.4 <sup>a,c</sup>
Microalbumin (mg/L)	46.9 $\pm$ 26.2	83.6 $\pm$ 55.5 <sup>a</sup>	70.9 $\pm$ 33.8	45.4 $\pm$ 32.9
Immunoglobulin (mg/L)	23.9 $\pm$ 14.6	21.5 $\pm$ 29.1	37.3 $\pm$ 26.4	33.5 $\pm$ 29.1
Transferrin (mg/L)	3.2 $\pm$ 10.2	10.8 $\pm$ 27.8	8.2 $\pm$ 15.9	8.1 $\pm$ 12.4

<sup>a</sup>*P* < 0.05, *vs* group 1; <sup>c</sup>*P* < 0.05, *vs* group 2. Group 1: GFR declined from baseline by 0%-10%; Group 2: GFR declined from baseline by 11%-20%; Group 3: GFR declined from baseline by 21%-40%; Group 4: GFR declined from baseline by > 40% and/or SCr was increasing.

4, hepatolithiasis in 3, hepatocellular carcinoma in 38, and cholangiocarcinoma in 6. All hepatocellular cancer patients met the UCSF criteria (a single tumor  $\leq$  6.5 cm in diameter, or 2 or 3 tumors, none exceeding 4.5 cm in diameter and whose sum of tumor diameters did not exceed 8 cm). The demographic data of these patients are shown in Table 1. There were 102 (71.3%) recipients in Group 1, 35 (24.5%) in Group 2, 6 (4.2%) in Group 3, and none in Group 4 at baseline. At entry into this study, all recipients had normal levels of BUN, SCr, and GFR with detectable microproteinuria in fresh urine. There

were no significant differences in body mass index, or pre-transplant MELD score.

Through measurements of GFR by the blood clearance of <sup>99m</sup>Tc-DTPA at entry into the study and at the follow-up visits, we found there was a downward trend in renal function over time, and the reductions in GFR were significantly different across all groups (Table 2). The value of GFR was 97.4  $\pm$  12.7 mL/min per 1.73 m<sup>2</sup> in Group 1 (decreased 7.3%  $\pm$  2.6% from baseline), and 85.6  $\pm$  17.9 mL/min per 1.73 m<sup>2</sup> in Group 2 (*P* < 0.001 *vs* Group 1) (decreased 16.7%  $\pm$  10.1% from baseline), and

62.3 ± 20.5 mL/min per 1.73 m<sup>2</sup> in Group 3 ( $P < 0.001$  vs Group 1;  $P < 0.001$  vs Group 2) (decreased 32.5% ± 12.9% from baseline). In Group 4, the value of GFR was 49.6 ± 13.2 mL/min per 1.73 m<sup>2</sup> ( $P < 0.001$  vs Group 1;  $P < 0.001$  vs Group 2;  $P = 0.002$  vs Group 3) (decreased 52.4% ± 20.8% from baseline). The SCr significantly increased only in Group 4 (173.7 ± 28.5 μmol/L) when compared with the other groups ( $P = 0.017$  vs Group 1,  $P = 0.021$  vs Group 2,  $P = 0.035$  vs Group 3). Similar trends were found for BUN. However, the urinary β<sub>2</sub>m ( $P < 0.01$ ) and α<sub>1</sub>m ( $P = 0.043$ ) were significantly different between the 4 groups of patients. This study also indicated that microalbumin, immunoglobulin and transferrin had no significant differences in the 4 groups of patients.

In Group 1, the mean level of urinary α<sub>1</sub>m at entry and at subsequent follow-up visits was 22.6 ± 21.1 mg/L, which was significantly lower than that in Group 2 (38.9 ± 25.4 mg/L,  $P < 0.001$ ), and in Group 3 (42.3 ± 35.9 mg/L,  $P < 0.001$ ). In Group 4, the level of urinary α<sub>1</sub>m (65.1 ± 30.4 mg/L) was significantly higher than that of all the other groups ( $P < 0.001$  vs Group 1;  $P < 0.001$  vs Group 2;  $P = 0.002$  vs Group 3). The findings in this study suggested that the increase in urinary α<sub>1</sub>m occurred long before the elevation of SCr or BUN, and the reductions in GFR were closely correlated with the increases in α<sub>1</sub>m ( $r^2 = -0.728$ ,  $P < 0.001$ ). The urinary α<sub>1</sub>m, as a marker of tubular damage, was sensitive in assessing subtle changes in renal function caused by CNI nephrotoxicity.

The mean level of urinary β<sub>2</sub>m during follow-up visits was 0.2 ± 0.1 mg/L in Group 1, 0.4 ± 0.2 mg/L in Group 2 ( $P = 0.041$  vs Group 1) and 1.0 ± 1.3 mg/L in Group 3 patients ( $P < 0.001$  vs Group 1;  $P < 0.001$  vs Group 2). The level of urinary β<sub>2</sub>m in Group 4 patients (4.2 ± 2.5 mg/L) was further increased compared to other subgroups ( $P < 0.001$  vs Group 1;  $P < 0.001$  vs Group 2;  $P < 0.001$  vs Group 3). The urinary β<sub>2</sub>m concentration gradually increased from Group 1 to Group 4. The results of this study showed that the increase in urinary β<sub>2</sub>m, as a marker of tubular epithelial cell dysfunction, occurred long before the elevation of SCr or BUN, and was also closely correlated with the reduction in GFR ( $r^2 = -0.787$ ,  $P < 0.001$ ). The urinary β<sub>2</sub>m was also sensitive for detecting subtle changes in renal function in patients exposed to CNI-based immunosuppression regimens. Additionally, monitoring both β<sub>2</sub>m and α<sub>1</sub>m could improve the sensitivity and specificity of measurement of CNI-related nephrotoxicity.

## DISCUSSION

The tremendous success of CNIs in reducing acute rejection episodes and early immunologic graft injury has not been accompanied by a benefit in long-term recipient survival<sup>[15,16]</sup>. CNI nephrotoxicity in liver transplantation is a significant concern and appears to be progressive over time when CNI exposure is maintained<sup>[17]</sup>. Microproteinuria has been used as an early marker of nephrotoxicity to detect small changes in the function of tubular epithelial cells in many pathological conditions<sup>[10]</sup>.

The persistence of microproteinuria may result from drug toxicity or pretransplant renal diseases after liver transplantation. Therefore, recipients with normal serum creatinine at baseline and detectable microproteinuria were selected as subjects in this study.

Follow-up data of this study demonstrated that there was a downward trend in renal function over time, with the persistence of microproteinuria. The urinary concentration of β<sub>2</sub>m and α<sub>1</sub>m significantly increased with the subtle change in renal function in all study groups, but the levels of SCr and BUN significantly increased only when renal function was severely reduced by CNI nephrotoxicity (in Group 4, renal function declined 52.4% ± 20.8% from baseline). A similar study also found microproteinuria occurred long before the elevation of SCr<sup>[18]</sup>. The subsequent reductions in GFR were closely correlated with elevated α<sub>1</sub>m ( $r^2 = -0.728$ ,  $P < 0.001$ ) and β<sub>2</sub>m ( $r^2 = -0.787$ ,  $P < 0.001$ ) in the study groups. The results of this study were similar to another report<sup>[19]</sup>, indicating that tubular epithelial dysfunction defined by elevation of tubular injury biomarkers (β<sub>2</sub>m or α<sub>1</sub>m) was very common when CNI exposure was maintained. Additionally, as β<sub>2</sub>m is unstable in fresh urine, fewer patients were found to have β<sub>2</sub>m in the urine than α<sub>1</sub>m in this study. This problem can partly be overcome by maintaining the urine pH value (by adding basic buffer to the urine) to prevent the degradation of β<sub>2</sub>m. This study suggested that urinary β<sub>2</sub>m and α<sub>1</sub>m are sensitive urinary markers for detecting CNI-related nephrotoxicity in liver transplant recipients.

In conclusion, monitoring of patients with SCr requires a higher laboratory effort and the use of gender-specific cut-off values. Measurement of microproteinuria is easily available, non-expensive, and convenient in daily clinical practice. The urinary β<sub>2</sub>m or α<sub>1</sub>m can be used as an early, sensitive and simple diagnostic indicator for detecting CNI-related renal dysfunction. Furthermore, it should be used as the screening method after liver transplantation to prevent the progressive deterioration of subclinical renal dysfunction.

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## COMMENTS

### Background

Deterioration of renal function with calcineurin inhibitor (CNI) therapy has been widely reported in liver transplant recipients. Monitoring of renal function, however, is still dependent on somewhat old technologies: serum creatinine (SCr), blood urea nitrogen (BUN), total urine output. Whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients has not been unequivocally addressed.

### Research frontiers

Measurement of SCR, BUN, and total urine output are particularly problematic, as they do not have sufficient specificity, sensitivity, or accuracy to allow appropriate and timely prevention of the deterioration of renal function. In this field, the research goal is to identify more sensitive indicators in the early diagnosis of CNI-related nephrotoxicity in liver transplant recipients.

### Innovations and breakthroughs

Recent reports have highlighted that nephrotoxicity of CNIs contributes to renal function deterioration in liver transplant recipients. This is the first study to investigate whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients.

### Applications

By using  $\beta$ 2-microglobulin ( $\beta$ 2m) and  $\alpha$ 1-microglobulin ( $\alpha$ 1m) as markers for early diagnosis of CNI-related nephrotoxicity, this study may present a future screening method for preventing the progression of CNI-related renal dysfunction in liver transplant recipients.

### Terminology

Microproteinuria: it is a hallmark of the early changes in glomerular and proximal tubular function and includes  $\alpha$ 1m,  $\beta$ 2m, immunoglobulin, microalbumin and transferrin.

### Peer review

Microproteinuria was studied in these study groups at entry and at subsequent follow-up visits and was correlated with glomerular filtration rate. It revealed that microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients. This manuscript is very interesting and may present a future screening method to prevent the progression of CNI-related subclinical renal dysfunction after liver transplantation.

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