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Brief communication

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

K. Budding[a,*], M. Rossato[a,b], E.A. van de Graaf[c], J.M. Kwakkel-van Erp[c], T.R.D.J. Radstake[a,b], H.G. Otten[a]

[a] Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
[b] Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
[c] Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht, The Netherlands

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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

* Corresponding author at: Laboratory of Translational Immunology, University Medical Center Utrecht, F.03.821, P.O. Box 85500, 3508 GA Utrecht, The Netherlands.
E-mail address: k.budding@umcutrecht.nl (K. Budding).

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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