의학석사 학위논문

Urinary Neutrophil Gelatinase-Associated Lipocalin as a Predictive Biomarker for the Progression of Renal Injury in Liver Transplant Recipients using Calcineurin Inhibitors

간이식 후 칼시뉴린 억제제 복용환자에서 신손상 예측 인자로서의 소변 Neutrophil Gelatinase-Associated Lipocalin(NGAL)의 유용성

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ABSTRACT

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Introduction: Calcineurin inhibitors (CNI) are commonly used in liver transplant (LT) recipients, though a known side effect is nephrotoxicity. Neutrophil gelatinase-associated lipocalin (NGAL) is a marker that is expressed in the kidneys after renal ischemia. We sought to evaluate the feasibility of NGAL as an early predictor of renal impairment in patients under CNI therapy.

Methods: Urine samples were obtained cross-sectionally from LT patients who visited the outpatient clinic from February 2016 to June 2016. Glomerular filtration rate (GFR) at the time of urine sampling was compared with that at 5-7 months later. Patients were divided into 3 groups according to the GFR at the time of urine sampling and the severity of renal dysfunction ('Normal' [GFR: > 90 mL/min/1.73 m^2], 'Mild' [GFR: 60-89 mL/min/1.73 m^2], 'Moderate' [GFR:

30-59 mL/min/1.73 m^2]). They were then further divided into 2 groups according to urinary NGAL level ('NGAL-High' [urinary NGAL > 25 ng/mL], 'NGAL-Low' [urinary NGAL < 25 ng/mL]). 'Progression of renal injury' was defined as a decrease in GFR greater than 5 mL/min/1.73 m^2 in the 'Mild' or 'Moderate' groups, or if the patient was shifted to either the 'Mild' or 'Moderate' group from the 'Normal' group during the follow-up period.

Results: Fifty-one patients were enrolled in this study. Mean NGAL level was higher in the 'Moderate' group than in the 'Normal' and 'Mild' groups (18.38 ± 14.31 vs. 7.74 ± 8.13, $p < 0.01$). A proportion of 'NGAL-High' was also significantly higher in the 'Moderate' group than in the 'Mild' group (40% vs. 5%, $p = 0.03$). In the 'Mild' and 'Moderate' groups, 'NGAL-High' was shown to be a significant risk factor for 'Progression of renal injury' after follow-up by multivariate analysis (hazard ratio: 72.44; confidence interval : 1.283-4088.25), p=0.04).

Conclusion: Increased NGAL can be a marker of low GFR, as well as a predictor of renal injury after short-term follow-up in patients with mild to moderate renal impairment.

Keywords: Renal impairment, Immunosuppressants, NGAL, Liver transplant

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INTRODUCTION

Calcineurin inhibitors (CNI) are the most commonly used immunosuppressant in liver transplant (LT) recipients. However, one of the known side effects of CNI is nephrotoxicity. The incidence of acute kidney injury (AKI) has been reported to be about 5-50% in patients under CNI therapy. The incidence of end-stage renal disease in this setting has been reported to be about 9.5%.(1-3) CNI causes vasoconstriction of the afferent and efferent glomerular arterioles and reduction of blood flow, and shows a dose-dependent response. Improvement of renal function can be achieved by dose reduction in more than 50% of patients, and the use of alternative immunosuppressants can help prevent renal impairment.(2, 4, 5)

Creatinine is a commonly used marker for kidney injury. However, several other early biomarkers involving specific nephron segments reflect kidney injury before the rise of creatinine (Figure 1). Neutrophil gelatinase-associated lipocalin (NGAL) is one of those markers that is upregulated and expressed from the proximal and distal tubules after renal ischemia. NGAL has been reported to be a faster predictor of AKI than creatinine in patients who have undergone heart surgery with admission to the intensive care unit (ICU).(6, 7). Tacrolimus, a CNI, is known to cause toxicity of proximal and distal tubules (Figure 1).(8) However, there are few reports on the significance of NGAL in the LT setting.

We hypothesized that NGAL could be an early biomarker for renal impairment in LT recipients under CNI therapy. In this study, we investigated the relationship between urinary NGAL and the changes in glomerular filtration rate (GFR) approximately 6 months after LT.

MATERIALS AND METHODS

Patient and GFR calculation

From February of 2016 to June of 2016, urine samples were collected from patients who visited the Seoul National University Hospital outpatient clinic after liver transplantation and who consented to participate in this study. The patients were selected randomly regardless of the transplantation type, age, gender, GFR, immunosuppressant use, and follow-up periods. Exclusion criteria included pediatric patients, patients with GFR less than 30 mL/min/1.73 m^2, and patients needing renal replacement therapy. GFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation.(9) Five to 7 months later, the patients revisited the outpatient clinic and the GFR as well as the difference from the previous GFR were calculated. Other clinical data were collected from the medical records. The Institutional Review Board approved the study protocol (1512-123-729) and informed consent was obtained. This study was performed in accordance with the Helsinki Declaration of 2000.

Urine analysis

Spot urine samples were centrifuged at 2500 rpm for 10 minutes. Supernatant was removed and aliquots were stored at -80 ºC at the human resource bank and subsequently thawed for the enzyme-linked immunosorbent assays (ELISA) test. The NGAL ELISA kit (Human Lipocalin-2/NGAL

Quantikine ELISA kit (DLCN20), R&D systems, MN, USA) was used to obtain NGAL measurements.

Patient group and NGAL level

The patients were divided into 3 groups according to their GFR at the time of urine sampling, and the severity of renal function was classified according to the categorical values of the Kidney Disease Outcomes Quality Initiative (KDOQI) Chronic Kidney Disease (CKD) classification: 'Normal' (GFR: > 90 mL/min/1.73 m^2); 'Mild' (GFR: 60-89 mL/min/1.73 m^2); and 'Moderate' (GFR: 30-59 mL/min/1.73 m^2). The patients were then further divided into 2 groups according to urinary NGAL level. 'NGAL High' was defined as NGAL > 25 ng/mL and 'NGAL Low' was defined as NGAL < 25 ng/mL.

'Progression of renal injury' as an outcome

We defined 'progression of renal injury' (PRI) as a decrease in GFR of more than 5 mL/min/1.73 m^2 after a follow-up period of 5-7 months in the 'Mild' to 'Moderate' group, or if a patient was shifted to the 'Mild' or 'Moderate group' from the 'Normal' group. Since the KDOQI recommends that the upper reporting limit be placed at 90 mL/min/1.73 m^2, all values above this threshold must be referred to as > 90 mL/min/1.73 m^2.

Risk factor analysis

The potential risk factors for PRI during follow-up were investigated. The factors included age, gender, type of transplantation, hepatocellular carcinoma, underlying liver disease, trough level of tacrolimus, follow-up period after LT, spot urinary NGAL level, and GFR at the time of urine sample.

Statistical Analysis

Spearman's correlations were used for the correlations between urine biomarkers and clinical data, including GFR, GFR differences, and immunosuppressant trough level. One-way analysis of variance test and Tukey's range test as a post hoc analysis were performed for comparison of the 3 groups. Chi-square tests and Student's t-test were used according to the type of variables, and multiple linear regression analysis was used to adjust the clinical factors. Chi-square test and multiple linear regression analysis were performed to determine the significant cut-off value of NGAL.

Immunosuppression and follow-up

Data on the patients' current immunosuppressant regimens were collected retrospectively. Generally, the immunosuppression regimens are comprised of basiliximab (an interleukin-2 receptor antagonist) induction, plus a triple regimen including CNI, mycophenolate mofetil, and a steroid. The steroid was

tapered off within 3 to 6 months. The tacrolimus and cyclosporine doses were adjusted according to individual clinical need, with target whole blood trough levels around 8-12 ng/mL and 200-300 ng/mL, respectively, for the first month after LT, followed by 5-8 ng/mL and 100-200 ng/mL thereafter.

Outpatient follow-up was usually conducted once a week for the first month after discharge, then monthly for a year, and then gradually lengthened to every 3 or 4 months, with additional visits as clinically necessary. A complete laboratory investigation, including liver function tests and blood CNI trough level measurement, was conducted at each follow-up.

RESULTS

Demographics

A total of 51 patients were enrolled in the study. The demographics of each group are described in Table 1. The mean postoperative period was 32.7±25.3 (range: 4-130) months and there was no difference between the groups. The 'Moderate' group was significantly older with a greater male proportion than the 'Normal' group. Preoperative GFR, GFR at the time of urine sampling, and GFR after follow-up were lower in the 'Mild' and 'Moderate' groups than that in the 'Normal' group. Mean NGAL level was significantly higher in the 'Moderate' group than in the 'Mild' group. All patients took CNI and 3 patients were also taking cyclosporine. However, there was no difference in the type of immunosuppressant and tacrolimus trough level between the 3 groups. PRI occurred in 11 patients (21.56%) overall.

NGAL level as a marker for moderate renal impairment.

Urinary NGAL level was shown to be a marker for moderate renal impairment, but not for mild renal impairment, as the mean NGAL level was significantly higher in the 'Moderate group'. Moreover, the proportion of 'NGAL High' was significantly higher in the 'Moderate' group (Table 1). However, there was no difference between the 'Normal' and 'Mild' groups in terms of NGAL level. Furthermore, NGAL showed a negative correlation with

GFR (r=-0.333, *p*=0.036) in patients in the ‘Mild’ and ‘Moderate’ groups (Figure 2).

NGAL as a predictor of PRI after short-term follow-up

A total of 11 patients (21.56%) showed PRI during the follow-up period. There was no significance in the proportion of PRI between the 3 groups, and there was no significant risk factor for PRI in univariate and multivariate analyses. However, ‘NGAL High’ was the single significant risk factor in multivariate analysis when cases were limited to ‘Mild’ or ‘Moderate’ (hazard ratio: 72.435; confidence interval: 1.283-4088.258, *p*=0.037) (Table 2). The ‘Normal’ group was not included in this analysis because no patients in the ‘Normal’ group were categorized as ‘NGAL High’. In the ‘Mild’ and ‘Moderate’ groups (n=40), the number of patients with ‘NGAL High’ was 6 (15%), and PRI occurred in 3 patients (50%) among the 6 ‘NGAL High’ patients. Therefore, the sensitivity and specificity of ‘NGAL high’ for PRI was 50% and 82.4%, respectively.

Table 1. . Patient characteristics according to the GFR at the time of urine sampling

['Normal' (GFR, >90 ml/min/1.73m^2), 'Mild' (GFR: 60-89 ml/min/1.73m^2), 'Moderate' (GFR: 30-59 ml/min/1.73m^2), 'NGAL High'(Urinary NGAL > 25ng/ml)]

	'Normal' (n=11)	'Mild' (n=30)	'Moderate' (n=10)	*p* value
Age	48.45 ± 10.01*	58.83 ± 7.46	61.5 ± 6.9*	0.001
	(34-66)	(42-71)	(53-70)	0.001*
Male (%)	6 (54.5%)*	23 (76.7%)	10 (100%)*	0.049
				0.001*
LDLT	9 (81.8%)	22 (73.3%)	6 (60%)	0.529
HCC	7 (63.6%)	19 (63.3%)	6 (60%)	0.98
Underlying liver disease				0.304
HBV (%)	9 (81.8%)	19 (63.3%)	5 (50%)	
Others (HCV, alcoholic, Fulminant, autoimmune, cryptogenic)	2 (18.2%)	11 (36.7%)	5 (50%)	
Immunosuppressant				NA
Tacrolimus	10 (90.9%)	22 (73.3%)	7 (70%)	
Tacrolimus + mTORi	1 (9.1%)	8 (26.7%)	1 (10%)	
Cyclosporin A	0	0	2 (20%)	
MDRD GFR (ml/min/1.73m^2)				
Preoperative GFR	130.7 ± 34.9	90.61 ± 22.85	56.38 ± 28.79	0.001
GFR at the time of urine sample	106.87 ± 9.96	75.02 ± 8.84	48.38 ± 11.08	0.001
5-7 months after sample	103.3 ± 13.85	76.79 ± 12.39	52.74 ± 14.86	0.001

Differences of GFR				
Preoperative GFR – GFR at the time of urine sample	-1.84 ± 11.64	3.91 ± 14.48	5.22 ± 12.72	0.365
GFR at the time of urine sample – 5-7month after urine sample	3.57 ± 10.46	-1.73 ± 9.02	-4.36 ± 9.35	0.142
The number of patient with "Progression of renal injury"	2 (18.18%)	7 (23.3%)	2(20%)	0.696
Post-operative period after liver transplatation (months)	30.73 ± 25.75	33.27 ± 26.9	24 ± 24.7	0.630
Tacrolimus level (ng/ml)	5.0 ± 1.85	4.69 ± 2.28	4.96 ± 2.23	0.899
Urinary NGAL (ng/ml)	10.57±8.18	7.74 ± 8.13*	18.38 ± 14.31*	0.015
	(2.48-24.5)	(2.44-39.34)	(2.84 – 38.96)	0.001*
Urinary "NGAL High"	0	2(5%)*	4(40%)*	0.026*

* Statistically significant group in post-hoc analysis (Tukey's range test) after one-way analysis of variance and *p* value.

Table 2. Multivariate analysis of factors predicting "Progression of renal injury" in patient with mild to moderate renal impairment group

Variable	Category	Hazard ratio (95% CI*)	*p* value
Age	≤ 65	1.225 (0.091–16.481)	0.878
	> 65	Reference	
Gender	Male	9.798 (0.279-344.151)	0.209
	Female	Reference	
Type of liver transplant	LDLT	Reference	0.130
	DDLT	8.539 (0.53–137.555)	
non HCC vs HCC	non HCC	Reference	0.128
	HCC	4.913 (0.482-50.118)	
HBV vs Others	HBV	Reference	0.412
(HCV, alcoholic, fulminant	Others	3.09 (0.209-45.746)	
autoimmune, cryptogenic)			
Follow up period more than 1 year	≤ 1 year	2.828 (0.333-24.03)	0.341
	> 1 year	Reference	
Tacrolimus trough level (ng/ml)		1.122 (0.683-1.844)	0.649
Urinary NGAL (ng/ml)	≤ 25 'NGAL low'	Reference	0.037

	> 25	‘NGAL high’	72.435 (1.283-4088.258)	
Patient GFR group	Mild		18.956 (0.722-497.861)	0.078
	Moderate		Reference	

*CI confidence interval.

Figure 1. The utility of biomarkers to detect injury to specific nephron segment and drugs that elicit site specific toxicity in the kidney. (8) Ref: Next-generation biomarkers for detecting kidney toxicity by Bonventre JV, et al. Nature biotechnology. 2010;289:139-57

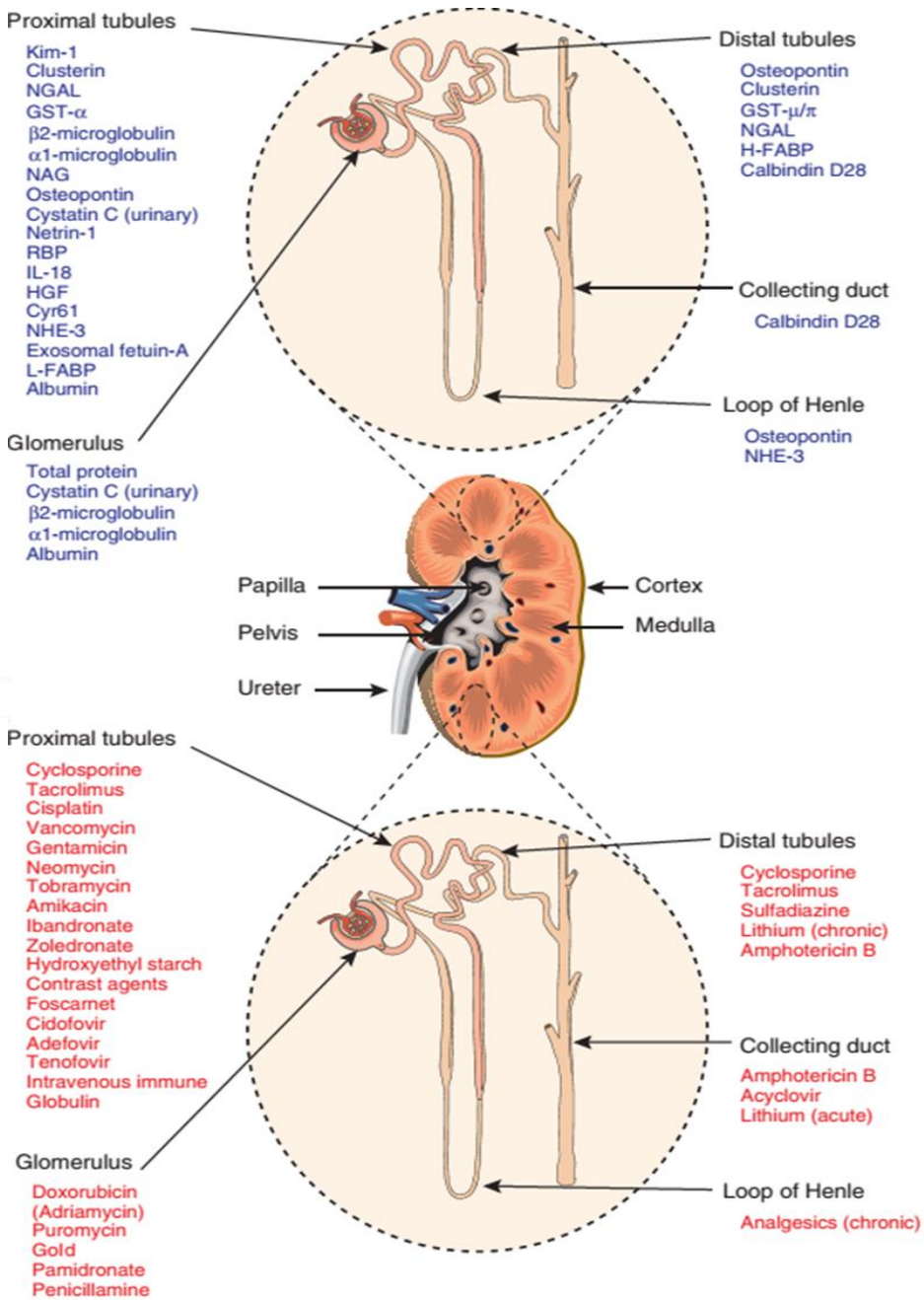


Figure 2. Scatter plot between urinary NGAL and GFR at the time of urine sampling in mild to moderate renal impairment group. (r=-0.333, *p*=0.036)

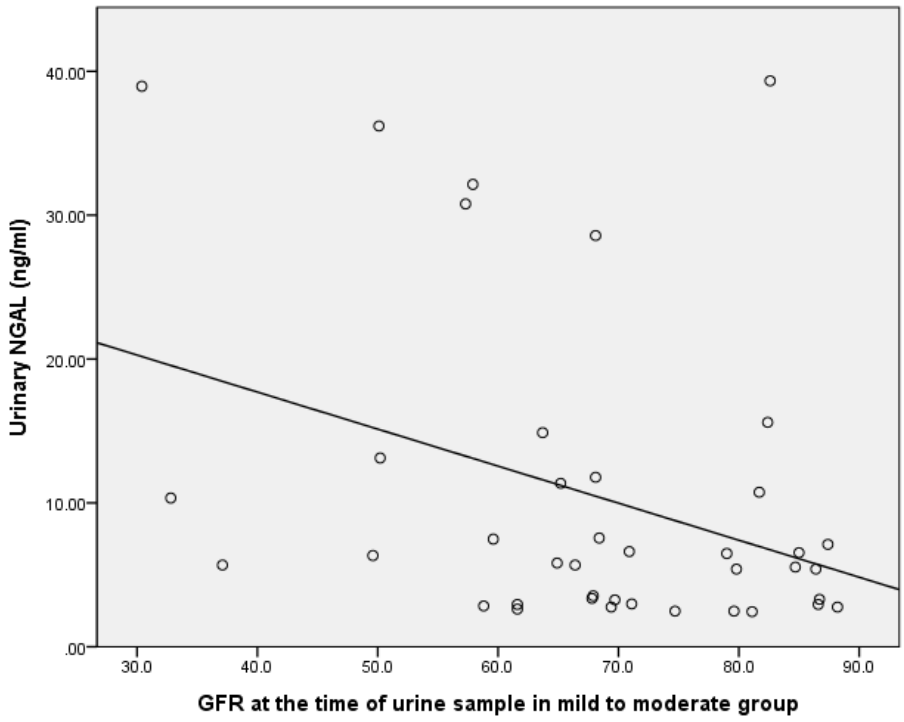


Figure 3. The structures of NGAL

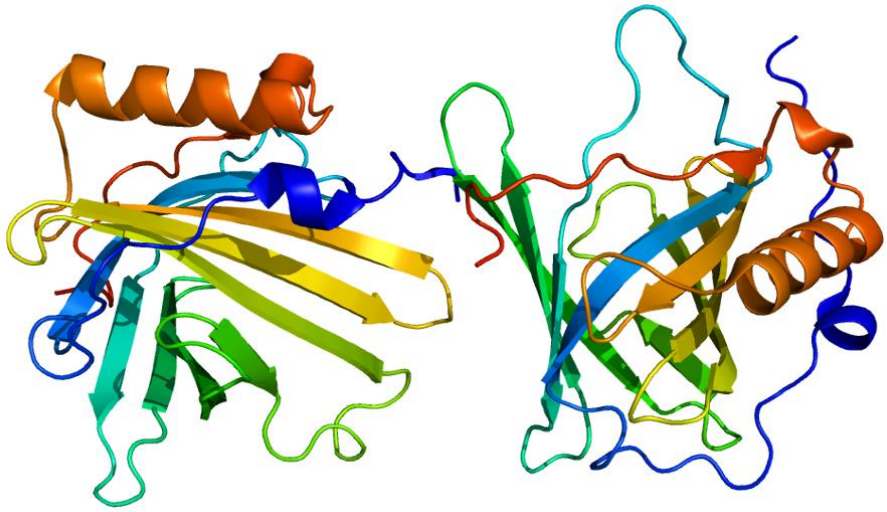
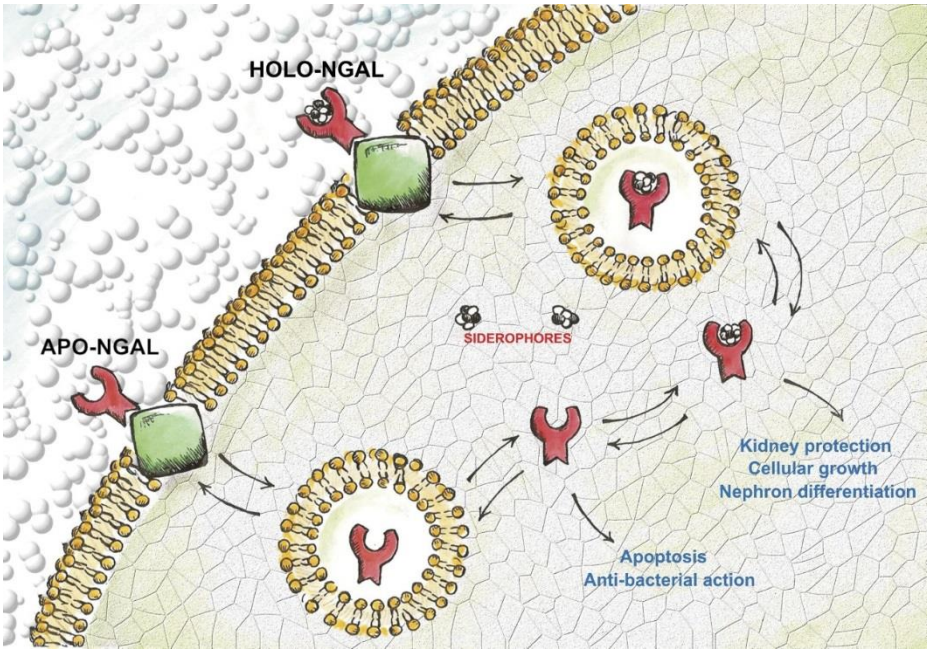


Figure 4. Schematic of NGAL cellular turnover and opposite effect of different complex with iron-siderophores.(15) Ref: NGAL as a marker of kidney damage by Bonventre JV, et al. American journal of kidney disease. 2008;52(3):595-605



DISCUSSION

The CNI has been widely used as an important immunosuppressant after organ transplantation to prevent allograft rejection. However, it has been established that long-term use of CNI is an important risk factor for nephrotoxicity.(1, 10) Many previous studies showed that a dose reduction of CNI resulted in the recovery of renal function. Indeed, Konberg et al. stated that the tapering of CNI with the addition of mycophenolate mofetil could result in not only a survival benefit in patients with renal impairment, but also improved renal function.(4, 5) Therefore, early recognition of the consequences of immunosuppression at the time of early stage renal damage may attenuate further injury, if simple, reliable markers for PRI can be used. Notably, levels of creatinine and blood urea nitrogen, the markers typically used to ascertain kidney function, can appear normal until the kidney is significantly damaged. In addition, the predictability of these markers can be decreased in patients with less muscle mass. As a result, investigations of alternative early biomarkers have been conducted or are in progress (Figure 1). (8)

One of these early biomarkers is NGAL. NGAL was first described as a protein in neutrophils with MMP-9(11) and as a member of the lipocalin protein family. It is made up of 20 small, extracellularly-secreted proteins and is a protease-resistant polypeptide chain of 178 amino acids with a molecular mass of 25 kDa (Figure 3). Gene expression is demonstrated in various organs, such as the uterus, prostate, lung, trachea, stomach, and colon, with the highest

expression in kidney tissue (12) The NGAL gene is regulated by the innate immune response (13) that prevents the growth of the bacterial strains that depend on siderophores for their iron supply. Initially, it was identified as bound to gelatinase in specific granules of the neutrophils, but NGAL may also be induced in epithelial cells in the setting of inflammation or malignancy. It is stable in the urine and freely filterable through the glomerulus. It is known to have a protective effect when there is ischemic tubular injury to the kidney and it has been known to be an important factor in the maintenance of tubular integrity in mouse models. (14) The cellular activities of NGAL are tightly influenced by bonds with specific surface receptors. After interaction with these receptors, NGAL is internalized inside the cell as a protein alone or in a complex form with iron-binding siderophores. These 2 forms have completely opposite effects (Figure 4) (15), which may explain the way that NGAL exerts strong antibacterial properties and, under particular conditions, can promote cellular apoptosis.

NGAL has been reported to be a useful marker of renal damage in the ICU. In these studies, the average sensitivity and specificity of NGAL measured 1 to 3 days prior to AKI diagnosis was 73-80%. (6, 7, 16, 17) In previous studies, the cutoff value of NGAL for predicting AKI was shown to have a wide range, from 25 to 61 ng/mL, and a significant AKI prediction time of within 7 days. The sensitivity and specificity were 73-100% and 45-100%, respectively, for contrast-induced nephropathy in cases of where urine NGAL was greater than

52.4-100 ng/mL. AKI developed in all of these cases within 7 days. There are also reports to show that NGAL level is associated with the severity of kidney dysfunction. Higher cystic growth and decreased GFR was associated with higher urine and plasma NGAL in patients with autosomal dominant polycystic kidney disease (18). Another study in patients with chronic glomerular nephritis demonstrated that mean urinary NGAL concentrations were higher in CKD patients. (378.28 ± 111.13 μg/L vs. 7.38 ± 3.26 μg/L in controls; p=0.01). NGAL concentrations were significantly correlated with serum creatinine concentrations (r=0.588, p-value=0.02), GFR (r=-0.528, p-value=0.04), and proteinuria (r=0.294, p-value=0.01) (19).

In this study, we hypothesized that a similar predictive feasibility of NGAL can be seen in the LT setting. We found that NGAL level was not different between the normal and mild renal impairment groups, but that the level was higher in the moderate group than in the mild renal impairment group. That means that NGAL level can be elevated in patients with relatively low GFR, and that "NGAL High" can be a predictor of future renal impairment in patients under CNI therapy, especially those with mild to moderate renal impairment. The patients' GFR group was not a significant factor for PRI in multivariate analysis. If NGAL level in the spot urine sample is high even in the context of normal or mildly elevated creatinine, we have to consider a protective strategy such as reducing or eliminating CNI therapy or other nephrotoxic medication in order to stop ongoing renal damage.

Patients were divided into 3 groups according to the categorical values for CKD per KDOQI guidelines. For the diagnosis of CKD, renal damage should be proven by objective criteria such as pathological abnormalities or markers of damage. However, in the present study, CKD was not proven by biopsy or urine abnormalities; instead, we used categorical variables to indicate CKD because the progression of CNI-induced renal injury occurs gradually in most transplant recipients. Previous studies focused on AKI according to the RIFLE criteria, which are based on clinical features and serum creatinine level, whereas we focused on the progression of renal injury as reflected by even minor changes. We identified the occurrence of PRI when the patient's GFR decreased by more than 5 mL/min/1.73 m^2 in order to avoid taking into account the minute changes in GFR that can occur outside of the context of kidney damage. According to the MDRD equation, when creatinine increases by 0.1 mg/dL, the GFR decreases about 5-10 mL/min/1.73 m^2 in patients with GFR less than 90 mL/min/1.73 m^2. Therefore, a GFR of 5 mL may be significant, but this needs to be validated in further sequential and long-term follow-up studies.

NGAL concentration measured in a spot urine sample can vary according to dehydration status and kidney function. In dehydrated conditions, NGAL level will be elevated compared to that in hydrated conditions. (20) Therefore, adjusted NGAL (NGAL level divided by urine creatinine) was suggested in several studies. However, there are advantages and disadvantages of using

adjusted NGAL. In patients with low GFR, NGAL level will appear higher since the excretion of creatinine is low, and urine creatinine is itself affected by hydration status and the patient's muscle volume. (20) Therefore, in this study, we collected urine samples from stable patients in the outpatient clinic and we assumed that the variations in hydration status were very minimal in this cohort.

Urine sampling is very convenient for both patients and physicians and carries no procedure-related risks. Therefore, this study suggests that the regular evaluation of NGAL in spot urine sampling during follow-up might be a simple and non-invasive tool to determine the optimal strategy to reduce further renal damage.

However, this study has some limitations. The changes in GFR were observed at one instance about 6 months after initial urine sampling. Renal function trends must be observed over a long-term period and at more time intervals. Furthermore, other important risk factors such as duration of exposure to CNI, combined immunosuppressants, and concomitant nephrotoxic medications were not considered in this study. Therefore, a future prospective observational study beginning just after LT with regular, long-term follow-up is necessary.

CONCLUSION

To conclude, urinary NGAL >25 ng/mL can be a marker for moderate renal impairment (GFR: 30-59 mL/min/1.73 m^2) and a predictor of PRI at six months later in patients with mild to moderate renal impairment (GFR: 30-89 mL/min/1.73 m^2). Renal protection strategies including CNI dose reduction should be considered in mild to moderate renal impaired LT recipients with NGAL levels > 25 ng/mL in spot urine sampling.

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국문 초록

서론: 칼시뉴린 억제제는 간이식후 가장 많이 쓰이는 면역억제제이나 부작용으로 인한 신손상의 위험이 높다. 소변 Neutrophil gelatinase-associated lipocalin (NGAL) 은 신장의 허혈성 손상시 증가하는 표지자로 알려져 있으며, 신손상이 발생시 혈청 크레아티닌보다 먼저 상승하는것으로 알려져 있어, 칼시뉴린 억제제 사용환자에서 NGAL 이 신손상의 표지자로서의 역할을 알아보기로 하였다.

방법: 간이식을 시행 받고 2016 년 2 월 부터 2016 년 6 월까지 서울대학교 병원 외래에 내원한 환자를 대상으로 소변을 수집하였다. 소변 체취 당시 사구체 여과율과 5 - 7 개월 후 사구체 여과을 비교 하였다. 환자는 내원 당시의 사구체 여과율을 기준으로 3 개의 그룹으로 분리하였다. ('Normal' [GFR: > 90 mL/min/1.73 m^2], 'Mild' [GFR: 60-89 mL/min/1.73 m^2], 'Moderate' [GFR: 30-59 mL/min/1.73 m^2]). 또한 소변 NGAL level 에 따라 두 그룹으로 분리 하였다. ('NGAL-High' [urinary NGAL > 25 ng/mL], 'NGAL-Low' [urinary NGAL < 25 ng/mL]). 환자의 신손상의 기준을 'Progression of renal injury' 로 하고 그 기준을 추적 관찰 기간인 6 개월 동안 사구체 여과율이 5 mL/min/1.73 m^2 이상 감소 하거나, 환자의 그룹이 'Normal' 그룹에서 'Mild' 또는 'Moderate' 로 감소한 경우로 하였다.

결과: 총 51 명의 환자가 이 연구에 포함되었다. 평균 NGAL 수치는 ‘Mild’와 ‘Moderate’ 그룹에 비해 ‘Moderate’ 그룹에서 높았다 (18.38 ± 14.31 vs. 7.74 ± 8.13, $p < 0.01$). ‘NGAL-high’ 의 비율 역시 ‘Moderate’ 그룹에서 ‘Mild’ 그룹에 비해 높았다 (40% vs. 5%, $p = 0.03$). ‘Mild’ 와 ‘Moderate’ 그룹에서의 다변량 분석시 ‘NGAL-High’는 경우 ‘Progression of renal injury’ 유의한 인자였다 (hazard ratio: 72.44; confidence interval : 1.283-4088.25), p=0.04).

결론:. 소변의 NGAL 의 상승은 감소된 GFR 의 표지자이며, 또한 ‘Mild’, ‘Moderate’ 의 신손상 저하를 갖는 칼시뉴린 억제제 사용 간이식 환자에서 단기간의 추적 관찰시 신손상 저하를 예측 할수 있을 것으로 보인다.

주요어: 신장 기능 악화, 면역 억제제, NGAL, 간 이식편, 간이식

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