Serum neutrophil gelatinase-associated lipocalin (NGAL) and interleukin-18 (IL-18) as predictive biomarkers for delayed graft function after kidney transplantation

Eun Young Lee

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Hyon-Suk Kim

The Master's Thesis
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Eun Young Lee

June 2011

This certifies that the Master's Thesis
of Eun Young Lee is approved.

Thesis Supervisor

Thesis Committee Member#1

Thesis Committee Member#2

The Graduate School
Yonsei University

June 2011

ACKNOWLEDGEMENTS

This thesis would not have been possible without my supervisor, professor Hyon-Suk Kim, who has been a great mentor and always encouraged me to achieve my full potential. It is an honor for me to show my most sincere gratitude to professor Myoung Soo Kim, who continuously paid attention to my subject. I owe my deep gratitude to doctor Yongjung Park whose timely supported in numerous ways and to the laboratory assistants at the clinical immunology, Yonsei University College of Medicine for the technical advice of experiment. With all my heart, I appreciate Mr. Soo Kyoung Suk, who has fully supported me.

Lastly, I offer my regards and blessings to my mother and father.

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ABSTRACT

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Eun Young Lee

Department of Medicine
The Graduate School, Yonsei University

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Early biomarkers for acute kidney injury after kidney transplantation has been studied because delayed graft function (DGF) is associated with increased risk of acute rejection and graft loss. We investigated the usefulness of serum neutrophil gelatinase-associated lipocalin (NGAL) and Interleukin-18 (IL-18) for the prediction of DGF after kidney transplantation. Fifty-nine kidney transplant recipients were included and they were separated into DGF and immediate graft function (IGF) groups. Serum samples were serially collected on the preoperative day as well as days 1, 5, and 14 after transplantation. NGAL and IL-18 were measured using enzyme-linked immunosorbent assays. After transplantation, serum levels of NGAL were significantly higher at any time in patients with DGF compared to those with IGF. Serum concentrations of IL-18 were not different between both groups. The ROC-AUC values of NGAL, IL-18, and creatinine on day 1 for the discrimination of DGF from IGF were 0.86, 0.63, and 0.65. On POD1, the sensitivities of NGAL and creatinine were respectively 78.6%, and 50.0% at 77.8% specificity, and the AUC values for any combinations including NGAL and that for NGAL alone were higher than that of creatinine. Serum NGAL is an early,

sensitive marker of graft dysfunction in kidney transplantation, while serum IL-18 showed limited predictive values. Therefore, adding serum NGAL for follow-up laboratory tests not only would facilitate early diagnosis of DGF but also could aid prompt decision making.

Key words: Neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), kidney transplantation, delayed graft function

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I. INTRODUCTION

Kidney transplantation is the renal replacement therapy of choice for most patients with end-stage renal disease. Although kidney transplantation may be successful, impaired kidney function could arise. The frequency of delayed graft function (DGF) varies from 4 to 10% in living donor kidney transplants and 5 to 50% in deceased donor transplants.[1] DGF can be usually caused from ischemic injury before and during kidney transplantation.[2] It may also be further aggravated by the reperfusion syndrome,[3] and is associated with the increased risk of graft loss and acute rejection.[4] Therefore, early assessment of kidney function following renal transplantation is crucial for predicting graft survival and early decision making. Unfortunately, serum creatinine concentration is not a reliable parameter for describing acute kidney injury (AKI).[5] Creatinine can be influenced by non-renal factors including muscle mass of the subjects, and its serum levels may not be increased until 50% of renal functions are lost. Moreover, creatinine does not reflect the degree of kidney function until a steady state has been reached, which may take

several days.[6] Accordingly, some translational studies have evaluated serum and urine biomarkers for the assessment of kidney function.[7]

Among those biomarkers, neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin family,[8] is expressed by neutrophil as well as tissues of kidneys, prostate, and the epithelia of the respiratory and alimentary tracts.[9] In recent publications, NGAL was detected in the blood and urine after AKI. Interleukin-18 (IL-18), a member of cytokines, has been known as a urinary marker of kidney injuries.[10] However, usefulness of serum IL-18 as a marker for detecting AKI is still controversial.[11-13]

In this study, we aimed to assess whether DGF after kidney transplantation can be predicted by NGAL and IL-18 levels in the serum samples collected serially from the recipients before and after transplantation.

II. MATERIALS AND METHODS

1. Study subjects

Of the 333 patients who underwent kidney transplantation from March 2006 to December 2008 in Transplantation Center of Severance Hospital, fifty-nine recipients were retrospectively included in this study after subjects with the following characteristics were excluded to eliminate various confounding factors; recipients with an HLA-identical in living related donor, mismatches of 6 HLA antigens in living related or deceased donor, recipients younger than 20 years of age or older than 55 years of age, donors younger than 20 years of age or older than 55

years of age, negative conversion of lymphocyte cross-matching report, positive pre/post-transplant hepatitis B or C positive recipients, diabetic recipients, and follow-up loss recipients.

Fifty-nine patients were separated into two groups: a DGF group (n=14) and immediate graft function (IGF) group (n=45). The patients with DGF had experience of dialysis within 1 week after transplantation or pathologic findings such as acute tubular necrosis of kidney graft.[14]

Serum samples of the subjects were collected on the preoperative day, the first postoperative day (POD 1), the fifth postoperative day (POD 5), and the fourteenth postoperative day (POD 14). Venous blood from all subjects was drawn in the morning after an overnight fast, and collected into Vacuette serum separate tube (Greiner-Bio-One, Kremsműnster, Austria). After sera were separated, all the samples were aliquotted and stored at -80℃ before assayed.

2. Assays for NGAL, IL-18, and creatinine

Serum NGAL levels were measured using enzyme-linked immunosorbent assay (ELISA) kit (KIT 037, BioPorto Diagnostics, Gentofte, Denmark) according to the manufacturer's instructions. The micro-wells of the ELISA plate were pre-coated with a monoclonal antibody against human NGAL. Horse-radish peroxidase-conjugated anti-NGAL antibody was added to each well. In order to create a calibration curve from which the NGAL concentration could be determined, 100 μL of each undiluted NGAL-calibrator (range 0-20 ng/mL) was added. Diluted serum

samples from study subjects were added to the remaining micro-wells to measure NGAL concentrations followed by incubation at room temperature for 30 minutes. Tetramethylbenzidine (TMB) as substrate was then dispensed to the micro-wells; 15 minutes later a stop solution was added. The ELISA plate was incubated at room temperature and washed between each step as prescribed by the manufacturer. In the first step, the biotinylated antibody was bound to NGAL in the samples; in the three subsequent steps, a color reaction was generated. Finally, the ELISA reader was set at 450 nm to quantify color intensity. The NGAL concentrations in the samples were calculated from the calibration curve generated by the NGAL-calibrators and its corresponding optical density (OD).

Serum IL-18 levels were assayed by Human IL-18 ELISA kit (Medical and Biologic Laboratories, Nagoya, Japan) in accordance with the instructions given by the manufacturer. The samples and standards were incubated for 60 minutes in 96 well plates pre-coated with anti-human IL-18 monoclonal antibodies. After washing, a peroxidase-conjugated anti-human IL-18 monoclonal antibody that recognizes different epitopes of IL-18 was added. After 60 minutes of incubation and another washing, the substrate TMB/H_2O_2 was allowed to incubate. The reaction was stopped after 30 minutes by adding an acid solution (H_2SO_4); resultant ODs were measured at 450 nm wavelength using a microplate reader. The concentration of human IL-18 was calculated from a dose response curve based on reference standards.

Serum creatinine concentrations were determined with a Hitachi 7600-210 Clinical Analyzer (Hitachi High-Technologies Co., Tokyo, Japan).

3. Statistical analysis

All statistical analysis were performed by PASW statistics 18.0 (formerly SPSS Statistics, SPSS Inc., Chicago, IL, USA) and Analyse-it software Method Evaluation Edition version 2.22 (Analyse-it Software Ltd., City West Business Park, Leeds, UK). Clinical characteristics and levels of markers between the study groups were compared using the Mann-Whitney U test for continuous variables and Chi-square test for categorical variables. Receiver operating characteristics (ROC) curves were generated to compare the performances of NGAL, IL-18, and creatinine for predicting DGF. In order to evaluate the performances of the markers with same evaluation basis, cut-off levels with a fixed specificity were determined from the results of the ROC analysis. Logistic regression analysis was performed to calculate the predicted probability values for the combinations of each markers with the presence of DGF as the binary dependent variable and the levels of a markers as the predictor variable. These values were used to estimate the ROC-area under the curve (AUC) for the respective markers' combinations. A *p*-value of less than 0.05 was regarded as statistically significant.

III. RESULTS

1. Characteristics and levels of NGAL, IL-18, and creatinine in the patient groups

Table 1 demonstrates clinical characteristics in recipients with DGF or IGF. Clinical parameters excluding age were not significantly different between the two groups (Table 1).

Table 1. Summary of clinical characteristics according to the study groups

Parameters	IGF	(n=45)	DGF	(n=14)	*p*-value
Age, mean (SD)	39.4	(9.8)	46.8	(6.7)	0.0097
Male, n (%)	29	(64.4)	6	(42.9)	0.1510
Deceased donor, n (%)	21	(46.7)	10	(71.4)	0.1051
Cause of ESRD					
Hypertention, n (%)	6	(13.3)	5	(35.7)	0.0604
Chronic glomerulonephritis, n (%)	10	(22.2)	1	(7.1)	0.2058
IgA nephropathy, n (%)	7	(15.6)	0	(0.0)	0.1160
Polycystic kidney, n (%)	2	(4.4)	1	(7.1)	0.6881
FSGS, n (%)	4	(8.9)	0	(0.0)	0.2479
Other, n (%)	16	(35.6)	7	(50.0)	0.3331

Abbreviations: SD, standard deviation; IGF, immediate graft function; DGF, delayed graft function; ESRD, end stage renal disease; FSGS, focal segmental glomerulosclerosis

At all times measured after transplantation, serum NGAL levels were significantly higher in patients with DGF compared to those with IGF. The median serum IL-18 levels were also higher in the DGF group, although statistically significant differences were not found (Table 2). Serum creatinine was not different between the two groups on the POD 1 (p=0.1030), but was higher in DGF group after day 5 from transplantation (p=0.0002).

Table 2. Median serum NGAL, IL-18, and creatinine levels according to the graft functions after transplantation

Reagent	Time	IGF	(n=45)[a]	DGF	(n=14)[a]	*p*-value
Serum NGAL (ng/mL)	Preoperative	837.7	(574.7-1141.3)	952.1	(647.1-1118.9)	0.3184
	POD 1	183.6	(145.2-232.5)	490.4	(238.3-722.6)	<0.0001
	POD 5	123.7	(93.4-178.9)	362.0	(184.5-502.1)	0.0001
	POD 14	108.1	(63.6-145.2)	194.6	(115.2-421.0)	0.0019
Serum IL-18 (pg/mL)	Preoperative	358.9	(238.7-497.1)	421.6	(362.0-467.5)	0.4436
	POD 1	265.4	(162.5-376.0)	343.1	(229.6-570.9)	0.1416
	POD 5	285.3	(143.6-367.5)	344.5	(242.4-511.4)	0.0823
	POD 14	249.0	(143.7-338.8)	320.1	(204.7-388.2)	0.2616
Serum Cr (mg/dL)	Preoperative	9.8	(7.4-12.7)	11.1	(9.3-14.1)	0.2090
	POD 1	4.9	(3.2-7.5)	7.6	(4.2-9.6)	0.1030
	POD 5	1.4	(1.0-2.0)	6.1	(4.0-8.5)	0.0002
	POD 14	1.2	(1.0-1.5)	2.3	(1.6-5.6)	<0.0001

[a], Data are shown as 'median (1st to 3rd quartiles)'.

Abbreviations: IGF, immediate graft function; DGF, delayed graft function; NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin-18; POD, postoperative day; Cr, creatinine

2. ROC analysis, cut-off determination and comparison of diagnostic performances

The ROC curves at POD 1 are shown in Figure 1, and the ROC-AUC values of NGAL, IL-18, and creatinine for predicting DGF on the respective collection days are summarized in Table 3. The AUC value of NGAL at POD 1 was higher than those of IL-18 and creatinine (p=0.0414 and p=0.0213, respectively). The AUC value of NGAL was not significantly different from those of IL-18 and creatinine at POD 5 (p=0.0714 for IL-18 and p=0.9746 for creatinine) and POD14 (p=0.0761 for IL-18 and p=0.1941 for creatinine).

The sensitivities for DGF prediction and corresponding cut-off levels of NGAL, and creatinine according to specificities are shown in Table 4. For instance, the sensitivities of NGAL, and creatinine at 77.8% specificity were 78.6%, and 50.0%; the cut-off levels at these sensitivities and specificities were 233.3 ng/mL, and 7.5 mg/dL at POD 1.

Figure 1. Receiver operating characteristics curves for serum biomarkers on the first postoperative day for predicting delayed graft function.

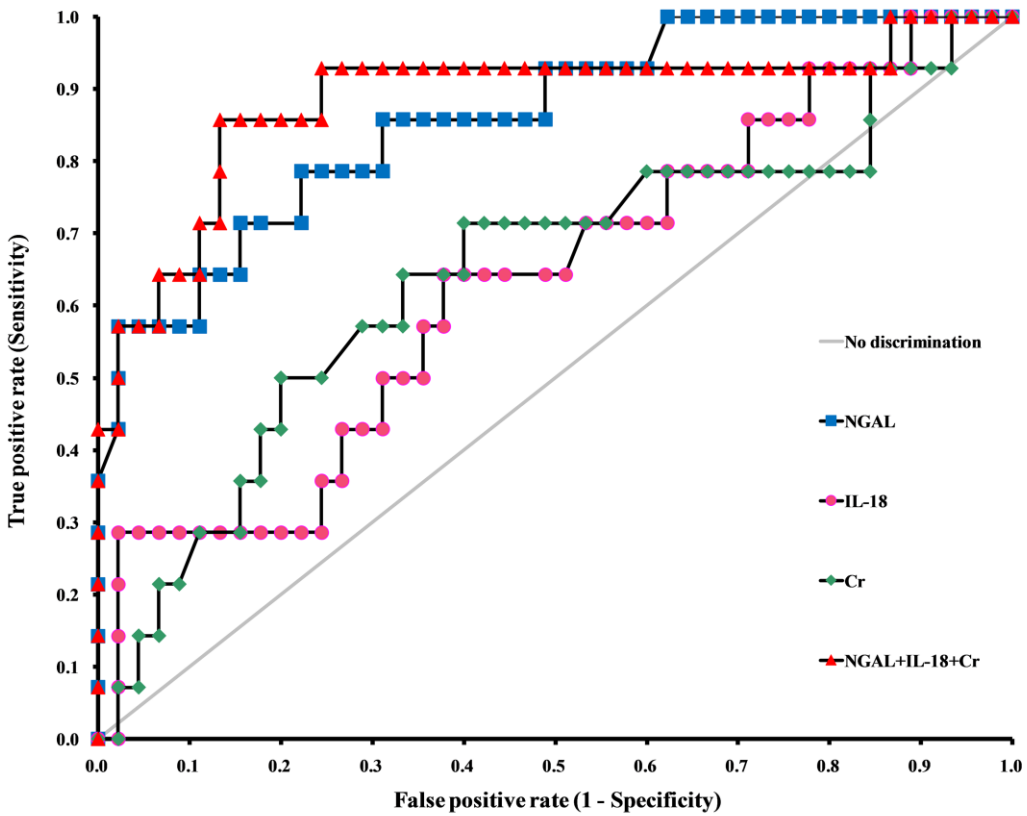

Table 3. Comparisons between AUC value of creatinine and those of the rest markers and their combinations

Combination panel	POD 1		POD 5		POD 14	
	AUC (95% CI)	*p*-value[a]	AUC (95% CI)	*p*-value[a]	AUC (95% CI)	*p*-value[a]
Cr	0.65 (0.46-0.83)	-	0.84 (0.66-1.00)	-	0.86 (0.74-0.98)	-
NGAL	0.86 (0.75-0.98)	0.0213	0.84 (0.70-0.98)	0.9746	0.78 (0.64-0.91)	0.1941
IL-18	0.63 (0.46-0.81)	0.8762	0.65 (0.48-0.83)	0.0481	0.60 (0.42-0.78)	0.0055
NGAL + IL-18	0.88 (0.79-0.98)	0.0027	0.86 (0.72-1.00)	0.6551	0.77 (0.63-0.92)	0.1649
NGAL + Cr	0.86 (0.71-1.00)	0.0888	0.89 (0.76-1.00)	0.1405	0.87 (0.75-0.98)	0.6120
IL-18 + Cr	0.64 (0.47-0.82)	0.9521	0.84 (0.67-1.00)	0.4465	0.87 (0.75-0.98)	0.2472
NGAL + IL-18 + Cr	0.89 (0.76-1.00)	0.0377	0.89 (0.75-1.00)	0.1622	0.87 (0.75-0.98)	0.8277

[a], vs the AUC values for the creatinine

Abbreviations: POD, postoperative day; CI, confidence interval; Cr, creatinine.

Table 4. Sensitivities and cut-off values of serum NGAL, and creatinine according to the specificities

Specificity (95% CI)	POD 1				POD 5			
	NGAL		Cr		NGAL		Cr	
	Cut-off[a]	Sensitivity (95% CI)[b]	Cut-off[a]	Sensitivity (95% CI)[b]	Cut-off[a]	Sensitivity (95% CI)[b]	Cut-off[a]	Sensitivity (95% CI)[b]
97.8	411.4	57.1	11.1	7.1	261.4	57.1	4.0	78.6
(88.2-99.9)		(28.9-82.3)		(0.2-33.9)		(28.9-82.3)		(49.2-95.3)
88.9	348.5	64.3	9.5	28.6	230.8	57.1	2.6	78.6
(75.9-96.3)		(35.1-87.2)		(8.4-58.1)		(28.9-82.3)		(49.2-95.3)
77.8	233.3	78.6	7.5	50.0	186.5	74.1	2.0	78.6
(62.9-88.8)		(49.2-95.3)		(23.0-77.0)		(41.9-91.6)		(49.2-95.3)
68.9	222.1	85.7	7.2	57.1	144.4	85.7	1.7	85.7
(53.4-81.8)		(57.2-98.2)		(28.9-82.3)		(57.2-98.2)		(57.2-98.2)

[a], Units are ng/mL for NGAL, and mg/dL for Cr.

[b], Units are percent.

Abbreviations: POD, postoperative day; Cr, creatinine; CI, confidence interval.

3. ROC analysis of combinations of NGAL, IL-18, and creatinine

Table 3 shows the AUC values of the individual markers or their combinations, those were compared to the AUC value of creatinine. The markers with *p*-value of less than 0.05 were regarded as having statistically different AUC values from that of creatinine. On POD 1, the AUC values for any combinations including NGAL and NGAL alone were higher than that of creatinine. On POD 5 and POD 14, the AUC values of any makers or combinations were not significantly higher than that of creatinine and the AUC values of IL-18 was even lower than that of creatinine.

4. Multivariate analysis

Multiple logistic regression was performed with the presence of DGF as the binary dependent variable and the levels of the three markers on POD 1 and age as the independent variables, because the ages between DGF and IGF groups were statistically different (p=0.0097). As a result, increased age and serum NGAL levels on POD 1 were associated with DGF (odds ratio for age=1.178, p=0.0341; odds ratio for NGAL=1.011, p=0.0021), while IL-18 and creatinine concentrations on POD 1 were not significantly related to DGF (p=0.0758 for IL-18 and p=0.2879 for creatinine).

IV. DISCUSSION

This study evaluated serum NGAL and IL-18 as new biomarkers for renal injury after kidney transplantation. NGAL has been evaluated in many studies; however, there are few studies on NGAL in serum samples. Furthermore, the usefulness of serum IL-18 for renal injury has been controversial. In our results, NGAL, IL-18, and creatinine were not significantly different between the DGF and IGF groups before kidney transplantation. These findings suggest that the kidney functions of the recipients before transplantation were not different between the two groups. On the POD 1, NGAL concentration was significantly higher in DGF group than in IGF group, while serum IL-18 and creatinine levels were not different between the two groups. DGF was able to be discriminated from IGF by creatinine levels after POD 5. These findings suggest that serum NGAL measured after transplantation can predict DGF earlier than IL-18 and creatinine. NGAL levels also showed large differences between patients with DGF and IGF on POD 1. Therefore, adding serum NGAL for follow-up laboratory tests not only would facilitate early diagnosis of DGF but also could aid prompt decision making.

ROC analyses also showed that NGAL performed better than serum creatinine at POD 1. The AUC value of NGAL at POD 1 was 0.86 (95% confidence interval [CI]=0.75 to 0.98); however, that of creatinine at POD 1 was 0.65 (95% CI=0.46 to 0.83), and those were statistically different (p=0.0213). Similar to our results, Hall et al. reported that the AUC value of urine NGAL at POD 1 for predicting dialysis

events was 0.82 (95% CI=0.72 to 0.92).[15] Irrespective of specimen type, the AUC value of NGAL seems to be similar; however, the diagnostic performances could be variable between studies due to the patients' characteristics, numbers of subjects enrolled, and the assays utilized. Therefore, further investigations with larger numbers of subjects might be necessary.

In our study, 14 patients with DGF and 45 with IGF were enrolled. Since the number of patients was small, the results of ROC analyses might have limited external validity. The optimal cut-off might be decided according to sensitivity and specificity, we compared marker's sensitivities with fixed specificity from ROC curve. The optimal cut-off for serum NGAL was 233.3 ng/mL with a sensitivity of 76.6% and a specificity of 77.8% at POD 1. The relatively low sensitivity and specificity of NGAL may result from the small number of subjects. For urine NGAL, Hall et al. reported a 77.0% sensitivity and 74.0% specificity with a cut-off of 350 ng/mL.[15] In the same study, 34 patients with DGF were included, which was larger than our study. Therefore, sensitivity and specificity at a specific cut-off might be evaluated with a larger number of subjects.

On POD 1, the markers having AUC value higher than creatinine were NGAL alone and the combinations including NGAL. The AUC value for the combination of NGAL, IL-18, and creatinine was highest among all of the combinations and individual markers. However, the AUC values for any combinations including NGAL and NGAL alone were not different from that of all three markers' combination (data was not shown). This means that NGAL alone and NGAL combined with IL-18 or creatinine would be as equally useful as the combination of

all three markers, and more useful than creatinine. On POD 5 and POD 14, the AUC values of any makers or combinations were not significantly higher than that of creatinine. Since NGAL and creatinine were able to predict renal dysfunction at POD 5 in our study, further researches with samples collected on POD 2, 3, and 4 may be necessary to estimate the predictive values for DGF of both markers on the respective days. With these values, physicians would be able to determine which test is more efficient to predict DGF on the respective days, because NGAL assays are still expensive for routine use.

IL-18 is a multifunctional cytokine that increases both innate and acquired immune responses and potentiates Th1 and Th2 reactions.[16] Urinary IL-18 is an early and accurate predictor of the need for dialysis in renal transplantation.[15,17] However, usefulness of serum IL-18 is still controversial.[11-13] In our study, serum IL-18 levels were higher in the DGF group than in IGF group after transplantation; however, statistically significant differences were not found.

We found that serum NGAL levels could predict kidney dysfunctions after transplantation. Most of the previous researches evaluated the usefulness of NGAL in urine samples, and only few studies performed with serum samples. Furthermore, in these studies, serum samples were collected only on a certain single day after the transplantation. In addition, they estimated the correlation between serum levels of NGAL and other marker (creatinine, glomerular filtration rate, high sensitivity C-reactive protein, etc.) in kidney transplantation recipients, but did not compare the predictive performances of NGAL in the renal insufficiency group to those in the group without renal insufficiency.[18,19] In our study, we analyzed serum NGAL levels

in the two groups according to graft function after kidney transplantation. Moreover, serum samples were sequentially obtained on the preoperative day as well as POD1, POD 5, and POD 14. Serum samples can be collected more easily than urine, because patients with renal dysfunctions would manifest decreased urine output. Besides, results with urinary NGAL could be unreliable in patients with hematuria after kidney transplantation due to assay interferences by hemoglobin. According to the previous study, NGAL concentrations were highly skewed towards the higher values in the presence of hemoglobin.[20]

In conclusion, serum NGAL exhibited to be an early, sensitive marker for kidney dysfunctions in renal transplantation, while serum IL-18 and creatinine showed limited value for the prediction of renal dysfunctions.

V. CONCLUSION

We investigated the usefulness of serum NGAL and IL-18 for prediction of DGF after kidney transplantation. Fifty-nine kidney transplant recipients were separated DGF group or IGF group. Serum samples were collected on the preoperative day as well as days 1, 5, and 14 after transplantation, and assayed for NGAL and IL-18. At any time after transplantation, levels of serum NGAL were significantly higher in patients with DGF compared to those with IGF. Levels of serum IL-18 were not significantly different between both groups. The ROC-AUC values and sensitivities of NGAL were better than those of IL-18 and creatinine on day 1. Serum NGAL is

an early, sensitive marker of kidney dysfunction in renal transplantation, while serum IL-18 is limited marker. Therefore, adding serum NGAL for follow up laboratory tests not only would facilitate early diagnosis of DGF but also could aid prompt decision making.

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ABSTRACT (IN KOREAN)

신이식 후 지연성 신기능 예측 표지자로서의 혈청 neutrophil gelatinase-associated lipocalin (NGAL)과 interleukin-18 (IL-18)

<지도교수: 김현숙>

연세대학교 대학원 의학과

이은영

신이식 후 지연이식편기능(delayed graft function)은 급성거부반응과 이식편 소실의 위험을 높이기 때문에, 신이식 후 급성 신손상(acute kidney injury)을 예측하기 위한 표지자에 대한 연구가 지속되었다. 본 논문에서는 신이식 후 지연이식편기능을 예측하는데 있어 혈청 neutrophil gelatinase-associate lipocalin (NGAL)과 interleukin-18 (IL-18)의 유용성을 평가하고자 하였다. 59명의 신이식 환자를 지연이식편기능군과 즉시이식편기능(immediate graft function)군으로 분류하였다. 신이식 수술 직전, 신이식 후 1일, 5일, 14일의 혈청 검체를 대상으로 NGAL과 IL-18을 측정하였다. 신이식 후 1일, 5일, 14일에 측정한 지연이식편기능군의 NGAL 농도는 즉시이식편기능군의 NGAL 농도보다 높았다. 반면 혈청 IL-18은 두 군간에 차이가 없었다. 신이식 후 1일의 NGAL, IL-18, creatinine의 ROC-AUC 값은 0.86, 0.63, 0.65였다. 신이식 후 1일, 특이도 77.8%에서 NGAL과 creatinine에 대한 민감도는 78.6%, 50.0%였고 NGAL 단독이나 NGAL을 포함한 표지자 조합의 AUC 값은 creatinine의 AUC 값보다 높았다. 혈청 NGAL은 신이식 후 신기능장애를 조기에 민감하게 예측할

수 있는 표지자로 생각되었고, 반면 혈청 IL-18은 제한적 표지자로 사료되었다. 따라서 혈청 NGAL을 추적검사에 포함시키는 것은 지연이식편기능을 조기진단할 뿐만 아니라 빠른 의사결정에도 도움이 될 것이다.

핵심되는 말: Neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18(IL-18), 신이식, 지연이식편기능