Transplant Immunology 42 (2017) 1–4

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Brief communication

Serum miRNAs as potential biomarkers for the bronchiolitis obliterans syndrome after lung transplantation

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ARTICLE INFO

Keywords:
Lung transplantation
Chronic lung allograft dysfunction
Bronchiolitis obliterans syndrome
Micro-RNAs
Biomarker

ABSTRACT

Lung transplantation (LTx) is the last treatment for patients suffering from end-stage lung diseases. Survival post-LTx is hampered by the development of the bronchiolitis obliterans syndrome (BOS) and diagnosis is often late. Given the urgent clinical need to recognize BOS patients at an early stage, we analyzed circulating miRNAs to identify possible stratification markers for BOS development post-transplantation. Therefore, pro-fibrotic (miR-21, miR-155), anti-fibrotic (miR-29a) and fibrosis-unrelated (miR-103, miR-191) miRNAs were analyzed in serum of end-stage lung disease patients and during LTx follow-up.

Significant elevated levels of serum miRNAs were observed for all investigated miRNAs in both chronic obstructive pulmonary disease and interstitial lung disease patients compared to healthy controls. The same miRNAs were also significantly increased in the serum of BOS + vs. BOS − patients. Most importantly, miR-21, miR-29a, miR-103, and miR-191 levels were significantly higher in BOS + patients prior to clinical BOS diagnosis.

We demonstrated that a selected group of miRNAs investigated is elevated in end-stage lung disease and BOS + patients, prior to clinical BOS diagnosis. Even if further research is expedient on the prognostic value of circulating miRNAs in BOS and lung conditions in general, these results strongly suggest that circulating miRNAs could be used as potential biomarkers for BOS development.

1. Introduction

Lung transplantation (LTx) is the last treatment option for patients suffering from end-stage lung diseases. Survival after LTx is severely hampered by the development of chronic lung allograft dysfunction, which can manifest in a restrictive form, restrictive allograft syndrome (RAS), or an obstructive form. The latter is defined as bronchiolitis obliterans (BO), and occurs in approximately 50% of LTx patients within 5 years after the transplantation [1]. BO is diagnosed via a surrogate marker, i.e. decline of the FEV_1 of 80% compared to baseline levels and is referred to as bronchiolitis obliterans syndrome (BOS). This diagnosis is often late and therefore there is urgent clinical need for novel biomarkers to identify patients at risk for BOS development at an earlier stage [2].

Micro-RNAs (miRNAs) are short non-coding RNAs, that inhibit gene expression at the post-transcriptional level by binding to the 3'UTR of target messenger-RNAs, thereby promoting their degradation or inhibiting translation [3]. Besides representing crucial endogenous regulators of gene expression within the cell, miRNAs can be found in biological fluids, including plasma, serum, breast milk etc., although their role in circulation is still largely unknown. The levels of circulating miRNAs were found to be either elevated or decreased in various transplantation settings, including kidney, heart, liver, and small intestine transplantation [4]. Interestingly, it is still unknown whether clinical parameters in LTx are also associated with levels of circulating miRNAs.

In this study we hypothesized that selected miRNAs could serve as stratification markers for patients who do or do not develop BOS after LTx and thereby function as novel diagnostic biomarker to identify patients at risk for BOS development. For this end, we analyzed the levels of different miRNAs in a cohort of LTx patients that did or did not develop BOS post-LTx. Furthermore, we investigated the levels of these

Abbreviations: BO, bronchiolitis obliterans; BOS, bronchiolitis obliterans syndrome; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; LTx, lung transplantation; miRNA, micro-RNA; RAS, restrictive allograft syndrome

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http://dx.doi.org/10.1016/j.trim.2017.04.002
Received 26 October 2016; Received in revised form 7 April 2017; Accepted 19 April 2017
Available online 28 April 2017

selected miRNAs in patients suffering from end-stage lung diseases. Our results show that a panel of selected miRNAs might prove to be beneficial to early identify patients who develop BOS post-LTx.

2. Patients and methods

2.1. Patients and sampling

All selected patients included in this study were transplanted at the Heart and Lung Center of the University Medical Center Utrecht in The Netherlands between May 2003 and September 2010. All patients gave informed consent and the study was approved by the medical ethical committee. Furthermore, all methods were carried out in accordance with the approved guidelines within our center.

Patient blood was drawn in serum tubes and centrifuged for 10 min at 2000 × *g*. Serum was isolated and stored at − 80 °C until further usage. For our analyses, we selected pre-LTx serum samples, drawn shortly prior to transplantation. For our follow-up experiments we used a quartile-based selection method [5]. We selected serum samples collected at 4 time points (quartiles) equally-distributed from the time of transplant until BOS diagnosis (quadrant-based selection). For clarification, the time between the transplantation date and the clinical diagnosis of BOS was divided by 4 to generate quartiles for each patient. Subsequently, serum obtained at each quartile was selected for further analysis. For each BOS + patient, a matched BOS − patient with similar follow-up length was selected in parallel. Therefore there is no difference in follow-up time between BOS + and BOS − patients, this is illustrated further in Table 1.

2.2. miRNA isolation and quantification

Serum RNA, including miRNAs, was extracted from 200 μl of serum isolated from patients and previously stored at − 80 °C, by using the miRCURY™ RNA Isolation Kit for Biofluids (Exiqon, Denmark) according to the manufacturer's instructions. cDNA was synthesized from 2,5 μl of serum-RNA by using individual miRNA-specific RT primers contained in the TaqMan Human miRNA assay in the presence of 3.3 U/μl MultiScribe RT enzyme (Lifetechnologies, USA), by using the following thermal cycler conditions: 10 min, 4 °C; 30 min, 16 °C; 20 min, 42 °C; 5 min, 85 °C. Circulating miRNA levels were quantified in duplicate from 3 μl cDNA, with TaqMan Fast Advance Master Mix and specific primers of the TaqMan Human miRNA assay, using the following amplification condition on the Quantstudio 12k flex Real-Time PCR system (Lifetechnologies, USA): 2 min, 50 °C; 20 s, 95 °C; 40 cycles of 1 s, 95 °C; 20 s, 60 °C. RTqPCR data were analyzed via the comparative threshold cycle method [6]. The abundance of each circulating miRNA was expressed as relative fold change (FC) as compared to the median level detected among all patients set as 1.

Table 1
Clinical and demographic profile of lung transplantation patients
Overview of selected BOS + and BOS − patients. No differences were observed between the matched BOS + and BOS- patients as indicated by the respective *p*-values. BOS: bronchiolitis obliterans syndrome, COPD: chronic obstructive pulmonary disease, CF: cystic fibrosis.

	BOS	Non BOS	*p*-Value
Total number	10	10	
BOS grade			
I	6	N.A.	
II	3	N.A.	
III	1	N.A.	
Onset of BOS (month)	35 (23–59)	N.A.	
Mean follow up (months)	43 (24–104)	60 (26–103)	0.105
Quartiles (months)			
I	9 (6–17)	9 (5–15)	0.861
II	18 (12 – 30)	18 (11–29)	0.986
III	27 (18–45)	28 (16–48)	0.832
IV	38 (24–61)	38 (24–63)	0.905
Type of transplantation			
Single	4	2	0.329
Bilateral	6	8	
Mean age (years)	43 (16–61)	43 (21–61)	0.957
Gender			
Male	2	2	1.000
Female	8	8	
Primary disease			
COPD	6	6	1.000
CF	4	4	

2.3. Statistics

Statistical analysis was performed using GraphPad Prism software version 6.02 (GraphPad Software, USA) and SPSS version 20 (IBM Corp., Armonk, NY). The normally distributed log2-transformed FC results were analyzed via usage of the Kruskal-Wallis test and two-way ANOVA and the Dunn's multiple comparisons test for multiple testing comparison with power of test set at $\alpha = 0.05$. Values for end-stage lung disease patients and healthy controls (HC) were tested for Gaussian distribution via the D'Agostino-Pearson omnibus normality test and subsequently analyzed via the Mann-Whitney test. A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Patients and miRNA selection

The cohort of end-stage lung disease patients consisted of patients suffering from chronic obstructive pulmonary disease (COPD, $n = 5$), cystic fibrosis (CF, $n = 5$), and interstitial lung disease (ILD, $n = 5$). All patients were treated with standardized immunosuppressive therapy consisting of tacrolimus, basiliximab, prednisone, and mycophenolate mofetil. Incidentally, patients that were defined as being at high risk for either CMV or EBV reactivation (CMV^-/EBV^- patient and CMV^+/EBV^+ donor) were treated with valganciclovir up until 6 months after transplantation. For follow-up analyses we included 10 BOS + and 10 BOS − patients matched for underlying disease prior to transplantation, age, and gender (Table 1), resulting in a total of 80 serum miRNA level determinations. None of the patients analyzed presented with episodes of acute rejection or infections. Also, no RAS was observed, as determined by international guidelines [1].

We hypothesized that ILD patients (diagnosis for lung allocation score: other pulmonary fibrosis) might present higher pro-fibrotic miRNA levels, because prolonged ILD is often associated with pulmonary fibrosis. Additionally, BOS is associated with extensive pulmonary fibrosis [2]. Therefore, we selected two pro-fibrotic miRNAs, miR-21 (5p, TaqManID: 000397) and miR-155 (5p, TaqManID: 002623), given their association with multiple fibrotic conditions, including different transplantation settings, in literature [7–9]. Based on previous knowledge on these conditions, we also selected an anti-fibrotic miRNA, miR-29a (3p, TaqManID: 002112), and control miRNAs unrelated to fibrosis, i.e. miR-103 (5p, TaqManID: 121218) and miR-191 (5p, TaqManID: 002299) [10]. All selected miRNAs are identified as potential biomarkers for various diseases, confirming there relative abundancy in serum [9,11–14].

3.2. Levels of selected serum miRNAs are elevated in end-stage lung disease patients compared to healthy controls

The qPCR analysis revealed that all the selected miRNAs were significantly increased in the pre-transplant serum of patients suffering from end-stage lung diseases as compared to healthy controls (HC, Fig. 1A–E). Moreover, when stratified per type of lung disease, all the serum miRNAs investigated were significant elevated in both COPD and

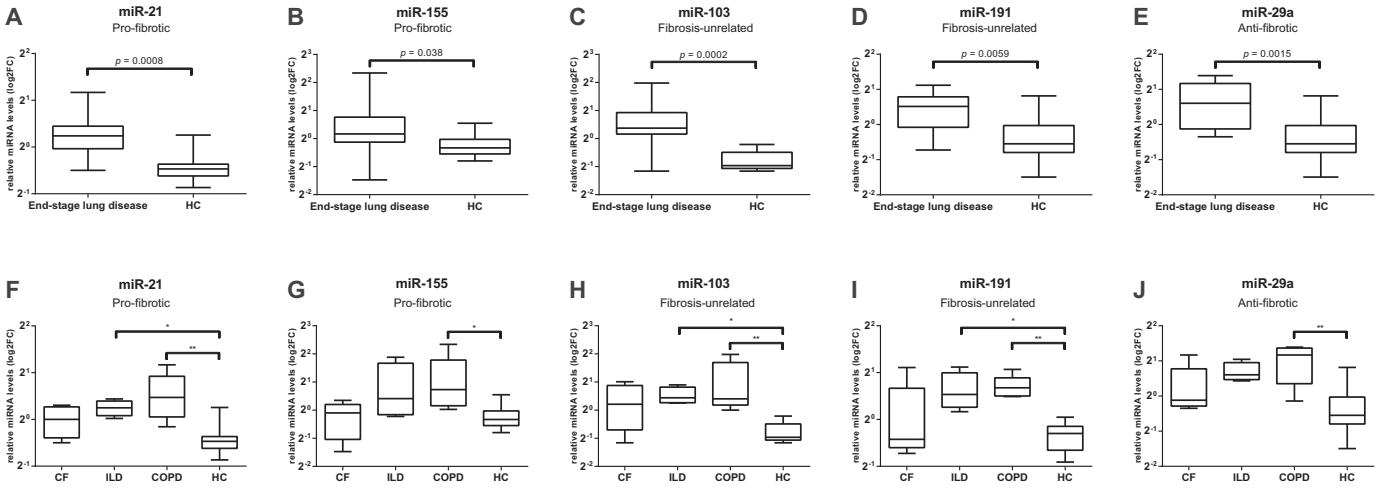

Fig. 1. Levels of serum miRNAs are elevated in end-stage lung diseases compared to healthy controls. The levels of serum miRNAs were analyzed by RTqPCR as described in the text in the serum of patients after lung transplant and represented as log2FC. Micro-RNAs were quantified in the serum of patients with end-stage lung diseases and are depicted as box and whisker plots, where boxes are indicating the median and the 25th to 75th percentiles and the whisker the min and max values within each diagnostic group. CF: cystic fibrosis, ILD: interstitial lung disease, COPD: chronic obstructive pulmonary disease, HC: healthy controls, FC: fold change, $*p < 0.05$, $**p < 0.01$.

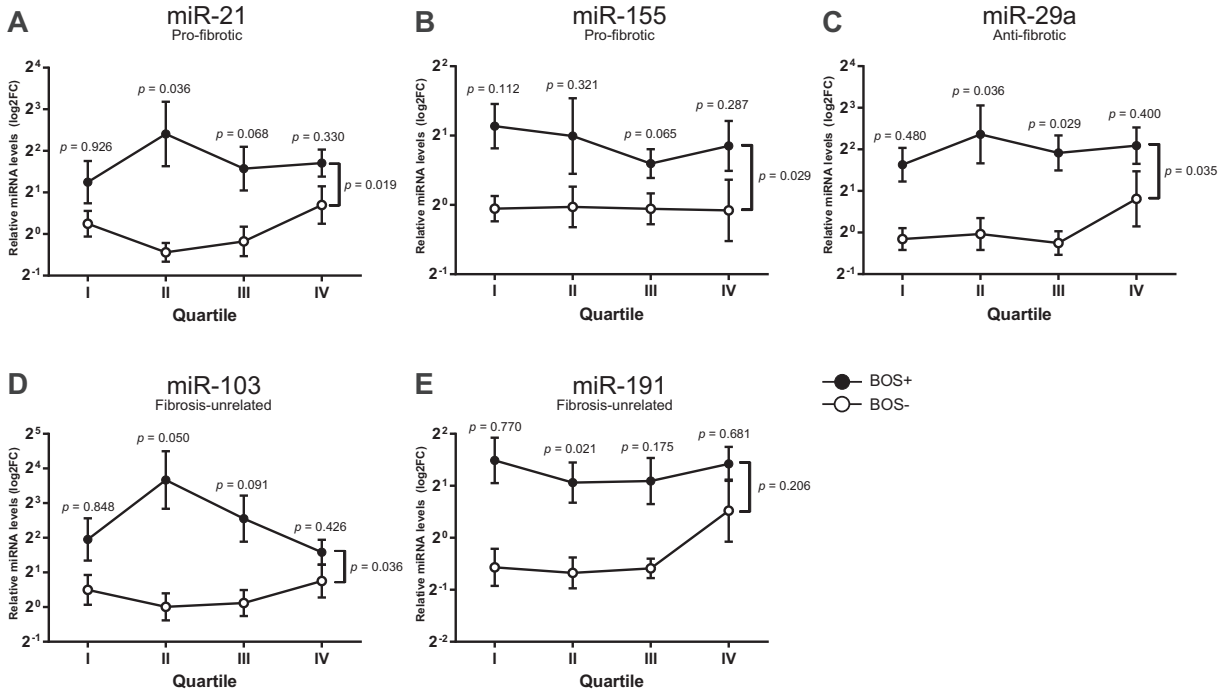

Fig. 2. BOS + patients present higher levels of selected serum miRNAs compared to matched BOS − patients. The impact of time and BOS development on the level of circulating miRNAs was analyzed by using the two-way ANOVA, setting time and disease as variables. Relative miRNA levels are depicted as log2FC (Mean + SEM). Taken together, all different miRNAs, except miR-191, were significant elevated in BOS + compared to BOS − groups ($p < 0.04$, individual p values depicted per graph). BOS +, $n = 10$, BOS −, $n = 10$ (A–E).

ILD patients compared to HC. No differences between the diagnostic clusters CF, COPD, and ILD were observed (Fig. 2F–J).

3.3. Selected serum miRNAs are elevated in BOS + patients compared to matched BOS − patients

We next investigated whether BOS patients had consistently high serum levels of miRNAs post-transplant or whether these levels increased further at the time of BOS development. To this end, serially obtained post-transplant sera were analyzed for miRNA levels. The time from lung transplant until the onset of BOS of each patient was divided into four equal quadrants and one sample taken in the middle of each quadrant was analyzed. Analysis of these serum samples demonstrated that the levels of all analyzed miRNAs, except for mir-191, showed a similar pattern, and were overall significantly increased in the serum of BOS + vs. BOS − patients. In addition, this elevation was already present in mir-21, mir-29a, mir103, and mir-155 prior to the clinical diagnosis of BOS (see Fig. 2).

4. Discussion

In this study, we investigated the presence of selected miRNAs in serum of both end-stage lung disease patients prior to LTx as well as in serum of a follow-up cohort post-LTx consisting of both BOS + and BOS − patients. Our results show that all selected pro-fibrotic, miR-21 and miR-155, anti-fibrotic, miR-29a, and fibrosis-unrelated, miR-103 and miR-191, miRNAs were elevated in end-stage lung disease patients. Given that the levels of circulating miRNAs are consistently higher in

patients with end-stage lung diseases, we investigated whether this was maintained after transplantation and/or exacerbated in case of BOS development. We show that selected miRNAs, including mir-21, mir-29a, mir-103, and mir-155, are elevated in patients who develop BOS and that these elevated levels are measurable prior to any clinical manifestations.

Despite the relatively small cohort sizes, we showed that the presence of an inflammatory lung disease is associated with increased levels of circulating miRNAs. Interestingly, the ILD patients investigated did not exhibit elevated levels of pro-fibrotic or diminished levels of anti-fibrotic miRNAs compared to other end-stage lung diseases, despite the pulmonary fibrosis which is often observed in these patients.

Intriguingly, both pro- and anti-fibrotic miRNAs, as well as fibrosis-unrelated miRNAs, were found increased in the circulation of BOS + patients and in patients suffering from other forms of inflammatory lung diseases not related to transplant complications. Micro-RNAs are actively secreted from cells via exosomes. Also, it is proposed that miRNAs can be released in the extracellular compartments upon cell injury and cell death, e.g., due to the prolonged exposure to an immune-activated environment [4], often present in the pathogenesis of BOS and end-stage lung diseases [1]. These inflammatory processes diminish upon transplantation [2], and this might be reflected by lower levels of serum miRNAs, as observed in BOS − patients. On the contrary, patients that cannot counteract the chronic inflammation even after LTx and are thereby characterized by an increased abundance of circulating miRNAs, might be the ones developing BOS. Even if the discriminative value of either pro- or anti-fibrotic miRNAs is limited, the combination of selected miRNAs could perhaps improve this distinction. However, a full miRNA profiling, including lung-enriched and inflammatory candidates, and deeper investigations are desirable to further establish these observations and to prove these hypotheses.

The overall increase of serum miRNAs in BOS + patients could be detected before the actual diagnosis of BOS, suggesting that the detection of elevated circulating miRNAs might serve as early biomarkers for BOS development. Further supporting this concept, serum miRNAs have been proposed as potential prognostic biomarkers in different transplantation settings [4,15,16]. Due to cohort constrains we were not able to assess potential correlations between the miRNAs investigated in this study and other biomarkers, including inflammatory cytokines. Previously, our group identified sCD59 as a biomarker for BOS development [17]. However, for these patients the miRNA analysis did not improve the distinction between being at risk for BOS development, which could be attributed to small patient numbers. Therefore, the additive value of miRNAs in the clinical diagnosis of BOS remains unclear.

Dysregulated expression of other miRNAs was observed in peripheral mononuclear cells of BOS patients with donor specific-HLA antibodies, further stressing the importance of miRNA regulation in this transplant complication [18] and their potential value as biomarkers for this condition validated. LTx patients identified as being at risk for BOS development by using such miRNA-biomarkers could therefore undergo alternative treatment regimens, which could potentially restrain disease progression and delay clinical manifestations. Overall miRNA abundancy in serum was comparable to miRNA levels obtained from bronchoalveolar lavage fluid samples and acquired Ct values were all within measurable range (data not show). As circulatory miRNAs are easily accessible and highly stable in human blood, they would represent profoundly good biomarkers for routine diagnostic follow-up after transplantation procedure [10].

In summary, we demonstrated that a selected group of miRNAs is elevated in BOS + patients compared to BOS- patients and this difference is present prior to the clinical diagnosis of BOS. This work opens new perspectives for future research aimed to further elucidate the prognostic value of circulating miRNAs in BOS and lung conditions in general.

Author contributions and disclosure statement

KB performed the research; MR, EAG, and HGO participated in data analysis; EAG, and JMK contributed patient serum; KB, MR, EAG, and HGO participated in research design; KB, MR, EAG, TRDJR, and HGO wrote the paper. All authors provided final approval of the version to be published and declare to have no conflict of interest.

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