Urinary N^1,N^{12}-diacetylspermine as a biomarker for pediatric cancer: a case control study

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Author contributions

Conception and design by K. Y, K. H, A. H, H. U, and M. K[3]. Provision of study materials or patients by K. Y, A. H, M. K[4, 5], T. T, C. S, W. S, S. M, M. O, A. T, A. Y, and Y. N. Collection and assembly of data by K. Y, K. H, H. A, M. K[4,5], M. K[3], T. T, C. S, W. S, S. M, M. O, A. T, A. Y, Y. N and Y. K. Data analysis and interpretation by K. Y, K. H, A. H, H. A, Y. K, K. H, H. U, and M. K[3]. The manuscript was mainly written by K. Y, H. A, M. K[4, 5], H. U, and M. K[3]. All authors approved the Manuscript.

Abstract

Purpose: Minimally invasive examinations are particularly important in pediatric patients. Although the significance of urinary N^1,N^{12}-diacetylspermine (DiAcSpm) as a tumor marker (TM) has been reported in many types of adult cancers, its usefulness in pediatric cancers has not been reported. This may be due to urinary DiAcSpm level variations with age. This study aims to measure the normal levels of urinary DiAcSpm in healthy individuals and investigate its usefulness as a TM in childhood cancer.

Methods: Urinary samples were collected from pediatric patients with and without cancer. The urinary DiAcSpm levels were measured, and the values were compared.

Results: A total of 32 patients with cancer and 405 controls were enrolled in the study. Of the 32 patients, 13 had neuroblastoma, 9 had malignant lymphoma (ML), and 10 had leukemia. In the control group, the urinary DiAcSpm values markedly fluctuated among those with young age, especially infants; meanwhile, the values converged among those aged roughly 10 years and above. The sensitivity of DiAcSpm was significantly different among the three types of cancers: neuroblastoma (30.8%), ML (77.8%), and leukemia (40%).

Conclusion: The urinary DiAcSpm value is a useful TM for both screening and follow-up of ML.

Keywords: Malignant lymphoma, urinary tumor marker, polyamine, N1,N12-diacetylspermine, pediatric cancer

Introduction

Urinary N^1,N^{12}-diacetylspermine (DiAcSpm) has attracted research attention as a tumor marker [1] in many types of cancers, including urogenital malignancies [1-3], breast cancer [4], colorectal cancer [1, 5], lung cancer [6-9], pancreatobiliary cancer [10], ovarian cancer [11], and hepatocellular carcinoma [12]. DiAcSpm comprises 0.46% of the total polyamines in the urine. These polyamines are essential for normal cell proliferation, gene expression, membrane stabilization, apoptosis, and organogenesis. Malignant transformation of normal cells requires an increase in cell proliferation; thus, polyamine concentration increases during malignant transformation [5, 13, 14]. Russell et al. first reported that polyamines excreted in the urine of patients with cancer were higher than those in healthy individuals in 1971 [15], which led to a surge in research on polyamine analysis to determine whether the level of urinary polyamine would be an indicator of malignancy [1]. However, polyamines as tumor markers were not useful because of the individual differences in polyamine excretion, which led to false-positive and false-negative results. Moreover, polyamines are increased in patients with benign diseases [1]. Hiramatsu et al. found that DiAcSpm, a species of polyamine, has potential as a tumor marker because its levels vary only minimally between individuals, and although they are present only in low percentages, they are regular constituents of urine in healthy individuals [4, 16].

Although less invasive examinations such as urinary tumor markers are more desirable than blood or imaging tests for the diagnosis or follow-up of pediatric patients, the usefulness of urinary DiAcSpm for

pediatric cancer has not been reported. This may be because its level varies with age, and no cut-off value has been established [17].

The aim of this study was to investigate the usefulness of DiAcSpm as a tumor marker for childhood cancer. First, the normal amount of urinary DiAcSpm in healthy individuals was measured to determine the cut-off values. The urinary DiAcSpm value of patients with pediatric cancer, including neuroblastoma, malignant lymphoma, and leukemia, was then analyzed and compared to that of controls.

Patients and Methods

Patients and ethical concerns

This study enrolled pediatric patients with cancer who were treated at Nagoya University Hospital and Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (Komagome Hospital) between May 2009 and January 2018. The control group comprised patients with no known cancer who were admitted to Nagoya University Hospital, Komagome Hospital, and Aichi Children's Health and Medical Center between May 2009 and January 2018, and children of officials at Komagome Hospital in 2009. All patients were under 18 years of age. This study was approved by the Ethics Committee of Nagoya University Hospital (Approval no: 2015-0349 and 2015-0477) and by the Ethics Committee of Komagome Hospital (Approval no: 2009-697) and by Ethics Committee of the Tokyo Metropolitan Institute of Medical Science (Approval no: 12-20 (2012)).

Written informed consent was obtained from each patient or their parents.

Urine collection

Urine samples were collected from both the patients and controls. For pediatric patients with cancer, urine was collected once before treatment and then as appropriate during the course of treatment. The following information was collected from the patients' medical records: sex, age, type of cancer, degree of progression, treatment and its effect, and fluctuation of known tumor markers.

For the controls, urine was collected only once before treatment. The following information was collected from the patients' medical records or via interviews with the parents: age, sex, and diagnosis.

Measurement of urinary DiAcSpm

Urine samples were analyzed at the Stem Cell Project, Tokyo Metropolitan Institute of Medical Science, which is a collaborating institution for this study. Urinalysis was conducted as described previously [7, 8, 17, 18]. The urine samples were supplemented with 3 mmol/L NaN_3 and stored at -20°C until analysis. Urinary DiAcSpm was measured using a colloidal gold aggregation procedure using AutoDiAcSpm® reagent (Alfresa Pharma Co., Osaka, Japan) on a JCM BM-6010 automatic biochemical analyzer (JEOL, Tokyo, Japan).

Urine creatinine levels were measured enzymatically using NESCAUTO® VL Ⅱ CRE reagent (Alfresa

Pharma Co., Osaka, Japan) on a JCM BM-6010 automated biochemical analyzer. The value of DiAcSpm was expressed as the creatinine normalization value.

Determination of the standard age-matched value of urinary DiAcSpm

The urinary DiAcSpm values in the control group were measured. The linear regression and 95% confidence intervals were calculated after the urinary DiAcSpm value was plotted on the vertical axis against age in months on the horizontal axis. This range was defined as the standard age-matched value.

Investigation of the usefulness of urinary DiAcSpm as a tumor marker of pediatric cancer

The pretreatment DiAcSpm value in patients with pediatric cancer was measured, and the values exceeding the standard age-matched value were judged as “positive.” Sensitivity and specificity were determined for each cancer type. For highly sensitive cancers, the clinical course and known tumor markers were compared with changes in the value of DiAcSpm. DiAcSpm was considered useful as a tumor marker when the clinical course and the value of DiAcSpm correlated.

Statistical analysis

Linear regressions and 95% confidence intervals were calculated using Stata (Stata Co., Texas, USA). This

range was defined as the standard age-matched value. The patients in the cancer and control groups were compared via univariate analyses using Fisher's exact test using IBM SPSS Statistics 25 (IBM, New York, USA). Statistical significance was set at $p < 0.05$.

Results

Patients and controls

The overall cohort comprised 437 patients. Of these, 405 and 32 were enrolled in the control and patient groups, respectively. The majority of the patients in the control group were preoperative patients scheduled for varying surgeries for the treatment of inguinal hernia, umbilical hernia, and undescended testis. In the control group, 55 patients were under 1 year old, 110 were 1-3 years old, 106 were 4-6 years old, 82 were 7-10 years old, 47 were 11-15 years old, and 5 were over 16 years old. Meanwhile, of the 32 pediatric patients with cancer, 13 had neuroblastoma, 9 had malignant lymphoma, and 10 had leukemia. The patient characteristics are shown in Table 1.

Table 1

Determination of the standard age-matched value of urinary DiAcSpm

The level of DiAcSpm in each spot urine sample obtained from the control group is plotted in Figure 1. Urinary DiAcSpm levels varied markedly among younger patients. Analysis of the nonlinear relationship indicated that

Figure 1

the regression line achieved using the following natural logarithm was valid.

"log(y) = -0.166619(x) + 8.16239" (x; months, y; the value of DiAcSpm).

The R-squared value of this equation is 0.7610, and this linear regression is considered appropriate.

Following this, a 95% confidence interval was determined; the upper and lower limit lines along with the regression line are added in Figure 1. In the control group, 22 cases were out of this range, and the specificity was 94.6%.

Sensitivity and specificity of DiAcSpm for pediatric cancer

The pretreatment DiAcSpm levels in patients with cancer are shown in Figure 2. Of the 32 patients, 15 had positive DiAcSpm. Specifically, seven of the nine patients with malignant lymphomas, including three patients with Burkitt lymphoma with prominently high values, were "positive," while the other two patients were "negative." Four of the ten patients with leukemia were "positive," and the other six patients had DiAcSpm near the upper limit, although this value was within the reference range. Only 4 of the 13 patients with neuroblastoma were "positive." In two cases of N-MYC amplified neuroblastoma, one was "positive" and the other was "negative."

Figure 2

The sensitivity and specificity of DiAcSpm for all three types of pediatric cancers were 46.9% and 94.6%, respectively, and the sensitivities of DiAcSpm for malignant lymphoma, neuroblastoma, and leukemia were

Table 2

77.8%, 30.8%, and 40%, respectively, as shown in Table 2. There were significant differences in the positive ratio in patients with each type of cancer, such as neuroblastoma, malignant lymphoma, and leukemia, compared with the patients in the control group.

Correlation between the urinary DiAcSpm level of patients with malignant lymphoma and their clinical course

Since the sensitivity of urinary DiAcSpm was high in malignant lymphoma, correlations between the urinary DiAcSpm value and the known tumor marker value (soluble interleukin-2 receptor; sIL-2R) or imaging findings were investigated. The characteristics of patients and the values of DiAcSpm with malignant lymphoma are presented in Table 3. Because the standard value of sIL-2R at our hospital was changed during the research period, the evaluation was made based on whether or not it was within the range of the standard value without using the numerical value. Except for one patient who changed hospitals, all patients with malignant lymphoma underwent successful chemotherapy, with no tumors noted on post-treatment imaging. As the treatment progressed, the values of sIL-2R and DiAcSpm also dropped to the standard value. The other patient had a small tumor left in the post-treatment imaging (patient 9), but the value of sIL-2R was negative, while the value of DiAcSpm remained positive. The patient died after an immediate relapse. Considering that one of the two patients whose pre-treatment DiAcSpm was “negative” had positive sIL-2R (Patient 4), the combination of urinary DiAcSpm and sIL-2R was useful for both screening and follow-up of malignant

Table 3

lymphoma.

Discussion

The findings of this study support the clinical value of urinary DiAcSpm as a tumor marker in pediatric cancer. First, the normal value of DiAcSpm was determined according to age. We found that the DiAcSpm level varied significantly among young patients, and this converged as age increased. This is because DiAcSpm is a polyamine that is associated with normal cell proliferation, gene expression, membrane stabilization, apoptosis, and organogenesis. It is thought to be highly variable in young healthy children [5, 13, 14]. When a patient is approximately 10 years of age, the DiAcSpm value reaches the adult level. Therefore, high DiAcSpm values should be interpreted with caution, which can be done using our standard DiAcSpm reference range. There is no existing report on the normal values of DiAcSpm with respect to age. Thus, our study is the first report of this kind.

Seven of the nine patients with malignant lymphomas were positive for urinary DiAcSpm, and three had extremely high levels. Changes in DiAcSpm levels correlated with the course of treatment and the value of sIL-2R. In one patient with lesions on post-treatment imaging (patient 9), DiAcSpm remained positive, while

sIL-2R was negative. This patient was prone to chemotherapy resistance and had repeated recurrences. Therefore, it was considered that the value of DiAcSpm which is associated with cell proliferation and gene expression did not decrease. The results of this study indicate that the combination of urinary DiAcSpm and sIL-2R might be highly sensitive for post-treatment evaluation of malignant lymphomas. Based on reports showing the prognostic value of urinary DiAcSpm in lung cancer [6-8], it may also be a prognostic indicator of malignant lymphoma. This study suggests that urinary DiAcSpm may be a useful marker for pediatric malignant lymphoma. However, there are few reports of malignant lymphoma in adults.

Although patients with neuroblastoma had a high absolute value of DiAcSpm, the sensitivity of DiAcSpm was 30.8%, which was not high. The sensitivity of established urinary markers in neuroblastoma, including homovanillic acid and vanillylmandelic acid, is as high as 75%-100% [19], which is markedly higher than that of DiAcSpm in this study. Therefore, urinary DiAcSpm seems to have low value as a diagnostic marker for neuroblastoma. However, when DiAcSpm level was followed in individual neuroblastoma patients, a rapid increase of DiAcSpm level seemed to correlate with a short progression-free survival period [20]. This suggests that DiAcSpm may be a potential prognostic indicator for neuroblastoma, although age-related decrease of basal DiAcSpm level has to be taken into consideration especially when a long-term follow-up may be a matter. Although there are many reports on the use of urinary DiAcSpm as markers for adult cancer, it is not majorly used in pediatric cancer because the normal range varies widely, and the difference in growth between

fetal-derived tumors and normal cells cannot be distinguished.

In the case of leukemia, there is no known urinary marker; thus, the sensitivity is not low (40%). Urinary DiAcSpm remains a potential diagnostic marker for leukemia and should be studied further.

Several studies have suggested the involvement of the MYC gene in the mechanism by which urinary DiAcSpm increases [20-22]. Polyamines such as DiAcSpm are synthesized from ornithine using ornithine decarboxylase (ODC) as an enzyme, and ODC is a rate-limiting enzyme in polyamine biosynthesis. Moreover, the ODCs of the polyamine pathway are transcriptional targets of c-MYC or N-MYC genes [20-22]. Therefore, it was thought that increased expression or amplification of the MYC gene led to high ODC expression and subsequently high polyamine levels [20]. Because N-MYC and c-myc are implicated in neuroblastoma and lymphoma, respectively [21], DiAcSpm may be a useful marker. As shown in Figure 2, the DiAcSpm levels of three patients with malignant lymphoma were extremely high, and all three of them had Burkitt lymphoma. As shown in Table 3, these three patients had IgH-MYC translocation and c-MYC expression at oncogenic levels. This study clearly indicates a correlation between the increased expression of c-MYC and urinary DiAcSpm. In addition, in two reported cases of N-MYC-amplified neuroblastoma, one patient had urinary DiAcSpm levels below the upper limit of the 95% confidence interval, while it was beyond the upper limit in the other patients, as shown in Figure 2. There was some uncertainty regarding the correlation between amplification of the N-MYC gene and the value of urinary DiAcSpm.

The value of DiAcSpm can be measured through urinalysis and can therefore reduce the potential discomfort of pediatric patients. Although the concentration of urine excreta may change depending on the circadian rhythm, it is difficult to collect urine at a fixed time in children. This problem can be solved by creatinine normalization [17]. The amount of DiAcSpm excreted in the 24-hour urine sample corresponds to the amount of DiAcSpm expressed in nmol / g • creatinine. Therefore, creatinine normalization is useful and appropriate for estimating daily DiAcSpm excretion from spot urine samples [17]. In addition, the value of urinary DiAcSpm does not fluctuate depending on the time from collection to measurement, similar to that of hemoglobin and bilirubin [4. 17]. This stability provides another advantage of urinary DiAcSpm as a tumor marker to ensure reliable results.

The limitation of this study is that the total number of patients was small. We could not determine whether the value of urinary DiAcSpm is positively associated with any stage of cancer. This should be addressed in studies involving larger cohorts.

Conclusions

Urinary DiAcSpm has the potential to be a tumor marker for pediatric malignant lymphoma and leukemia. Specifically, it is a useful tumor marker for both screening and follow-up of malignant lymphoma. However, because the value of urinary DiAcSpm varies in young patients, it should be used with caution when screening

for cancers that are often found in relatively young age groups.

Compliance with Ethical Standards

Conflict of interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Figure Legends

Figure 1. Correlation between the urinary DiAcSpm value and age in the control group.

The top curve shows the upper limit of the 95% confidence interval, the middle curve shows the regression line, and the bottom curve shows the lower limit of the 95% confidence interval.

Figure 2. Pretreatment values of urinary DiAcSpm in each patient.

The shape of the plotted point changed depending on the type of cancer. ● indicates neuroblastoma, ▲ indicates leukemia, and ■ indicates malignant lymphoma. The range of the two green lines is within the range of the 95% confidence interval of the control group, and the red line is the regression line of the control group.

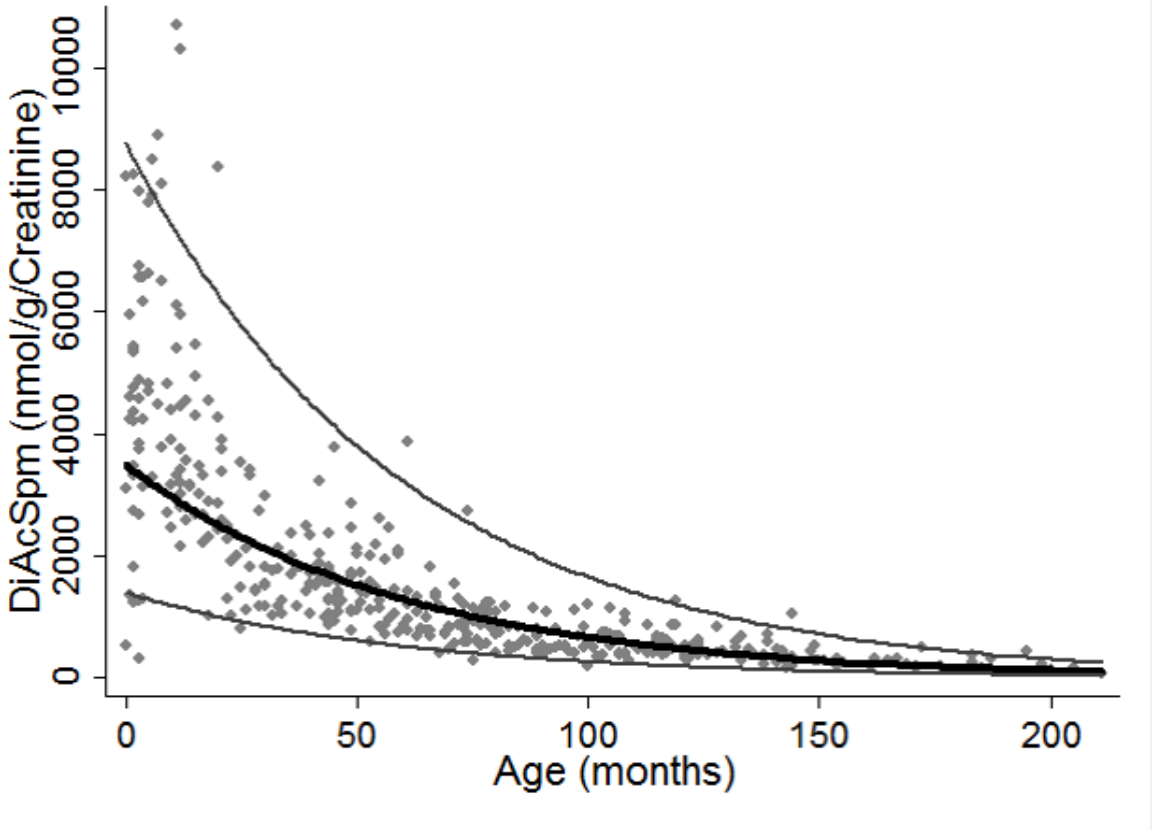
DiAcSpm (nmol/g/Creatinine)
0
2000
4000
6000
8000
10000
0
50
100
150
200
Age (months)

Figure 1

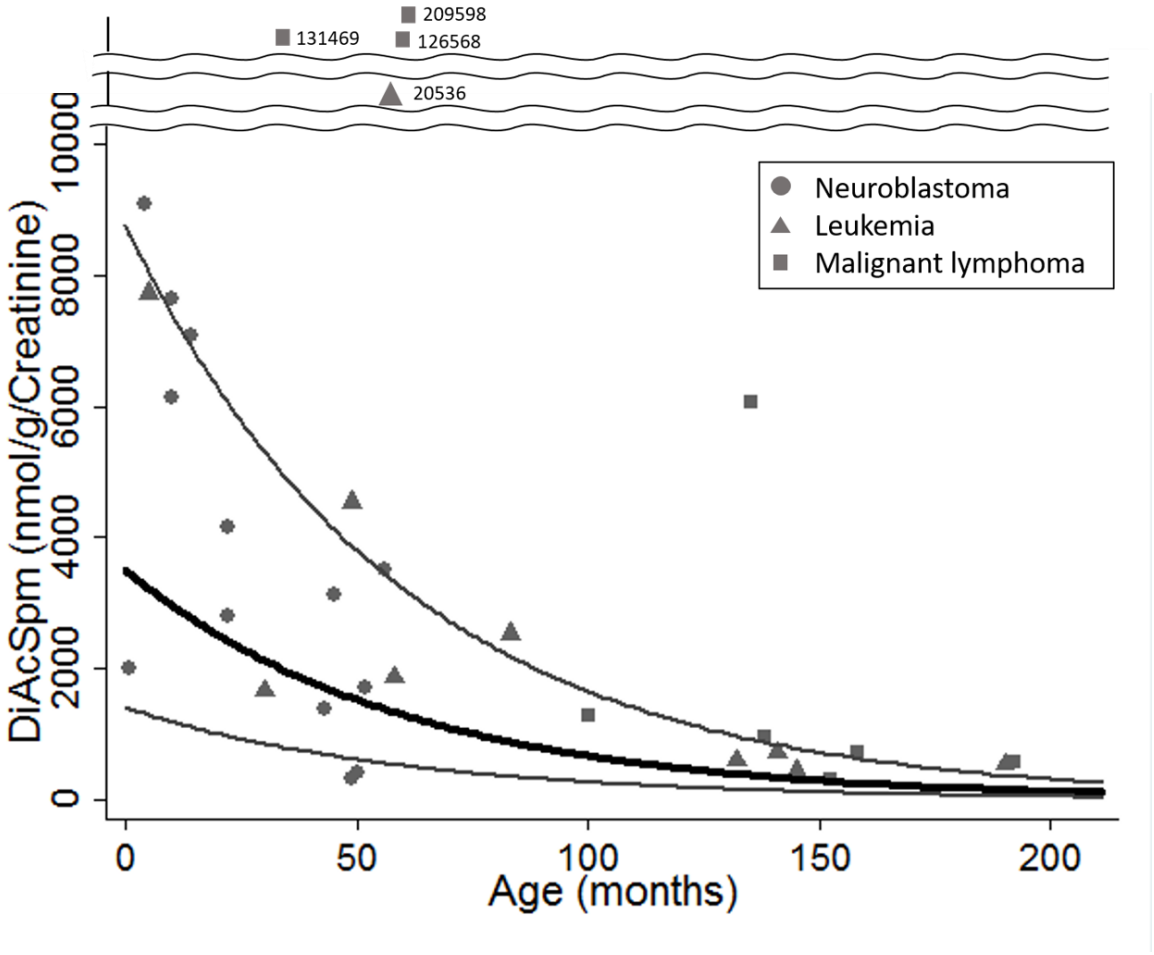
209598
131469
126568
20536
Neuroblastoma
Leukemia
Malignant lymphoma
DiAcSpm (nmol/g/Creatinine)
0
2000
4000
6000
8000
10000
0
50
100
150
200
Age (months)

Figure 2

Table 1. Patient characteristics

Type of cancer	Number of patients	Number of male patients	Age, months median (range)
Neuroblastoma	13	6	22 (1-56)
Malignant lymphoma	9	8	135 (35-192)
Leukemia	10	4	73 (5-190)

Table 2. Sensitivity of the urinary DiAcSpm value

Type of cancer	Total number of patients	Number of patients who tested positive	Sensitivity (%)	P value
Neuroblastoma	13	4	30.8	0.006
Malignant lymphoma	9	7	77.8	0.000
Leukemia	10	4	40.0	0.000
Total	32	15	46.9	

Table 3. Characteristics and clinical course of patients with malignant lymphoma

	Age at diagnosis (Months)	Type of lymphoma	IgH-MYC translocation	Ann Arbor stage	Pre-treatment			Post-treatment		
					DiAcSpm (value)	sIL-2R	Tumor location	DiAcSpm (value)	sIL-2R	Tumor location
1	35	B-L	(+)	Ⅲ	Positive (131000)	Positive	Right lower quadrant Pleural fluid	Negative (1810)	Negative	None
2	64	B-L	(+)	Ⅳ	Positive (127000)	Positive	Intrapelvic LN, Liver, Ascites, Bone marrow	NA	Positive	None
3	66	B-L	(+)	Ⅲ	Positive (210000)	Positive	Intrapelvic LN, Pleural fluid	Negative (540)	Negative	None
4	100	DLBCL	NA	Ⅱ	Negative (1290)	Positive	Small intestine Cervical LN	Negative (494)	Negative	None
5	135	TLBL	NA	Ⅲ	Positive (6070)	Positive	Anterior mediastinum Axillary LN	Negative (167)	Negative	None
6	138	BLBL	NA	Ⅲ	Positive (962)	NA	Jawbone, Rib, Subcutaneous	NA	NA	NA
7	151	H-L	NA	Ⅰ	Negative (256)	Negative	Cervical LN	Negative (61.0)	Negative	None

8	170	H-L	NA	Ⅱ	Positive (738)	Positive	Anterior mediastinum, Supraclavicular LN	Negative (227)	Negative	None
9	192	DLBCL	NA	Ⅲ	Positive (599)	Positive	Anterior mediastinum Axillary LN	Positive (470)	Negative	Anterior mediastinum 10mm×10mm

sIL-2R, soluble interleukin-2 receptor; B-L, Burkitt lymphoma; DLBCL, Diffuse large B cell lymphoma

TLBL, T cell lymphoblastic lymphoma; BLBL, B cell lymphoblastic lymphoma; H-L, Hodgkin's lymphoma; LN, lymph node

NA, Data not available; Positive, above the standard value; Negative, below standard value; None, there were no more tumors detected

value, nmol / g · creatinine