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Urinary neutrophil gelatinase associated lipocalin as a biomarker in ifosfamide induced chronic renal failure

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Abstract. – OBJECTIVE: Neutrophil gelatinase associated lipocalin (NGAL) have been used with great success in acute renal failure and in some cases in chronic nephrotoxicity. In this work, we aimed to investigate urinary NGAL as an early marker of chronic renal failure (CRF).

PATIENTS AND METHODS: We investigated urinary NGAL of 29 children treated with ifosfamide chemotherapy and compared them with those of 12 healthy children. Urinary β_2 microglobulin, serum cystatin C, and creatinine clearance analyses were also studied.

RESULTS: The median age was 11 years (4-21) and median remission time was 4.3 years (1.8-14.4). The cumulative dose of ifosfamide was 36 g. Glomerular filtration rate was decreased in 41.4% and urine β_2 microglobulin levels and serum cystatin C levels were elevated in 31% of the patients. As the remission time increased, serum creatinine and cystatin C levels were also increased. The sensitivity for β_2 microglobulin and cystatin C in demonstrating CRF was 35.2% and 23% and specificity was 33.2% and 50% respectively. The 24-hour urine NGAL cut-off level for demonstrating CRF was found to be 1.065 ng/mL/24 hours. The sensitivity and specificity for this cut-off value were 83% and 77%, respectively.

CONCLUSIONS: NGAL levels were significantly higher in the study group as compared with the control group. Although ifosfamide treatment was suggested to be safe with no complication of renal failure under a dose of 80 g/m^2, chronic renal failure and deficits in glomerular and tubular function could be seen when the remission time increased. Elevated NGAL levels may be a good option in determining CRF.

Key Words:
Ifosfamide, Nephrotoxicity, β_2 microglobulin, Cystatin C, NGAL.

Introduction

Although ifosfamide is an effective drug for the treatment of cancer, nephrotoxicity, both during the treatment and post-treatment is one of the most important complications. Ifosfamide-induced acute nephrotoxicity commonly appears in the form of proximal tubular dysfunction and decreased glomerular filtration rate (GFR)[1-4]. Ifosfamide nephrotoxicity can present only with aminoaciduria and glycosuria as well as with acute or chronic renal failure (CRF), Fanconi syndrome, renal tubular acidosis and nephrogenic diabetes insipidus[1,2,5,6]. It may also develop as subclinical nephrotoxicity and in this case it can only be diagnosed with very sensitive tests such as for β_2 microglobulin, cystatin C, and retinol-binding protein. In recent years, parallel to the improvement in cancer treatment and the increase in the number of survivors, early detection and treatment of nephrotoxicity leading to serious clinical conditions like chronic renal failure and dialysis has become important. Thus, beta2 microglobulin, cystatin C, glomerular filtration rate and creatinine were markers used in the early detection of nephrotoxicity.

However, blood urea nitrogen (BUN) and creatinine will not increase above the normal range until 60% of total kidney function is lost. At that point, NGAL is a promising marker for detecting early nephrotoxicity, as shown in recent years[7,8]. Various studies have proven that it is the most sensitive marker indicating acute renal failure and renal ischemic injury. Conditions that can result in a 2 fold increase in serum creatinine and can be detected 2-3 days after the event are detected within 2 hours with NGAL[7,8]. In a study conducted on 45 children with chronic renal failure, NGAL was suggested as a better marker than cystatin C and GFR presenting with CRF[9]. In our study, the importance of NGAL in demonstrating CRF was investigated in children surviving from cancer who were treated with ifosfamide.

Patients and Methods

Patients who were treated with ifosfamide chemotherapy at the Hacettepe University Chil-

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dren's Hospital Pediatric Oncology Unit for various types of cancer were enrolled in the study. Ethical approval was obtained (2007-URB54). Patients who had a nephrotoxicity and renal cancer involvement history, infection on physical examination, vomiting and acute feeding insufficiency, elapsed time < 1 year after the last chemotherapy and who collected urine under 2 ml/min after a 24 hour period (with 25% variability) were excluded. The study group consisted of 29 children and 12 healthy children who served as controls.

Demographic data of all patients were collected and included age at diagnosis, time of remission, length, weight, body surface area of the subjects. The date at diagnosis, cumulative dose of ifosfamide, and concomitant chemotherapeutic drugs were also recorded (Table I). Urine samples were collected for 24 hours to avoid diurnal variation.

The renal functions were evaluated with the parameters in Table II. Serum cystatin C, spot urine β_2-microglobulin and NGAL levels in 24-hour urine were evaluated.

Control group consisted of 12 patients without any known prior infection or nephrologic disease. Informed consent was obtained from the patients and parents. The study was approved by the local Ethical Committee of the University.

Table I. The demographic findings of patients.

Demographics	The number and percentage of patients (n/%)
Total number of patients (n)	29
Female/Male	14/15
Age (year/range)	11 (4-21)*
Remission duration (year/range)	4.3 (1.8-14.4)*
Diseases (n/%)	
Neuroblastoma	9 (31.0%)
Ewing's sarcoma	5 (17.2%)
Osteosarcoma	1 (3.4%)
Rhabdomyosarcoma	7 (24.1%)
Hepatocellular carcinoma	1 (3.4%)
PNET	4 (13.8%)
Hodgkin's disease	2 (6.9%)
Cumulative drug doses (n/range)	
Ifosfamide dose (g) (n=29)	36 (6-72)*
Cisplatin dose (mg) (n=21)	600 (270-1700)*
Cyclophosphamide dose (g) (n=13)	6 (0.18-15) *
Carboplatin dose (mg) (n=8)	215 (100-720)*

*Median value; PNET: primitive neuroectodermal tumor.

Biochemical Parameters from Serum and Urine Samples

For biochemical analyses, all blood samples were drawn in the morning after at least 12 h of fasting. At the same time we collected first morning urine samples from the patients. 24 hour and fasting urine samples and serum samples were stored at –80°C until the time of the assay. Serum cystatin C levels were studied with an immunonephelometric method (N-Latex Cystatin C, BH Systems, Dade Behring Marburg GmbH). Urine β_2 microglobulin levels were analyzed from fresh urine samples with a chemiluminescence method (IMMULITE 200, Siemens Health Diagnostics, Deerfield, IL, USA).

Method of Human NGAL ELISA Measurement

Urine samples were stored at –40°C until the time of analysis. The concentration of Lipocalin 2 levels in urine samples was analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Biovendor, Modrice, Czech Republic) according to the manufacturer's instructions. In brief, urine samples were

Table II. Laboratory findings of patients.

	Values and range (n)		
	Ifosfamide group (n: 29)	Control group (n: 12)	p
Serum urea (mg /dL)	15 (8.6-28.7)	10.5 (3-14)	0.96
Serum creatinine (mg/dL)	0.54 (0.32-1.93)	0.75 (0.60-1.0)	0.0001
GFR [creatinine clearance (ml/min/1.73 m^2)]	98 (33-208)	136 (93-239)	0.0001
Urine β2 microglobulin (ng/mL)	188 (4-11890)	70.15 (21-128)	0.034
Serum cystatin C (mg/L)	1.08 (0.72-2.51)	0.73 (0.67-0.95)	0.0001
24-hour urine (n/%) NGAL (ng/mL/24 hours)	1.04 (0.02-34.10)	0.21 (0.08- 0.48)	0.002

The data were given as median.

diluted (1/10) with dilution buffer and analyzed without delay. Standards, control samples and diluted patient samples were all pipetted onto polyclonal anti-human lipocalin 2 antibody coated microplate wells. Plates were incubated for 1 hour at room temperature. After washing each well three times, biotin labeled polyclonal anti-human lipocalin 2 antibody was added and incubated for 1 hour at room temperature. After incubation, plates were washed three times and streptavidin-HRP conjugate was added and incubated again for 30 minutes at room temperature. After washing three times, substrate solution was added to the wells, stored for 10 minutes at room temperature and the reaction was stopped with stop solution. Absorbance values were read at 450 nm with an automatic ELISA reader (Synergy HT, Bio-Tek Instruments, Neufahrn, Germany). The concentrations were calculated by converting the optical density readings against a logarithmic curve. These values were multiplied with the dilution factor[10] and results were expressed as ng/mL. The calculated overall intra-assay coefficient of variation was 7.71%, and the calculated overall inter-assay coefficient of variation was 9.75%. The sensitivity of the assay was 0.02 ng/mL. We measured all samples in duplicate.

Creatinine clearance was calculated using the following formula for 24 hour collected urine: Creatinine clearance (mL/min) = urine creatinine (mmol/L) × urine volume (mL) × 1.73 m^2/plasma creatinine (mmol/L) × time of collection (min) × BSA of the patient.

Nephrotoxicity Criteria

Clinical nephrotoxicity was considered as a 0.5 mg/dl increase in serum creatinine or a decrease of creatinine clearance < 90 ml/min/1.73 m^2. American National Kidney Foundation defined the chronic renal failure criteria currently used as a guide in 2002[10]. Laboratory nephrotoxicity has been recognized as an increase in β_2 microglobulin levels equal or more than twice the upper limit of the normal value[11]. Our study is based on the same criteria.

Statistical Analysis

The data were analyzed by using SPSS 15.0 software package. Demographic data were presented in mean, median and Standard Deviation. Data normality was measured by Kolmogorov Smirnov test. The possible association between qualitative variables was measured with the Chi square (χ^2) test. The data were expressed as means ± SD. SPSS for Windows 10.0 was used for statistical analyses. p values < 0.05 were accepted as statistically significant. Kruskal Wallis and Mann-Whitney-U tests were used for comparing groups with each other and with the control group. Pearson correlation coefficient test was performed for correlation between the cumulative doses and pathologic severity. Comparison between means was performed with the Student's t test for independent data. Multiple linear regression was employed to study the relationship between Receiver operating characteristics (ROC) curve was used for evaluation of the optimal sensitivity and specificity for the measurement of NGAL levels. Sensitivity (also called the true positive rate) measures the proportion of actual positives which are correctly identified. The sensitivity of a test is defined as the proportion of people that are known to have the disease who will test positive for it. Specificity (also called the true negative rate) measures the proportion of actuel negatives which are correctly identified. The specificity of a test is defined as the proportion of people that are known not to have the disease who will test negative for it. ROC curve was formed using the NGAL levels in patients with GFR under 90 mL/min/1.73 m^2 in order to obtain a NGAL cut-off value with the highest sensitivity and specificity level.

Results

There were 29 patients in the study group that were treated with ifosfamide. The demographic characteristics and cumulative drug doses of the patients are shown in Table I. Laboratory find-

Table III. The correlations in the ifosfamide treated group as regard to demographic data and laboratory tests.

	Serum creatinine	Serum cystatin C	Creatinine clearance	Urine NGAL
Age	0.552			
Serum urea		0.396	–0.396	
Creatinine clearance	–0.425			–0.507
Urine β_2 microglobulin	0.398	0.458	–0.522	

ings of patients, including median levels of serum urea, creatinine, creatinine clearance, urine β_2 microglobulin and serum cystatin C are shown in Table II.

The patients with low GFR had significantly higher serum creatinine, cystatin C, urine β_2 microglobulin, and NGAL levels, respectively (p = 0.021, p = 0.043, p = 0.006, p = 0.001).

The patients with high urine β_2 microglobulin levels had significantly higher serum urea and cystatin C levels as compared to patients with lower urine β_2 microglobulin, respectively (p = 0.010, p = 0.002), while the level of GFR was significantly lower (p = 0.007). Serum cystatin C and urine β_2 microglobulin levels were significantly higher compared with the control group, respectively ($p < 0.001$, p = 0.035).

The correlation of patients for demographic and laboratory data are given in Table III. Urine β_2 microglobulin levels were positively correlated with serum urea, creatinine, and cystatin C levels, respectively (r = 0.453, r = 0.398, r = 0.458), and was negatively correlated with serum creatinine clearance, respectively (r = –0.552. Urine NGAL was negatively correlated with creatinine clearance (r = –0522). The sensitivity for β_2 microglobulin and cystatin C was 35.2%, 23% and specificity was 33.2%, 50%, respectively in demonstrating CRF.

Urine (24-hour) NGAL levels were measured to demonstrate the course of CRF. The cut-off value for for NGAL was found to be 1.065 ng/mL/24 hours. The sensitivity and specificity were 83% and 77%, respectively, for this cut-off value (Figure 1). NGAL levels were significantly higher in the study group as compared with the control group (p = 0.003).

Control group consisted of 12 children with no known renal disease and of similar age. There were 5 female and 7 male patients. The median age was 10 years[5-16]. The laboratory data was presented in Table II.

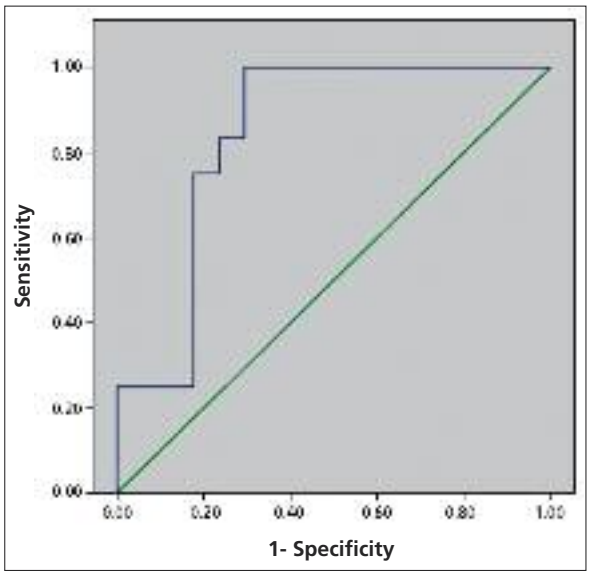

Figure 1. ROC curve was generated to evaluate the sensitivity and specificity of 24-hour urine levels of NGAL in demonstrating chronic renal failure in the ifosfamide treatment group; cut-off value: 1.065 ng/mL/24 hours; area under the curve: 0.84, p = 0.002.

Discussion

Early detection of CRF and preventing renal damage is directly correlated with lower number of cancer survivors undergoing dialysis. In this context, we evaluated the role of NGAL in demonstrating CRF earlier and found that an NGAL cut-off value 1.065 ng/mL/24 h had a sensitivity and specificity as 83% and 77%, respectively. Proximal tubular damage related to ifosfamide has been reported to be more frequent and severe in children aged less than 5 years due to the more limited rate of hepatic cytochrome P450 activation[12]. The median age of children in the ifosfamide group was 11 years old (4-21 years) in our study. We found an increasing serum creatinine levels parallel with the age of the patients increase. This indicates that at older ages, tubulopathy can be reversible after ifosfamide treatment; however, glomerulopathy is not. We also found that as the duration of remission increased, serum creatinine and cystatin C levels also increased. This condition may be related to the decrease in compensation capability of the kidneys with time.

Although the range of cumulative doses of ifosfamide causing nephrotoxicity[13] was reported to be > 80 g/m^2, it is emphasized that one should be aware of effects as the cumulative dose[12] reaches 60 g/m^2. A significant relationship was detected between high cumulative ifosfamide dose and GFR, as detected in 148 patients with cancer and a cumulative drug dose of 62 g/m^2 in a multicenter study in England[13]. The median cumulative drug dose was 36 g/m^2 (6-72) in our study. When the study group was divided into two groups as cumulative drug doses higher or lower than 30 g/m^2, there was no significant dif-

ference between the groups with regard to serum urea, creatinine, cystatin C, urine β_2 microglobulin, NGAL or GFR levels. Although cumulative ifosfamide doses below 80 g/m^2 were found to be safe in this study, these results suggest that this may lead to nephrotoxicity and predispose to chronic renal insufficiency even at subclinical cumulative drug doses.

The use of cisplatin with ifosfamide has been reported to increase the risk of kidney damage[12]. As carboplatin does not convert to toxic metabolites in renal tubule cells, it is not as nephrotoxic as cisplatin. However, single and high dose carboplatin-ifosfamide combinations were reported to be as nephrotoxic as cisplatin-ifosfamide combinations[14]. In our study, the cumulative cisplatin dose was 600 mg/m^2 (270-1700) and carboplatin dose was 215 mg/m^2 (100-720). We could not demonstrate any significant difference between patients who concomitantly received cisplatin or carboplatin, or did not receive these treatments, in terms of renal function tests and nephrotoxicity indicators. Although these results suggest that the combination treatment of carboplatin or cisplatin with a cumulative ifosfamide dose below 80 g/m^2 did not show any additional contribution to nephrotoxicity, the low number of patients could have affected this finding.

A decrease in GFR was detected in 25% of patients who received 14 g/m^2 of ifosfamide treatment and 17-50% of younger patients who received lower doses[15]. After completion of ifosfamide treatment, an average of 35 ml/min/1.73 m^2 decrease in GFR was detected[13]. In another study, although there was no significant difference between mean GFR levels of patients one and ten years after treatment, a variable clinical course was observed in tubular phosphate reabsorption and there was a need for electrolyte supplementation[1]. Skinner et al[16] investigated ifosfamide nephrotoxicity 1-28 (median 2 months) months after therapy in children between 2.1-16.2 (median 6.9) years old. Ten of these patients (45%) had low GFR, 6 had proximal tubular toxicity and renal tubular acidosis, and one patient had distal tubular toxicity and nephrogenic diabetes insipidus. Patients who received higher than a 60-100 g/m^2 dose were reported to be in a high risk group. In another study[1], nephrotoxicity was investigated in 12 children 1-10 years after ifosfamide treatment with a median age of 5.9 years (2.1-13.7) and a cumulative ifosfamide dose of 104 g/m^2 (53-153) and no significant difference was detected between patients in median total nephrotoxicity score. A decrease in GFR was observed in two patients (< 60 ml/min) and renal tubular damage was seen in 4 patients. Chronic nephrotoxicity in children due to ifosfamide has been reported to be 30-60%[17]. In our study, we detected a decrease in GFR in 41.3% of patients receiving ifosfamide treatment. Low GFR, high serum creatinine, cystatin C, β_2 microglobulin and NGAL levels may suggest a close follow-up of these patients for renal functions in the future.

In CRF, serum β_2 microglobulin began to accumulate as GFR decreased before and earlier than the increase in serum creatinine level. Thus, urine or serum β_2 microglobulin levels are better markers than serum creatinine in calculating GFR and demonstrating disturbance in renal function[18,19]. It has been reported that 4% of high-risk sarcoma patients treated with ifosfamide developed Fanconi-like syndrome in the beginning and five days after treatment. All patients demonstrated acute, reversible subclinical nephrotoxicity and a 70 fold increase were detected in serum β_2 microglobulin levels. Severe β_2 microglobulinuria has been reported[2] to be a prognostic marker in developing chronic nephrotoxicity. Renal functions were evaluated in 60 children after cisplatin and ifosfamide therapy. They were divided into two groups: those who received less than or greater than 80 g/m^2 ifosfamide treatment. Urine β_2 microglobulin level was reported to be the most specific marker in demonstrating ifosfamide nephrotoxicity[20]. In our study, 31% of the patients had high urine β_2 microglobulin levels in the ifosfamide group. Patients with elevated urine β_2 microglobulin levels had higher cystatin C and serum creatinine levels and lower GFR than patients with low urine β_2 microglobulin levels in the ifosfamide treated group. This shows that urine β_2 microglobulin levels are positively correlated with other tests indicating renal failure.

Cystatin C is considered to be a good marker in childhood for determining GFR compared to serum creatinine. It correlates better with renal maturation, reaches adult levels after the first year of life and is not affected by age, sex or body muscle mass[18,21,22]. In a study including 258 children with leukemia or solid tumors[23], serum cystatin C was used in demonstrating GFR and compared with serum creatinine and creatinine clearance. In this study, 92 children were on chemotherapy treatment, 108 children were in remission, 40 children had no renal dis-

ease, and 18 children had chronic renal disease. The study showed that there was a significant correlation between serum creatinine and serum cystatin C levels, and cystatin C levels were rapidly increased with multimodal treatment, cyclophosphamide, ifosfamide, cisplatin and methotrexate. It was more elevated in patients with CRF and on chemotherapy. It has been shown that serum cystatin C may be used to demonstrate GFR during and after treatment in children with cancer [23].

In our study, serum cystatin C was found to be elevated in 31% of the patients treated with ifosfamide. In the ifosfamide group, patients who had high serum cystatin C levels also had high urine β_2 microglobulin and serum urea levels and this was correlated with increased serum cystatin C level. This suggests that serum cystatin C correlates with tests demonstrating renal failure that show a high specificity in demonstrating renal failure.

NGAL was initially reported to be a specific and sensitive marker in diagnosing acute renal failure. A recent study[9] evaluated its effectiveness in CRF. In another study including 45 children with CRF, NGAL was well correlated with serum cystatin C and GFR and showed an even better performance as GFR decreased. In our study, the NGAL cut-off value was 1.065 ng/mL/24 h and sensitivity and specificity for this cut-off value was 83% and 77%, respectively. NGAL levels in the ifosfamide group were significantly higher than the control group.

Conclusions

Our results were similar with the rates of impairment in renal function tests such as creatinine, β_2 microglobulin and cystatin C. NGAL may be a good indicator for demonstrating CRF in cancer survivors and can be used as a diagnostic test on long term follow-up.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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