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Original Article / *Özgün Makale*

A preliminary study of tracking B-cell kinetics in patients with lung transplantation by monitoring kappa-deleting recombination excision circles

Akciğer nakli olgularında B hücre kinetiğinin kappa silici rekombinasyon eksizyon halkalarının monitörizasyonu ile izlenmesine yönelik bir ön çalışma

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ABSTRACT

Background: This study aims to evaluate humoral immune system response by measuring copy numbers of kappa-deleting recombination excision circles *(KREC)* gene segment from B lymphocytes in patients with lung transplantation.

Methods: Between September 2015 and November 2016, a total of 11 patients (8 males, 3 females; mean age: 45.4±12.0 years; range, 23 to 59 years) who underwent lung transplantation with different primary indications were included. The copy numbers of *KREC* gene segment were quantified using real-time polymerase chain reaction method in peripheral blood samples collected pre- and post-transplantation. The samples of the patients were compared with the *KREC* levels in deoxyribonucleic acid extracted from blood samples of healthy children.

Results: There was no significant change in *KREC* levels between pre- and post-operation (p=0.594 and p=0.657), although the median values indicated that the highest increase in the *KREC* levels (7×10^5-12×10^5; 85-170) was on Day 7 of transplantation. There was a positive correlation between the *KREC* levels (mL in blood) and lymphocytes at 24 h after transplantation (p=0.043) and between *KREC* copies per 10^6 of blood and age on Day 7.

Conclusion: Our preliminary results suggest that *KREC* levels as an indicator of B lymphocyte production are elevated after lung transplantation. A prognostic algorithm by tracking B cell kinetics after post-transplantation for long-term follow-up can be developed following the confirmation of these preliminary results with more patient samples.

Keywords: B lymphocyte, immune system, kappa-deleting recombination excision circle, lung transplantation.

ÖZ

Amaç: Bu çalışmada, akciğer nakli olan hastaların B lenfositlerinden kappa-silici rekombinasyon eksizyon halkası (*KREC*)'nın kopya sayısı ölçülerek, hümoral immün sistem yanıtı değerlendirildi.

Çalışma planı: Ekim 2015 - Kasım 2016 tarihleri arasında farklı primer endikasyonlar nedeniyle akciğer nakli yapılan 11 hasta (8 erkek, 3 kadın; ort. yaş: 45.4±12.0 yıl; dağılım, 23-59 yıl), çalışmaya alındı. Nakil öncesi ve sonrasında periferik kan örneklerinden elde edilen *KREC* gen segmentinin kopya sayısı, gerçek zamanlı polimeraz zincir reaksiyon yöntemi ile analiz edildi. Hastalardan alınan örnekler, sağlıklı çocuklardan alınan kan örneklerindeki deoksiribonükleik asitteki *KREC* düzeyleri ile karşılaştırıldı.

Bulgular: Ameliyat öncesi ve sonrası *KREC* düzeylerinde anlamlı bir farklılık olmamakla birlikte (p=0.594 ve p=0.657), nakil sonrası 7. günde *KREC* düzeylerinin (7×10^5-12×10^5; 85-170) medyan değerlerinde en yüksek artış izlendi. Nakil sonrası 24. saatte *KREC* düzeyi (kanda mL) ve lenfositler arasında (p=0.043) ve 7. gündeki 10^6 kanda bulunan *KREC* kopya sayısı ile hasta yaşları arasında (p=0.011) pozitif bir korelasyon bulundu.

Sonuç: Ön çalışma sonuçlarımız, akciğer naklinden sonra B lenfosit üretiminin bir göstergesi olarak *KREC* düzeylerinin yükseldiğini ortaya koymaktadır. Bu ön çalışma sonuçlarının daha fazla sayıda hasta numunesi ile doğrulanmasından sonra, nakil sonrası uzun süreli takip için B hücre kinetiğini izleyerek bir prognostik algoritma geliştirilebilir.

Anahtar sözcükler: B lenfositi, immün sistem, kappa silici rekombinasyon halkası, akciğer nakli.

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Lung transplantation (LTx) is considered a therapeutic instrument for adults with chronic, end-stage lung disease.[1] The number of LTx cases has increased worldwide rapidly, while the number of annual transplants plateaued at around 1,400/year in the mid 1990s. In recent years, the number of transplants has reached to around 4,500/year.[2] Lung transplantation has been performed in different centers in Turkey since 1998. Since then, there are several attempts, but the first successful LTx was performed in 2009.[2]

The lung is the one of the largest transplantable organs with an extensive vasculature that is exposed to the entire cardiac output. The lung has also a vast intrinsic immune apparatus, which includes large populations of both antigen-presenting cells and effector cells, and the respiratory tract is in constant contact with extrinsic inhaled antigens, leading to local inflammatory reactions with upregulation of alloantigen expression on bronchial epithelium and activation of T lymphocytes.[3]

Production of B lymphocytes in solid organ transplants to follow the response of the recipient's immune system can be monitored by measuring kappa-deleting recombination excision circles *(KRECs)* as in hematopoietic stem cell transplants.[4,5] The KREC is an extrachromosomal circular product. It separates from deoxyribonucleic acid (DNA) due to light chain gene rearrangement in B lymphocyte development, and it is diluted each time the cell is divided.[4] During the maturation processes of B-cell receptor (BcR) heavy and light chains, antigen receptor genomic rearrangements generate functional receptors. This process is necessary, as the gene complexes encoding the BcR components do not contain a functional first exon, while including multiple variable (V), diversity (D), and joining (J) genes. In the antigen-independent differentiation phase, stepwise rearrangements are introduced into the genome to couple one of each segment together to form a functional first exon. B-cell maturation and *KRECs* are the products of recombination events determining the allelic and isotypic exclusion of the immunoglobulin (Ig) kappa *(IGK)* locus.[6] Although Igs have two types of light chain, kappa (κ) and lambda (λ), B lymphocytes express only one of these two types of chains. About 60% of the B lymphocytes in human express ϰ chain, while 40% have λ chain.[7] When this gene rearrangement is successfully completed in the cell, the B cell enters the germinal center and undergoes somatic hypermutation, resulting in memory or plasma cells with an Igϰ light chain.[5,8]

In the present study, we used real-time polymerase chain reaction (PCR) assay to quantify B lymphocyte mobilization through detection of *KRECs*. The primer and probe final concentrations were described previously by Sottini et al.[9] Standardization of the test procedure was achieved by carrying out specific optimization procedures. We aimed to investigate the possibility of using the *KREC* levels, as an indicator of B cell kinetics, in follow-up of patients with LTx. We, therefore, report preliminary results from 11 patients in the short term and discuss potential studies in the future.

PATIENTS AND METHODS

This prospective study was conducted at Kartal Koşuyolu High Specialization Education and Research Hospital, Department of Thoracic Surgery and Immunology Department of Faculty of Medicine, Yeditepe University between September 2015 and November 2016. We obtained samples from 22 patients, among whom we were able to make a complete analysis of 11 patients (8 males, 3 females; mean age: 45.4±12.0 years, 23 to 59 years) who underwent LTx during the study period were included. Peripheral blood samples were collected at pre-transplantation, 24 h post-transplantation, and on Day 7 after transplantation into ethylenediaminetetraacetic acid (EDTA) containing blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were transferred to the Immunology Department, Faculty of Medicine, Yeditepe University in 2 h after collection in special transportation boxes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Life Sciences, USA) using density gradient centrifugation. Then, PBMCs were frozen at -20°C for subsequent DNA extraction. The DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Lloyd St N, Manchester, UK) in accordance with the manufacturer's instructions.

Preparation of *KREC* signal joints and reference gene (T cell receptor alpha constant- TCRAC)

Lymphocyte subpopulations and the population of naïve B cells defined by CD19 phenotype gradually decreases with age/age-related accumulation of immune experience resulting from contact with foreign antigens. B cells are manifested by a gradual shift from immature to mature status, with the total number of B lymphocytes gradually decreasing since the neonatal period along with an increasing ability to respond to foreign antigen.[10]

In almost all studies where *KREC* level is measured, *TCRAC* is used as the reference gene in the quantitative PCR method.[11-15] T cell receptors (TCR) are very important in Ig synthesis and major histocompatibility complex (MHC)-dependent antigen recognition via B cell. Each TCR gene locus contains V and J gene segments. In addition, as in the Ig gene locus, D segment is present in β and δ regions. The receptor formation of the T cell occurs similar to the V (D) J gene rearrangement in the Ig genes.[7] While the gene segment (TREC) carrying the signal segment in the T cell division does not duplicate itself, the coding segment *(*TCRAC*)* is transferred to newly formed cells. The gene rearrangement mechanism in T cells is similar to formation of immunoglobulins. Since TCRAC has two copies in each cell, the quantitative results obtained in molecular studies are evaluated by dividing it into two.[9,14-17]

Five healthy children (3 males, 2 females, 36.6±33.5 month) between six month and three years of age were selected for preparation of the standart curve in real-time PCR. They were recruited during routine pediatric outpatient visits for conditions not related to immunological or infectious diseases. All healthy control patients underwent a thorough clinical evaluation and were screened for a normal blood cell count.

The DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The fragment of SJ *KRECs* was prepared by amplifying the DNA extracted from the blood of each child with specific primers (forward: 5'-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3' and reverse: 5'-AGG AGC CAG CTC TTA CCC TAG AGT-3') and the specific primers for TR alpha constant *TCRAC* (forward: 5'-TGG CCT AAC CCT GAT CCT CTT-3' and reverse: 5'-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3') using conventional PCR. After optimization studies, we determined an initial heating at 95°C for 2 min for *KRECs*, followed by 45 cycles of denaturation at 95°C for 10 sec, primer annealing at 58°C for 30 sec, and elongation at 72°C for 1 min. Additionally, final elongation step was performed at 72°C for 10 min by 1 cycle. Also, we used the same protocol for optimal PCR condition in *TCRAC* gene, except for primer annealing temperature being optimized at 62°C for 30 sec. We evaluated whether the correct genes after conventional PCR were obtained by gel electrophoresis for both genes (*KRECs* 90 bp, TCRAC 80 bp). The Qiaquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) was used to purify amplification after gel electrophoresis.

Real-time PCR for *KRECs* and *TCRAC*

The absolute numbers of patient *KRECs* and *TCRAC* at different periods were detected by a real-time PCR assay performed on the real-time PCR (Applied Biosystem Step One Plus) in duplications. The SJ *KREC* forward primer (5'-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3') and reverse primer (5'-AGG AGC CAG CTC TTA CCC TAG AGT-3') and probe (5'-VIC-TCT GCA CGG GCA GCA GGT TGG-TAMRA-3'), and *TCRAC* (forward 5'-TGG CCT AAC CCT GAT CCT CTT-3', reverse 5'-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3' and probe 5'-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3') were designed. Primer specificities were checked by Basic Local Alignment Search Tool (BLAST) search (http://www.ncbi.nlm.nih.gov/blast/), which confirmed their uniqueness.

We used a previous protocol designed by Sottini et al.[9] Briefly, PCR reactions were developed in a final volume of a 20 μL mixture, consisting of 10 μL of 2× TaqMan Universal PCR master mix (AppliedBiosystems) and primers and probes for SJ *KRECs* at the final concentration of 900 nM and 200 nM, respectively. Amplification of the *TCRAC* reference gene and *KREC* gene was performed in the same plate with the same concentrations of specific primers and probes. The *KREC* and *TCRAC* copy numbers were obtained by extrapolating the respective sample quantities from the standard curve obtained by serial dilutions (10^7, 10^5, 10^3, and 10^1) which were amplified in each PCR plate. A standard curve was included in every PCR reaction. The number of *KRECs* per 10^6 PBMC was calculated using the following formula:

$$\frac{\text{mean of } KREC \text{ quantity} \times 10^6}{\text{mean of } TCRAC \text{ quantity}/2}$$

The mean quantity of *TCRAC* was divided by two, as there are two *TCRAC* gene copies in each cell; i.e., one for each chromosome. This value, together with the lymphocyte plus monocyte count (which were the cells obtained in PBMC preparation) in 1 mL of blood, was utilized to calculate the absolute number of *KRECs* per mL of blood (copies/mL) that is = (*KRECs* per 1×10^6 PBMC) × (lymphocyte plus monocyte count in 1 mL of blood)/10^6. Each experimental procedure was accepted, only if the standard curve slopes ranged between -3.55 and

-3.30, PCR efficiencies varied from 91 to 100%, and correlation coefficients (R^2) were higher than 0.998.[9]

Treatment protocols

In immunosuppressive treatment after transplantation, in addition to triplet therapy comprising tacrolimus, mycophenolate mofetil, and prednisolone, an induction therapy with basiliximab 20 mg was administered on the day of transplant and four days post-transplant. After the end of the transplantation, continuous infusion was started with tacrolimus at a dose of 1 mg/kg/d and, then, continued with oral administration of 0.025 mg/kg every 12 h. Mycophenolate mofetil 1,000 mg was administered in the operating room during the transplant. Methylprednisolone 250 mg was administered prior to implant and after the reperfusion and, then, for the first postoperative days at a dose of 125 mg intravenously three times, 1 mg/kg methylprednisolone daily for one week and subsequently tapered to prednisone 0.5 mg/kg/d. Thirty days after the LTx, methylprednisolone was weaned by 5 mg every two weeks and treatment was switched to prednisone 20 mg/day by six to eight weeks. The dose of prednisone was reduced to 5 mg/day within three months.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The Shapiro-Wilk test was used to test the normal distribution assumption of parameters. The Kruskal-Wallis test was used to compare the non-normally distributed quantitative attributes of diagnosed groups. The Mann-Whitney U test was utilized to compare different groups' non-normally distributed parameters such as sex, and forced expiratory volume in 1 sec (FEV1) values of the patients; intragroup comparisons of *KREC*, blood parameters, age versus *KRECs* overtime and sex versus *KRECs* overtime were performed using the Wilcoxon signed-rank test. The Spearman correlation analysis was used to investigate the dependence between variables. A p value of <0.05 was considered statistically significant.

RESULTS

Demographic characteristics and laboratory findings of the patients are summarized in Table 1.

KRECs in patients before and after transplantation

Real-time PCR results for *KRECs* were obtained first by comparing with the standard curve from *KREC* levels of DNA obtained from healthy pediatric blood other than plasmid DNA. For both genes, the calculation was made with the mean values of the patients according to the number of DNA copies in different dilutions. As a result, the mean 24-h *KRECs*/10^6 PBMC was higher than before transplantation (5×10^5; 1×10^5) and on Day 7, *KRECs*/10^6 PBMC was higher than the measurement

Table 1. Patient characteristics

Patients	Sex	Age/year	Prim. diagnosis	Other diagnosis	FEV1 (%)	Smoking/year
P9	F	57	IPF	DM	62	No
P10	M	39	IPF	DM	78	No
P11	M	36	Bronchiectasis	No	32	No
P12	M	49	COPD	No	23	30
P13	M	41	Silicosis	No	27	No
P15	M	32	Bronchiectasis	No	N/A	No
P16	F	53	IPF	No	N/A	No
P17	M	51	COPD	No	13	30
P19	M	59	IPF	TBC	44	No
P20	F	23	CF	TBC	23	No
P22	M	59	COPD	TBC	26	16

FEV1: Forced expiratory volume in 1 sec before transplantation; IPF: Idiopathic pulmonary fibrosis; DM: Diabetes mellitus; COPD: Chronic obstructive pulmonary disease; TBC: Tuberculosis; CF: Cystic fibrosis; N/A: Not applicable; Samples were taken from 22 patients and 11 were included in the study.

Table 2. KREC production of patients before and after transplantation

Patients	Sample times	10^6 (PMBC)	KRECs (mL)
P9	Preop	38×10^5	800.71
	Postop 24th h	1×10^5	3.78
	Postop 7th day	0.3×10^5	1.47
P10	Preop	7×10^5	182
	Postop 24th h	2×10^5	0.02
	Postop 7th day	5×10^5	40188
P11	Preop	1×10^5	92.13
	Postop 24th h	41×10^5	164.49
	Postop 7th day	452×10^5	13567.16
P12	Preop	7×10^5	205
	Postop 24th h	4×10^5	94.42
	Postop 7th day	10×10^5	15.52
P13	Preop	0.5×10^5	10.87
	Postop 24th h	1×10^5	16.93
	Postop 7th day	18×10^5	170.23
P15	Preop	74×10^5	2756.15
	Postop 24th h	0.9×10^5	3.63
	Postop 7th day	69×10^5	768.67
P16	Preop	2×10^5	91
	Postop 24th h	10×10^5	224.59
	Postop 7th day	12×10^5	88.79
P17	Preop	17×10^5	365.45
	Postop 24th h	1×10^5	17.27
	Postop 7th day	3×10^5	32.91
P19	Preop	0.3×10^5	11.53
	Postop 24th h	380×10^5	3421.31
	Postop 7th day	155×10^5	7910.7
P20	Preop	51×10^5	1553
	Postop 24th h	153×10^5	1687.43
	Postop 7th day	246×10^5	2461.46
P22	Preop	0.02×10^5	0.11
	Postop 24th h	7×10^5	85.4
	Postop 7th day	1×10^5	53.64

KRECs: Kappa-deleting recombination excision circles; PMBC: Peripheral blood mononuclear cell; Preop: Pre-operation; Postop: Post-operation; Numerical data are shown in mean values.

at 24 h (45×10^5; 5×10^5), indicating that B cell kinetics tended to increase after transplantation. However, the difference was not statistically significant at different time points ($p>0.05$) (Table 2).

KRECs in different disease groups

All patients were divided into groups according to their primary indications, including idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), and others. There was no significant correlation between *KREC* numbers among these groups ($p>0.05$) (Table 3 and Table 4).

KRECs according to age

Eleven patients were divided into two different age groups and, on Day 7, *KREC* copies per 10^6 of blood

Table 3. Statistical comparison of KRECs (10^6 PMBC) between the three primary indication groups

	Primary indications						p^1
	IPF		COPD		Others[b]		
KRECs (10^6 PMBC)	Mean±SD	Median	Mean±SD	Median	Mean±SD	Median	
Preop	$15\times10^5\pm19\times10^5$	7×10^5	$6\times10^5\pm8\times10^5$	4×10^5	$32\times10^5\pm37\times10^5$	26×10^5	**0.521**
Postop 24th h	$4\times10^5\pm4\times10^5$	2×10^5	$98\times10^5\pm187\times10^5$	5×10^5	$49\times10^5\pm71\times10^5$	21×10^5	**0.931**
Postop 7th day	$6\times10^5\pm6\times10^5$	5×10^5	$40\times10^5\pm76\times10^5$	2×10^5	$121\times10^5\pm220\times10^5$	158×10^5	**0.075**
Preop-postop 24th h p^2	0.593		0.715		0.465		
Preop-postop 7th day p^2	0.593		1.000		0.144		
Postop 24th h-postop 7th day p^2	0.285		0.144		0.068		

KRECs: Kappa-deleting recombination excision circles; PMBC: Peripheral blood mononuclear cell; IPF: Idiopathic pulmonary fibrosis; COPD: Chronic obstructive pulmonary disease. SD: Standard deviation; Preop: Pre-operation; 1 Kruskal-Wallis test; 2 Wilcoxon sign-rank test; b Other primary indication; p>0.05; bronchiectasis, silicosis and cystic fibrosis.

Table 4. Statistical comparison of KRECs (mL) between the three primary indication groups

	Primary indication						p^1
	IPF		COPD		Others[b]		
KRECs (mL)	Mean±SD	Median	Mean±SD	Median	Mean±SD	Median	
Preop	$0.035\times10^5\pm0.038\times10^5$	182	$0.014\times10^5\pm0.017\times10^5$	108.3	$0.11\times10^5\pm0.13\times10^5$	822.6	**0.618**
Postop 24th h	$0.008\times10^5\pm 0.01\times10^5$	36.8	$0.09\times10^5\pm0.1\times10^5$	89.9	$0.04\times10^5\pm0.08\times10^5$	90.7	**0.750**
Postop 7th day	$1\times10^5\pm2\times10^5$	88.8	$0.2\times10^5\pm0.3\times10^5$	43.3	$0.4\times10^5\pm0.6\times10^5$	1615.1	**0.375**
Preop-postop 24th h p^2	0.285		1.000		0.715		
Preop-postop 7th day p^2	1.000		1.000		0.465		
Postop 24th h-postop 7th day p^2	1.000		1.000		0.068		

KRECs: Kappa-deleting recombination excision circles; IPF: Idiopathic pulmonary fibrosis; COPD: Chronic obstructive pulmonary disease. SD: Standard deviation; Preop: Pre-operation; 1 Kruskal-Wallis test; 2 Wilcoxon sign-rank test; b Other primary indication; p>0.05; bronchiectasis, silicosis and cystic fibrosis.

were 836×10^5 for the age group less than 45 years of age, and 37×10^5 for the age group over 45 years of age. There was an only positive correlation between *KRECs* copies per 10^6 of blood and age on Day 7 ($p<0.05$) (Table 5).

KRECs according to sex

There were only three female patients. The analysis of *KREC* results according to sex did not show any significant differences in the number of *KRECs* ($p>0.05$) (data not shown).

Correlation between *KRECs* and percentage of FEV1

The FEV1 percentage is an important parameter during the course of clinical follow-up of primary disease and transplant follow-up for patients undergoing LTx. We examined the relationship between the means of *KRECs* and FEV1 percentage. Pre-transplantation for patients were two separated groups according to the percentage of FEV1 under 50 and over. There was no significant correlation between *KRECs* and FEV1 percentage ($p>0.05$).

Table 5. KRECs results according to age groups

	Age groups				
	<45 years		>45 years		p^1
KRECs (10^6PMBC)	Mean±SD	Median	Mean±SD	Median	
Preop	$22\times10^5\pm32\times10^5$	4×10^5	$13\times10^5\pm15\times10^5$	7×10^5	**1.000**
Postop 24th h	$96\times10^5\pm150\times10^5$	21×10^5	$5\times10^5\pm3\times10^5$	4×10^5	**0.584**
Postop 7th day	$836\times10^5\pm1808\times10^5$	112×10^5	$37\times10^5\pm5\times10^5$	1×10^5	**0.011***
Preop-postop 24th h p^2	0.345		0.500		
Preop-postop 7th day p^2	0.116		0.345		
Postop 24th h-postop 7th day p^2	0.249		0.500		

KRECs: Kappa-deleting recombination excision circles; PMBC: Peripheral mononuclear blood cell; IPF: Idiopathic pulmonary fibrosis; COPD: Chronic obstructive pulmonary disease. SD: Standard deviation; Preop: Pre-operation; 1 Mann-Whitney U test; 2 Wilcoxon sign-rank test; * p<0.05.

Table 6. Evaluation of lymphocyte and leukocyte levels in patients before and after transplantation

	Lymphocytes		Leucocytes	
	Mean±SD	Median	Mean±SD	Median
Preop	2.05±1.11	1.6	10.14±3.74	9.4
Postop 24th h	0.41±0.19	0.5	22.15±11.89	21.9
Postop 7th day	1.08±1.55	0.5	12.55±4.84	13.1
Preop-postop 24th h p	0.004*		0.008*	
Preop-postop 7th day p	0.154		0.100	
Postop 24th h-postop 7th day p	0.233		0.026*	

SD: Standard deviation; Preop: Pre-operation; Postop: Post-operation; Wilcoxon signed-rank test; * p<0.05.

Table 7. Comparison of mean leukocyte and lymphocyte levels and KRECs results in patients before and after transplantation

Leukocytes	KRECs (10^6 PBMC)	KRECs (mL)
Preop	0.884	0.947
Postop (24th h)	0.060	0.043*
Preop (7th day)	0.853	0.298
Lymphocytes	**KRECs (10^6 PBMC)**	**KRECs (mL)**
Preop	0.413	0.293
Postop (24th h)	0.431	0.159
Postop (7th day)	0.673	0.538

KRECs: Kappa-deleting recombination excision circles; PMBC: Peripheral mononuclear blood cell; Preop: Pre-operation; Postop: Post-operation; Spearman's Rho correlation test; * p<0.05.

KRECs and blood count results of patients

When the lymphocyte absolute values of 11 patients who underwent LTx were compared at 24 h and Day 7 and after the transplantation, the decline in the mean lymphocyte count at 24 h was statistically significant compared to the preoperative period (p=0.004). When the absolute values of leukocytes are considered, there was a statistically significant increase at 24 h compared to pre-transplantation period (p=0.008). On Day 7 after transplantation, the mean of absolute leukocyte values decreased significantly compared to ones at 24 h (p=0.026) (Table 6). There was a positive correlation between the mean value of *KRECs* (mL) and the mean value of lymphocytes at 24 h after transplantation (p=0.043) (Table 7).

Four of the 11 patients died in the year following the transplantation with a one-year mortality rate of 36% and a survival rate of 64%. Unfortunately, we were not able to follow the patients for a longer period of time.

DISCUSSION

Solid organ transplants have started years ago in Türkiye. However, introduction of LTx in Türkiye was late for several reasons. One of the most important reasons was the donor shortage. The first pediatric heart-LTx patient was operated in 1998, but the first long-time survivor of LTx in Türkiye was a patient who underwent a single-LTx in 2009.[2] Therefore, the small sample size in the present study is associated with low number of LTx in our country.

Monitoring early immune response in organ transplants is critical for acute rejections that occur after transplantation. This is an area of interest for both surgeons and immunologists. In 2015, a symposium was held on antibody-mediated rejection in cardiac transplants at the International Society for Heart and Lung Transplantation (ISHLT) meeting. Outcomes of this symposium is discussed in detail in an article published by Colvin et al.[18] As indicated, development of new markers for better diagnosis and follow-up of antibody-mediated reactions are necessary in solid organ transplantations, and our preliminary study is a humble attempt in this respect.

Mansuroglu et al.[19] reported that cytoimmunological monitoring of patients is a non-invasive, easy and effective method in the early diagnosis of organ rejection after heart transplantation, particularly during hospitalization. The authors observed that changes in the CD4/CD8 ratio from the blood samples of the patients provided information about the infection or rejection.

In the present study, we examined the early immune response developed through B cells in patients undergoing LTx by current *KREC* assay procedure. The *KREC* is used as a marker for evaluating newly produced B lymphocytes. The *KRECs* consist of 50% of Ig light chain recombination of differentiated B cell progenitors and do not match their selves in subsequent B cell proliferation.[9] The *KRECs* can be assessed by molecular analysis for demonstrating the B cell outflow from bone marrow. With a very stable structure, the *KRECs* remain detectable in blood for a period of time and maintain it. The ratio between genomic coding part and signal part shows replication condition of subtypes of B lymphocytes and B cell neogenesis.[6,12] The quantification of *KRECs* was initially used to determine the number of developing B lymphocytes in the bone marrow of children with B-precursor or acute lymphoblastic leukemia treated with allogeneic human stem cell transplantation. However, as *IGK* gene deletion occurs physiologically in all B lymphocytes that fail to productively rearrange the *IGK* genes on one or both alleles, the number of *KRECs* has been proposed to be a quantitative marker of bone marrow output in all individuals.[8]

Piatosa et al.[10] described a pool of naïve CD19±IgD±CD27-B lymphocytes composing the largest B cell subset in all tested age groups (292 children and young adults aged 0 to 31 years, all of them healthy). While percentage of total B lymphocyte increases with age, it has been shown that naïve B lymphocyte subtypes were decreasing after age 2. Duchamp et al.[20] reported that the naive B-cells percentage declined between six months and eight years of age, and this definition of reference intervals for pediatric B-cell levels should facilitate the screening and diagnosis of various B-cell immunodeficiencies and the number of immature/transitional and naïve B cells remains stable during adulthood.[21,22] Therefore, in the present study, we used pediatric blood samples to set-up our protocol and showed any change in *KREC* results to ensure that the level of naïve B lymphocytes was high and independent of the age-increasing antibody response.

The *KREC* levels are determinant in developing immune response in pediatric patients with adenosine deaminase deficiency and in human immunodeficiency virus (HIV)-positive adult patients receiving retroviral treatment.[13,14] Detection of pre- and post-treatment quantity of *KRECs* is an indicator for reconstitution of the immune

system.[14] In some of the European countries, the quantity of *KRECs* in pilot studies as a newborn screening test is used for characterizing diseases by identifying lymphopenia for newborns having immunodeficiency.[16,17] In a recent study, Kwok et al.[23] reported that the level of *KRECs* obtained by real-time PCR should be used in the diagnosis of primary immune diseases.

There are several studies detecting the level of *KRECs* after hematopoietic stem cell transplantation for primary immune deficiencies. These studies have shown that *KRECs* levels can be used for long-term follow-up of the B cell production.[12,15] It was found that most of the detectable B cells were transporting signal part, supporting the B cell neogenesis. Thus, duration of long-term intravenous Ig treatment after transplantation can be shortened and development of a personal protective treatment is possible. Due to prescribing intensive diets suppressing immune system and long-term Ig replacement treatment after LTx, *KRECs* analysis may serve as a very important tool for those patients.[15] In our study, *KREC* analysis was performed in blood samples in three different time-periods; before transplantation, 24 h after transplantation and on Day 7. When the results were compared between these time-periods, there was a quantitative difference in the mean values, although no statistically significant difference was found. An increase was detected in the *KREC* levels (per 10^6 cell) at 24 h and on Day 7 after transplantation compared to the *KREC* value before transplantation. There was no significant difference between the *KREC* levels before transplantation and at 24 h; however, an increase was found on Day 7. Considering that immunosuppressive treatments administered to patients undergoing transplantation have an impact on B cell production,[24] we believe that *KREC* analysis can be performed with longer follow-ups to demonstrate early adaptive immune response in transplantation patients.

The properties of immune system changes as one gets older, and some components of the immune system is less effective in elderly. It is well known that the response against infection and vaccine decreases in this population. It is demonstrated that different B type cells change by age and CD27+IgD+ cells that are IgM memory cells decrease in elderly.[25] A study by Serena et al.[26] consisting 37 adult patients with immunodeficiency and 78 healthy adults evaluated the *KREC* levels (copy count/10^6) with monocyte and lymphocyte values (mL) analyzed by real-time PCR method and indicated that, in the patient group, the median value of *KRECs* was lower than in the control group and no change was observed according to sex and age category. Similar to our study, no change was observed in relation to sex in the control group, while the *KREC* level was stable as the age increased. In our patient group, the *KREC* level (10^6) of the patients over the age of 45 years and average copy amount (mL) on Day 7 was higher than in patients under the age of 45 years. Along with aging, decrease in co-stimulatory molecules affecting B cells, defects in incoming signals to B cell receptors and reduction in helper T cell functions which are necessary for germinal center structure lead to reduction in the high affinity antibody response of B cells.[27] This can be explained with the decreased number of pre-B cells in bone marrow, despite the lack of any decrease in immature B cell division and transportation to periphery related to the age.

There is a need for a newly produced T and B lymphocyte resource for maintaining immunity in patients with chronic lymphocytic leukemia (CLL) which is characterized by B lymphocyte accumulation and result from Ig heavy chain mutations in organs such as bone marrow, lymph nodes, and spleen related to the defects of humoral and cellular immune response.[28] The *KRECs* cannot be found in cells having cellular division more than once without making a discrimination between normal and leukemic populations. The aforementioned study consisting of early-stage naïve-patients and control group found that lower quantity of *KRECs* per 10^6 PBMC compared to the control group was related to a decrease in newly produced B cells through dominance of enlarging leukemic cells.[28] It was also emphasized that the decreased number of *KRECs* in blood indicated a real decline in newly produced B cells in patients compared to controls. No significant correlation was found in the comparison of the increased rate of lymphocytes with *KRECs* levels in CLL patients. In the present study, despite the lymphocyte count at 24 h in the patient group, no significant decrease was found compared to pre-transplantation values. These results support the idea that changes in lymphocyte counts for patients undergoing LTx may also be related to *KREC* levels, indicating the interpersonal differences.[28]

Neutrophils play a very critical role in the pathogenesis of COPD and the increased neutrophil levels have been shown to be associated with pulmonary involvement. Even so, the relationship between the amount of peripheral blood cells of other leukocyte groups and pulmonary disease, allergy, and markers of airway obstruction is unclear. For this purpose, in a study evaluating the effects of leukocytes in

peripheral blood on COPD symptoms and pulmonary functions, neutrophil counts were related to the increased symptoms related to chronic respiratory tract changes and decrease in FEV1 value.[29] In our study, the increase in neutrophil values between the time-periods could be associated with pulmonary diseases, as well as increased chemokines due to degeneration of fibrous tissue of the lung. There was a correlation only at 24 h between blood parameters and *KREC* levels, *KRECs*/leukocyte number and *KRECs*/hemoglobin. It also showed the presence of active inflammation. A maximum increase in leukocyte cells and its relation to the increased *KRECs* copy counts at 24 h explain the presence of inflammation. The FEV1 reflects the decrease in pulmonary functions and, thus, is an important marker in prognosis of pulmonary diseases. Acute rejection is typically characterized by decreased FEV1 value along with weakness, fever, cough, leukocyte increase, and gastrointestinal complaints. Tissue rejection may develop without any significant increase in the FEV1 value.[30] It is estimated that only 11% of the patients having a FEV1 value over 25%, which is accepted as the most important indicator for survival of patients undergoing transplantation, can survive more for a minimum of one year.[19] In the present study, a significant correlation could not be found between *KREC* levels of two groups having a FEV1 value under and over 50% before transplantation. Thus, we suggest that the patients should be followed at least until Day 7 of transplantation and further studies are necessary with the inclusion of FEV1 value after transplantation in long-term monitoring.

The main limitations to the present study include the limited number of patients due to lack of samples for all pre-determined time points, recent establishment of the LTx center, and limited research budget. Also, we were not able to follow patients for longer than a year.

In conclusion, the kappa-deleting recombination excision circle analysis is used for diagnosis of immunodeficiency disorders, particularly in demonstrating and observing immune system alterations after hematopoietic stem cell transplantation as an important tool for observation of B cell kinetics. Considering that survival rate is lower compared to other solid organ transplantations, long-term monitoring is of utmost importance in lung transplantation. In this preliminary study, the kappa-deleting recombination excision circle levels in relation to treatment modalities of the patients were not evaluated. Thus, measurement of kappa-deleting recombination excision circles within the B cell subtypes in future transplantations with more extensive patient groups would provide more insight into development of adaptive immune response following lung transplantation. The use of kappa-deleting recombination excision circles specific to different B cell subtypes as a routine biomarker may influence the prognosis and follow-up of the transplantation.

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Ethics Committee Approval: The study protocol was approved by the Yeditepe University Hospital Ethics Committee (date: 10.09.2015, no: 37068608-6100-15-1113). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from each patient.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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