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Role of circulating microRNAs in the immunopathogenesis of rejection following pediatric lung transplantation

Zhongping Xu, PhD^a, Wei Yang, PhD^b, Nancy Steward, MS^c, Stuart C. Sweet, MD^c, Lara Danziger-Isakov, MD, MPH^d, Peter S. Heeger, MD^e, and T. Mohanakumar, PhD^a

^aNorton Thoracic Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ, US

^bDepartment of Genetics, Washington University School of Medicine, St. Louis, MO, US

^cDepartment of Pediatrics, Washington University School of Medicine, St. Louis, MO, US

^dCincinnati Children's Hospital Medical Center, Cincinnati, OH, US

^eDepartment of Medicine, Recanati Miller Transplant Institute and Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, NY, US

Abstract

BACKGROUND—Acute rejection (AR) and development of chronic rejection, bronchiolitis obliterans syndrome (BOS), remain major limiting factors for lung transplantation (LTx). This retrospective study is to identify differentially expressed circulating microRNAs (miRNAs) that associate with development of AR and BOS in pediatric lung transplant recipients (LTxR).

METHODS—We determined the circulating levels of 7 selected candidate miRNAs in 14 LTxR with AR, 7 with BOS, and compared them against 13 stable pediatric LTxR at 1, 6, and 12 months following LTx. In addition, 6 AR, 7 BOS and 8 stable pediatric LTxR, 16 AR, 17 BOS and 16 stable adult LTxR were included for validation.

RESULTS—MiR-10a, -195, -133b were significantly lower in AR and miR-144, -142-5p, -155 were higher in AR compared to stable ($p < 0.05$). In addition, circulating levels of miR-134, -10a, -195, -133b were significantly lower and miR-144, -142-5p, -155 were higher ($p < 0.05$) with development of BOS. The receiver-operating characteristic demonstrated that miR-142-5p, miR-155, and miR-195 strongly discriminated patients with AR from stable LTxR ($p < 0.001$ for all comparisons): miR-142-5p (area under the curve (AUC)=0.854), miR-155 (AUC=0.876), and miR-195 (AUC=0.872). Further, miR-10a, miR-142-5p, miR-144, and miR-155 strongly discriminated BOS from stable LTxR ($p < 0.001$ for all comparisons).

Correspondence: T. Mohanakumar, PhD, Norton Thoracic Institute, St. Joseph's Hospital and Medical Center, 500 W. Thomas Rd., Ste. 500, Phoenix, AZ, 85013, United States, Tm.kumar@dignityhealth.org.

Authorship:

Author's contributions:

Z.P.X was responsible for study design, experiments, data analysis and interpretation, and article writing. W.Y. was responsible for the statistical analysis. L.D.I., P.S.H. and S.C.S. were responsible for study design, data interpretation, and article writing. N.S. was responsible for clinical samples collection. T.M. was responsible for study design, data interpretation, and article writing.

Disclosures statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclosure.

CONCLUSIONS—We demonstrated that differential expression of circulating miRNAs occurs in LTxR with AR and BOS, suggesting that they can provide not only important clues to pathogenesis but also may serve as potential noninvasive biomarkers for AR and BOS following pediatric LTx.

Introduction

Chronic allograft rejection, manifested histopathologically as obliterative bronchiolitis¹, is the single largest contributor to morbidity and mortality after lung transplantation (LTx), affecting up to 50–60% of patients who survive to 5 years after undergoing transplant². Acute rejection (AR) occurs in 30–50% of lung transplant recipients (LTxR) and is considered as the leading alloimmune-dependent risk factor for BOS^{3,4}. Although the onset of BOS can occur 1 or 2 years posttransplant, there are no diagnostic methods available during early posttransplant period. Therefore, there is a clear and urgent need to explore and develop new options for the early evaluation of patients at risk of AR and BOS after LTx.

MicroRNAs (miRNAs) have recently emerged as relevant factors in organ rejection because of their capacity to regulate a number of target genes that are key elements of both innate and adaptive immune responses^{5–7}. Anglicheau et al reported that miR-10a, -10b, 30c, 32, 142-3p, -223, and let-7c were aberrantly regulated in allograft biopsies undergoing AR in renal transplant recipients compared with those in stable allograft⁸. miR-326, -142-5p, -101, -144, -27a, -424, and -339-3p were increased in liver⁹ and heart¹⁰ transplant patients during AR. Recently, our group demonstrated that there were 74 miRNAs up-regulated and 6 miRNAs down regulated in LTxR with BOS when compared to stable¹¹, including miR-369-5p, miR-548d, miR-628-5p and miR-134. miRNAs are known to be highly stable in serum¹² and urine¹³, making them potential biomarkers for rejection status of solid organ allografts. However, at present there lacks functional evidences showing miRNAs are dysregulated in the serum of lung allografts with AR and BOS. The current study is to characterize the differentially expressed miRNAs in serum from pediatric LTxR and to define their impact on the development of AR and BOS following LTx.

Materials and Methods

This study was approved by the Washington University Human Studies Committee and all patients were enrolled following informed consent.

Patient selection

Forty-eight pediatric LTxR were selected from 7 academic medical centers that are members of the Clinical Trials in Organ Transplantation in Children (CTOTC-03) (Children's Hospital of Boston, Nationwide Children's Hospital, Baylor College of Medicine/Texas Children's Hospital, Washington University School of Medicine/St. Louis Children's Hospital, Children's Hospital of Philadelphia, Lucile Packard Children's Hospital at Stanford). Among these patients included, 20 had a diagnosis of biopsy-proved AR, and had no evidence of BOS 2 years after LTx (AR group); 7 were clinically diagnosed as BOS patients (BOS group) and 21 didn't develop AR and BOS (stable group). For the discovery pilot study, 14 AR, 7 BOS, and 13 stable patients were selected, and serum was collected at

1, 6, and 12 months after LTx (Table 1, Figure 1A). The pediatric subjects and samples for the discovery study were chosen as described in flowchart (Figure S1A). Within AR group, all 14 patients developed AR in 1 month (1.1 ± 0.3) after LTx, and successfully treated with no evidence of AR at 6 (5.9 ± 0.9) and 12 (12.3 ± 2.2) months after LTx. Further, these patients did not have any evidence for BOS. All of the 7 patients with BOS developed BOS at 12 months after LTx. All 13 stable patients maintained normal graft function at 1-year posttransplant. All biopsies were graded for rejection according to standard International Society for Heart and Lung Transplantation (ISHLT) criteria¹⁴. LTxR from the 3 groups of both discovery and validation studies were matched for ages, primary diseases, type of LTx, time from transplantation to index biopsy. All pediatric patients were treated with standard immunotherapy as tacrolimus, mycophenolate mofetil, and prednisone at baseline. All centers followed a uniform protocol for treatment of rejection (Table S1). There was no significant difference in PBMC cell number (per ml blood) between stable, AR and BOS group. We did not count the T, B cells separately. There were no patients with infection or treated with antibiotics at or before the sampling time points. All the patients had conventional graft histopathology together with concomitant assessment of miRNA expression in the allograft and serum taken at the time of biopsy. For validation, an independent cohort of 8 stable and 6 AR patients were enrolled, as well as 7 samples from different visit points of the same BOS cohorts because of limited pediatric BOS patients (Table S2). The pediatric subjects and samples for the validation study were chosen as described in flowchart (Figure S1A).

Adult LTx cohort included 16 AR, 17 BOS, and 16 stable patients (Figure S1B). These LTx were performed at Barnes Jewish Hospital/Washington University School of Medicine between January 2008 and June 2014. All of LTxR in 3 groups were matched for age, time from LTx, type of LTx, and underlying diagnosis (Table S3). The diagnosis of BOS was made by the criteria established by the ISHLT². The immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil, and prednisone.

TaqMan miRNA assay for individual miRNAs

Total serum RNA including small RNAs was isolated using Qiagen miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. In addition, 1 µg MS2 RNA as carrier RNA (Roche) was added during RNA purification steps in order to maximize the RNA yield and minimize purification efficiency variation. Individual miRNA analysis were performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA) as previously described¹¹. A synthetic RNA spike-in, *C. elegans* miR-39 miRNA mimic, was added prior to cDNA synthesis as a control for variation in reverse transcription efficiency. The PCR amplifications were carried out by incubation for 10 minutes at 95°C, followed by 40 amplification cycles at 95°C for 10 sec and 60°C for 1 min. All reactions were performed in duplicate. A cut off for unspecific amplification was set at the cycle threshold (C_t) = 35. miRNA with more than half of the C_t values > 35 per group were excluded from the analysis. The spike-in amplification values was used for quality control of each sample. Because U6 RNA and 5S rRNA are degraded in sera and a consensus housekeeping miRNA is lacking for quantitative RT-PCR analysis of serum miRNAs, we normalized miRNA concentration to serum volume. Relative expression was

calculated using the 2^{-Ct} method. Briefly, The relative expression of all the miRNAs [fold values ($2^{-(Ct - Ct_{\text{mean}})}$)] was obtained from the Ct value for each patient and each miRNA (Ct of the miRNA for a patient – mean Ct of the population)].

The candidate miRNAs were chosen for pilot screening based on previously published association with immune response, rejection, or transplantation^{8,11,15–17,18,19}. Twenty-five miRNA candidates were selected, including miR-518f¹¹, miR-369-5p¹¹, miR-10a^{11,18}, miR-98¹¹, miR-548d¹¹, miR-195¹⁵, miR-16¹⁵, miR-142-5p²⁰, miR-133b¹¹, miR-142-3p^{10,18,21}, miR-155¹⁸, miR-144^{10,12}, miR-223²², miR-148b²³, miR-148a^{23,24}, miR-152²³, miR-301a¹⁵, miR-134¹¹, miR-320^{25,26}, let-7a¹⁹, let-7b¹⁹, let-7c²², miR-21¹⁸, miR-31¹⁸, and miR-130a¹⁹.

Statistical Analysis

We applied a two-stage screening strategy (Figure 1B). In the prescreening stage for the cross-sectional study, longitudinal data with the linear longitudinal model were performed at a relaxed threshold without multiple testing corrections. Additional pair-wise comparisons were performed to find out at which timepoint for the expression differences of specific miRNAs levels. The candidate variants from 1st stage were then tested in the validation dataset, and Bonferroni procedure was used to adjust the significance level for p values at each locus to control the overall false positive rate at 0.05, with considering the number of tested variants. Results were shown as the mean $\pm$ standard deviation (SD). For evaluation of sensitivity and specificity, a receiver operating characteristics (ROC) curve was used to determine cut off points and to calculate the area under the curve (AUC) by using GraphPad PRISM 6.0.

Results

MiRNAs were selectively expressed in pediatric LTxR diagnosed with AR

The discovery pilot study contains a cohort of pediatric LTxR, including 14 AR, 7 BOS, and 13 Stable. Within the AR group, miRNA levels were measured at AR (1 month), posttreatment for AR (6 months), and 1 year posttransplant. MiRNA levels in stable and BOS groups were measured at time points similar to those of AR patients at time of AR (Figure 1A). We selected 25 candidate miRNAs for pilot longitudinal study screening. The expression levels of miR-518f, let7b, let-7c, miR-548d, miR-369-5p, and miR-301a were too low to provide conclusive results. We employed the remaining candidate miRNAs for our analysis. There was a significant down regulation of miR-10a, miR-195, and miR-133b levels and significant up regulation of miR-144, miR-142-5p, and miR-155 in serum from AR (1-month) compared to those after treatment of AR (6 months) and 1 year post-transplant (Figure 2). Other miRNAs were not significantly different over time in AR cohort, and all miRNAs levels showed no significant change over time in stable patients. These results, from our pilot discovery analysis, demonstrated that certain circulating miRNAs were differentially expressed in AR compared to those following treatment.

Pediatric LTxR diagnosed with BOS express differential miRNAs pattern

Expression levels of miR-134, miR-10a, miR-195, and miR-133b were significantly down regulated in the serum of BOS LTxR at 12 months compared to those prior to BOS. In contrast, miR-144, miR-142-5p, miR-155 showed significant upregulation in serum from BOS (Figure 2). There was no significant differential expression in any miRNAs measured at 12 months following transplant between AR and stable. These results demonstrated that miRNAs in serum were selectively regulated in BOS patients following pediatric LTx. In order to validate our findings we chose another independent set of both pediatric and adult LTxR who were either stable or diagnosed with AR or BOS.

Validation of differential serum miRNAs using pediatric and adult cohorts with AR

Another pediatric LTxR cohort including 6 AR, 7 BOS, and 8 stable was included for validation. In agreement with the pilot study, miR-10a, miR-195, and miR-133b levels were significantly lower (adjusted $p < 0.05$) in serum from AR when compared to stable. MiR-144, miR-142-5p, and miR-155 were significantly higher (adjusted $p < 0.05$) in AR compared to stable (Figure 3). To determine whether miRNAs were differentially expressed in pediatric recipients undergoing rejection are also differentially expressed in adult LTxR, 49 adult LTxR were enrolled, including 16 developed AR, 17 with BOS and the other 16 stable. We found that miR-10a was significantly lower (adjusted $p < 0.05$) in AR compared to stable; while miR-144, miR-142-5p, and miR-155 were significantly higher (adjusted $p < 0.05$) (Figure 4). There was a trend for reduction of miR-195, miR-133b expression, but not significant.

Validation of differential serum miRNAs using pediatric and adult cohorts with BOS

MiR-134, miR-10a, miR-195, and miR-133b were significantly lower (adjusted $p < 0.05$) in the independent pediatric LTxR with BOS. Expression of miR-144, miR-142-5p, and miR-155 was significantly higher (adjusted $p < 0.05$) in BOS compared to stable (Figure 3). We further validated these miRNAs in the adult LTxR cohort. MiR-134, miR-10a, miR-195, and miR-133b were significantly lower (adjusted $p < 0.05$) in BOS compared to stable; while expression of miR-144, miR-142-5p, and miR-155 was higher (adjusted $p < 0.05$) in BOS (Figure 4). In addition, miR-144 demonstrated significantly higher expression levels in adult BOS patients compared to adult AR patients (Figure 4); there was a trend of increased expression of miR-144 but without significant difference in pediatric LTxR between AR and BOS (Figure 3). miR-134 was significantly downregulated in pediatric BOS patients compared to AR patients (Figure 3), however we did not see any differences in the expression of miR-134 in the adult patients (Figure 4). Further, a cut-off value for the expression of 0.7 for miR-134 could discriminate AR and BOS ($p < 0.05$) in pediatric patients both in the discovery analysis and validation cohort.

ROC analysis of circulating miRNAs involved in AR

We performed a ROC analysis to calculate the relationship between sensitivity and specificity for combined pediatric LTxR cohorts (discovery and validation cohorts), 20 AR patients versus 21 stable patients. The ROC analysis showed that miR-142-5p, miR-155, and miR-195 could discriminate patients with AR from stable patients [miR-142-5p: AUC = 0.854, $CI_{95\%} = (0.718-0.99)$, sensitivity = 75%, specificity = 90.5%; miR-155: AUC =

0.876, $CI_{95\%} = (0.743-1.01)$, sensitivity = 81.2%, specificity = 85.7%; miR-195: AUC = 0.872, $CI_{95\%} = (0.738-1.005)$, sensitivity = 76.8%, specificity = 89.5%, $p < 0.001$ for all comparisons] (Figure 5 and Table S4). We further combined multiple miRNAs together to test if the ROC improves, and that higher AUC was found when miR-155 and miR-195 were combined (AUC = 0.945, $CI_{95\%} = (0.893-1.095)$, sensitivity = 92.5%, specificity = 96.6%, $p < 0.0001$, Figure 5 and Table S4).

ROC analysis of circulating miRNAs involved in BOS

Similarly, ROC curves were performed by comparing with pediatric patients with BOS vs. stable allograft function (combined patients from discovery and validation cohorts) to evaluate the relationship between sensitivity and specificity for 7 differentially expressed miRNAs. MiR-10a, miR-142-5p, miR-144, and miR-155 showed significant area-under-the-curve values (miR-10a: AUC = 0.811, $CI_{95\%} = (0.688-0.954)$, sensitivity = 72%, specificity = 82%; miR-142-5p: AUC = 0.821, $CI_{95\%} = (0.723-0.988)$, sensitivity = 77%, specificity = 80%; miR-144: AUC = 0.85, $CI_{95\%} = (0.765-0.998)$, sensitivity = 86%, specificity = 85%; and miR-155: AUC = 0.808, $CI_{95\%} = (0.734-0.957)$, sensitivity = 78%, specificity = 82%; $p < 0.001$ for all comparisons, Figure 6 and Table S4). Further combination of multiple miRNAs for ROC analysis shown that miR-142-5p+miR-144 combined gave the highest AUC (AUC = 0.938, $CI_{95\%} = (0.882-1.079)$, sensitivity = 92%, specificity = 96%, $p < 0.0001$, Figure 6 and Table S4).

Discussion

Dysregulated expression of miRNAs is associated with initiation and progression of pathological processes, including graft rejection¹⁹. We have previously reported miR-518f, -369-5p, -10a, -98, -548d, and -133b were differentially expressed in LTxR with BOS when compared to stable¹¹. In the present study, we demonstrated that miR-10a, -195, -133b were significantly lower in pediatric LTxR with AR compared to those in stable and miR-144, -142-5p, -155 were significantly higher in AR. In addition, circulating levels of miR-134, -10a, -195, -133b (lower) and miR-144, -142-5p, -155 (higher) showed strong correlation with pediatric LTxR BOS (Figure 2). We further validated these differential serum miRNAs using both pediatric and adult cohorts (Figure 3 and 4). Therefore, our results demonstrate that circulating miRNAs were dysregulated in LTxR undergoing AR and BOS after LTx, and may serve as potential non-invasive biomarkers of lung allograft rejection in pediatric lung transplant recipients.

Several of the miRNAs found in this report at a higher level in AR and BOS compared to stable have shown to play important roles in innate and adaptive immunity. For example, miR-155 was upregulated in activated mouse macrophages and induce a broad range of inflammatory mediators²⁷. Lind et al.²⁸ demonstrated that there is increased expression of miR-155 following the activation of the T-cell receptor, and has been proposed to contribute in immunoglobulin class switching in B cells. In addition, evolutionarily conserved miR-155 plays an important role in regulating T helper cell differentiation and the germinal center formation to produce an optimal T cell-dependent antibody response. Our results demonstrating that miR-155 levels in the circulation was present in higher abundance in

acute and chronic rejection compared to stable, strongly suggest an important role for miR-155 in inducing an immune response directed against a transplanted organ. This is supported further by reports demonstrating that intragraft level of miR-142 was also higher in AR biopsies and renal transplant recipients with chronic antibody-mediated rejection when compared to stable renal allograft biopsies²⁰. MiR-142 expression is mostly restricted to lymphoid cells and is expressed at a lower level in differentiated Th1 cells or Th2 cells compared to naïve T cells^{29,30}. Our investigation showed miR-142-5p to be higher in AR and BOS suggesting that upregulation of miR-142-5p in lymphoid cells is associated with acute and chronic rejection after LTx, though the underlying mechanisms remain unclear. Reduction of miRNA expression can result in the development of autoimmunity³¹. In LTxRs we noted that circulating miR-10a, miR-195, and miR133b levels were significantly lower in patients diagnosed with BOS. MiR-10a have been shown to be indispensable *in vivo* for global Treg function under homeostatic conditions³², indicating that reduced miR-10a expression will inhibit Treg function resulting in loss of peripheral tolerance leading to immune responses to lung associated self-antigens (SAGs) which has been proposed to play a role in BOS following LTx. MiR-195 was previously found to target regulatory factor X5 (RFX5) expression¹⁵, which can activate MHC class II members. Results presented here demonstrated that miR-195 was lower in serum of LTxR with AR and BOS, demonstrating that dysregulated miR-195 in AR and BOS could participate in augmenting antigen presentation of donor allo-Ag as well as SAGs. Further, we have demonstrated the lower expression of circulating miR-133b in AR and BOS which is involved in IL-17 production in $\alpha\beta$ and $\gamma\delta$ T cells³³. Alloimmunity evidenced by *de novo* development of anti-donor MHC could also result in activation of Th17 responses leading to immune responses to lung associated SAGs (K- α 1 Tubulin and Collagen V) and BOS. Therefore, miR-133b may exert a critical role for Th17 response and modulate the progression of alloimmunity-induced autoimmunity following LTx.

AR episodes have been shown to be an important risk factor for development of BOS following LTx¹⁴. The results presented with ROC curve demonstrated that circulating miRNAs (miR-155 and -195 together) can discriminate with greater than 96% specificity and 92% sensitivity between pediatric LTxR with AR and those without (Figure 5). ROC analysis also demonstrated that miR-142-5p and -144 combinations can discriminate pediatric LTxR diagnosed with BOS from stable with greater than 92% sensitivity and 96% specificity (Figure 6). Therefore, these results strongly suggest that circulating miRNAs will be of potential value as a diagnostic marker for the immune status of transplanted lungs.

There are several limitations in this study mainly the sample size is small due to the fact that obtaining blood samples from pediatric patients is a challenge. Results have to be cross-sectionally validated in a larger sample-size cohort by using multivariable predictive models including potential confounding clinical variables. Furthermore, we did not perform any functional investigation of the identified miRNAs. Finally, we analyzed for selected miRNAs based on our earlier reports demonstrating association with acute and chronic rejection, therefore at present we cannot exclude other potentially relevant miRNAs which may be important for lung allograft rejection in children.

In summary, our study for the first time, has defined selected miRNAs which are involved in immune regulation following pediatric LTxR. Target prediction and signal pathway analysis demonstrated that circulating miRNAs (miR-144, -134, -10a, -195, -142-5p, -133b, and -155) can be involved in regulating TGF β signaling, T and B cell activation, Treg function, and Th17 activation as well as augmentation of both donor and SAg presentation by antigen presenting cells. This selected panel of circulating miRNAs may also be useful as a potential noninvasive biomarker for diagnosis of lung allograft rejection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	acute rejection
AUC	area under the curve
BOS	Bronchiolitis Obliterans Syndrome
LTx	lung transplantation
LTxR	lung transplant recipient
miRNA	microRNA
ROC	receiver operating characteristics
SAgs	self-antigens

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Figure 1.

Experiment design and work flow. **(A)** Longitudinal study design in the pilot study of pediatric LTx patients. For the discovery pilot study, 14 AR, 7 BOS, and 13 stable patients were selected, and serum was collected at 1, 6, and 12 months after LTx. Within the AR group, all 14 patients developed AR in 1 month after LTx, and after treatment, there was no AR found after 6 and 12 months after LTx. There was no clinical diagnosis of BOS at 1 and 6 months in the 7 patients with BOS, while all these patients developed BOS at 12 months after LTx. All 13 stable patients maintained normal graft function at 1 year posttransplant. **(B)** Statistical methods used in data analysis.

Figure 2. Longitudinal study for differential expression of miRNAs in serum. Quantitative expression of miR-144, -134, -10a, -195, -142-5p, -133b, and -155 was assessed by TaqMan RT-PCR on serum RNA samples from 13 stable LTxRs, 14 LTxRs with AR and 7 BOS LTxRs. Relative expression of these miRNAs was calculated and observed statistically differentially expressed compared to the stable group in 1 month. * $p<0.05$, ** $p<0.01$.

Figure 3.

Validation of differential miRNAs using independent pediatric patients. Quantitative expression of miR-144, -134, -10a, -195, -142-5p, -133b, and -155 was assessed by TaqMan RT-PCR on serum RNA samples from 8 stable LTxRs, 6 LTxRs with AR and 7 BOS LTxRs. Relative expression of these miRNAs was calculated and observed statistically differentially expressed compared to the stable group. Bonferroni corrections were performed for adjustment to control false positive rate at 0.05, with consideration the number of tested variants. * $p<0.05$, ** $p<0.01$.

Figure 4.

Validation of differential miRNAs using independent adult patients. Quantitative expression of miR-144, -134, -10a, -195, -142-5p, -133b, and -155 was assessed by TaqMan RT-PCR on serum RNA samples from 16 stable LTxRs, 16 LTxRs with AR and 17 BOS LTxRs. Relative expression of these miRNAs was calculated and observed statistically differentially expressed compared to the stable group. Bonferroni corrections were performed for adjustment to control false positive rate at 0.05, with consideration the number of tested variants. * $p<0.05$, ** $p<0.01$.

Figure 5. ROC curves for miRNAs that are significantly different in AR patients as compared to stable patients. ROC curve was performed with AUC for miRNA-142-5p, -155, -195 and combination of miR-155 and miR-195.

Figure 6. ROC curves for miRNAs that are significantly different in BOS patients as compared to stable patients. ROC curve was performed with area under the curve (AUC) for miRNA-10a, -142-5p, -144, -155 and combination of miR-142-5p and miR-144.

Table 1

Demographics and clinical characteristics of pediatric LTx patients.

	Stable (N=13)	AR (N=14)	BOS (N=7)
Sex,male,N(%)	8, 62%	7, 50%	5, 71%
Age (years)	13 ± 6	11 ± 7	12 ± 5
Etiology			
Cystic fibrosis	9	8	6
Pulmonary hypertension	2	4	1
BO	0	1	0
Interstitial pneumonitis	1	1	0
Others	1	0	0
Sampling time (months)			
M1	1.0 ± 0.4	1.1 ± 0.3	0.9 ± 0.3
M6	6.3 ± 1.2	5.9 ± 0.9	5.7 ± 1.3
M12	11.8 ± 1.8	12.3 ± 2.2	12.7 ± 4.1
AR status			
Grade A1	0	0	4
Grade A2 or higher	0	14	0
Grade B	0	5	4
Time to BOS (months)	NA	NA	11 ± 5.7

Abbreviations: BO = Bronchiolitis Obliterans; BOS = Bronchiolitis Obliterans Syndrome.