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ORIGINAL ARTICLE

MiR-339 and galectin-3: diagnostic value in patients with airway obstruction after lung transplantation

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SUMMARY

Respiratory complications can be the cause of graft dysfunction after lung transplantation (LTx). MicroRNAs are small regulatory molecules—potential biomarkers of respiratory diseases and post-transplant complications. Galectin-3 is highly expressed in fibrosis of transplanted solid organs. The aim was to evaluate the expression of plasma miR-339 and galectin-3 concentrations in lung recipients including with airway obstruction after LTx. The study included 57 lung recipients (34 men and 23 women aged 10 to 74 years) were followed up to 5 years after LTx. The plasma microRNAs were detected by real-time PCR; galectin-3 levels were measured by ELISA. During follow-up in 30 recipients, post-transplant complications were detected: 12 (40.0%) cases of airway obstruction. The levels of miR-339 and galectin-3 were significantly higher in recipients with airway obstruction compare with 27 (47.3%) recipients without any complications ($P = 0.036$ and $P = 0.014$, resp.). Increasing miR-339 (above the 0.02 fold change) and galectin-3 (above the 11.7 ng/ml) threshold plasma levels in lung recipients is associated with high risk (RR = 7.14 ± 0.97 [95% CI 1.05–48.60], $P = 0.045$) of airway obstruction after LTx. A measurement of miR-339 expression in combination with galectin-3 level might be perspective to identify recipients at risk of airway obstruction after LTx.

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Introduction

Significant achievements have been made in the field of solid organ transplantation. According to the Registry of International Society for Heart and Lung Transplantation (ISHLT), over 4000 lung transplants are performed in the world annually [1]. The 5-year survival after lung transplantation (LTx) is inferior to that of other solid organ transplantation. Post-transplant complications leading to lung allograft dysfunction prevent improvement of long-term outcomes after LTx [2].

The lung transplant program in Russian Federation has developed actively since 2014 and supported in four transplant centers. The most of the LTx (about 70%) are performed at the Shumakov National Medical Research Center of Transplantology and Artificial Organs in Moscow.

The post-LTx management protocols are not standardized and depend on individual criteria of each transplant center. To date, the objective methods for verifying of structural changes in the lung graft airways are bronchoscopy with transbronchial biopsy and chest X-ray [3].

Diagnostics and treatment of lung graft dysfunction with airway obstruction in the early stages is extremely difficult because of the lack of clear signs of this.

A transbronchial biopsy is the definitive method for diagnosing graft dysfunction after LTx concern over procedural complications has limited use. Obtaining a biopsy involves examination of the local area, which does not guarantee exhaustive characteristic of the graft [4].

Multislice computed tomography is one of the most effective noninvasive tools for obstructive bronchopulmonary processes diagnostic. However, being a type of X-ray examination, it has some contraindications to use.

The recent studies are underway on biological agents that can be used as indicators of the risk of adverse events associated with obstructive processes leading to chronic pulmonary graft dysfunction.

MicroRNAs (miRNA) are a family of small noncoding regulatory molecules 18-25 nucleotides long. Over 2000 miRNAs are known to participate in the development and differentiation of hematopoietic cells, proliferation, and apoptosis, metabolic disorders, autoimmune diseases, and carcinogenesis [5].

It has been published that several miRNAs have potential value in solid organ transplantation. The significant upregulation of miR-27, miR-101, miR-142, miR-339, and miR-424 shown in recipients with acute rejection after heart and kidney transplantation. In the same time, investigated miRNAs are involved in different pulmonary tract pathologies: miR-27 regulates TGF-b function, which suppressed myofibroblast differentiation and attenuates bronchiolitis obliterans; miR-101 inhibits lung fibroblast proliferation and activation; downregulated in patients with idiopathic pulmonary fibrosis; miR-142 is involved in endothelial activation, inflammation, and antibody-mediated rejection; miR-142 high levels were found in patients with BOS**;** the role of mir-339 in the development of respiratory disorders (bronchial asthma, COPD) and obstructive processes in pulmonary tract has been shown; miR-424 upregulation was found in lung transplant recipients with fungal infections [6,7].

Galectin-3 is a new profibrogenic biomarker, belongs to the lectin family, and is involved in the pathogenesis of many relevant human diseases, including cancer, fibrosis, chronic inflammation, and scarring affecting many different tissues [8]. In the early stages of pulmonary fibrosis, the increasing serum levels of galectin-3 correlates with worsening lung function parameters and gas exchange [9,10].

The aim of this study was to evaluate the expression of plasma miR-339 and galectin-3 concentrations in lung recipients including with airway obstruction after lung transplantation.

Materials and methods

Patient characteristics

The study included 57 lung recipients who underwent transplantation from September 2014 to February 2020 at Shumakov National Medical Research Center of Transplantology and Artificial Organs.

Fifty-one patients (89.5%) got bilateral lung transplant (two of them got heart-lung transplantation); 6 (10.5%) recipients who underwent single lung transplantation. Among the lung recipients were 51 patients aged 18 to 74 (37 $\pm$ 14) years and 6 pediatric patients aged 10 to 17 (13 $\pm$ 2) years; 59.6% were male recipients.

Clinical findings and laboratory tests

Routine examination of recipients was carried out in accordance with the clinical guidelines of the Russian Transplant Society [11], which do not contradict the ISHLT consensus guidelines.

Instrumental diagnostic methods included computed tomography (CT) and chest X-ray, electrocardiography

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and echocardiogram, body plethysmography, bronchoscopy, transbronchial biopsy, and spirometry.

The post-transplant infectious complications were evaluated by quantitative examination of bacterial cultures from samples of bronchoalveolar lavage, sputum, pharyngeal smears, and blood.

The acute cellular rejection was verified by transbronchial biopsy and the clinical picture of acute respiratory failure with the presence of diffuse interstitial thickening in the lungs according to CT scan and chest X-ray.

Verification of obstructive airway processes was carried out based on data from video bronchoscopy and CT scan; the presence of noninfectious stenotic airway changes was evaluated (Fig. 1). All clinical and functional data were collected and entered in a specific database.

Immunosuppressive treatment

All recipients received basiliximab induction and immunosuppressive therapy with tacrolimus, mycophenolic acid, and methylprednisolone. The quantification of tacrolimus was performed by chemiluminescence immunoassay by Abbott's Architect i2000 according to the manufacturer's instructions.

Blood sampling and miRNA extraction

Blood samples from the lung transplant recipients were collected routinely 6 month after LT during physical examination or the moment of complications (1 to 4 samples; an average of 1.4 from each recipient). All blood collection tubes containing EDTA centrifuged for 10 minutes at 3000 rpm, after which blood plasma was separated from the cell sediment and immediately frozen at −20°C. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instruction. The complementary DNA (cDNA) templates were amplified by real-time RT-PCR using the miScript SYBR Green PCR Kit (Qiagen, USA) kit. Quantitative PCR (qPCR) was performed using CFX 96 (Bio-Rad, USA). A synthetic RNA spike-in, C. elegans miR-39 miRNA mimic was used as an internal control for the efficiency of RNA isolation, cDNA synthesis, and quantitative real-time PCR. MicroRNA copy numbers were normalized using C. elegans miR-39 too.

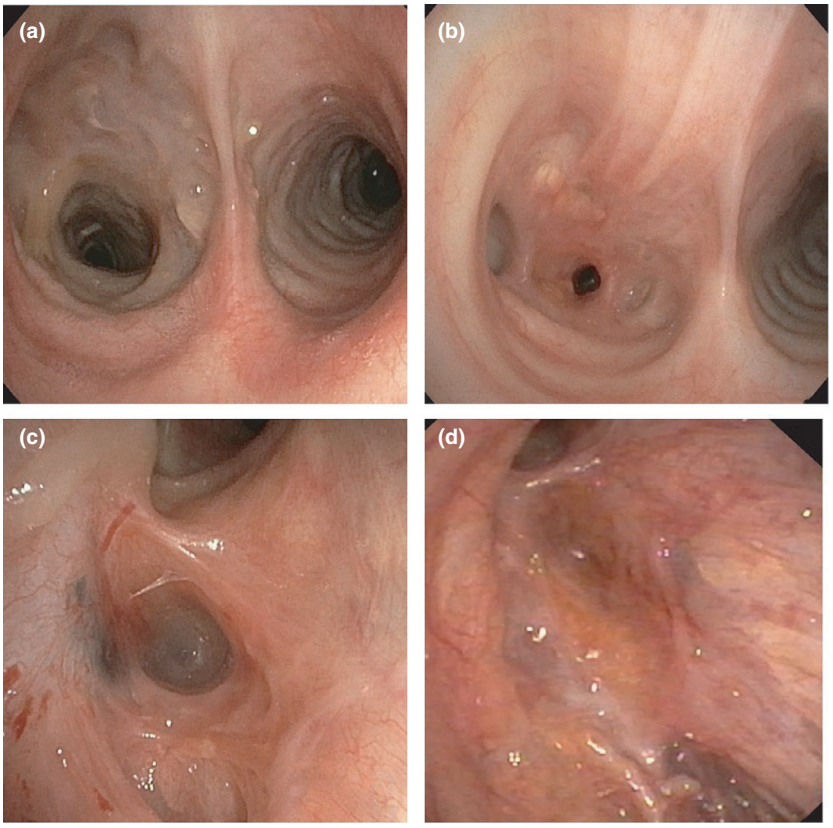

Figure 1 Endoscopic imaging of noninfectious stenotic airway changes: a) healthy airways; b) bronchial stenosis of 2–3 degrees; c) critical grade of bronchial stenosis; d) vanishing intermediate bronchus syndrome with airway occlusion.

Fold change of miRNA (miR-27, miR-101, miR-142, miR-339, and miR-424) levels was assessed by the $2^{-\Delta\Delta CT}$ method [12].

Measurement of galectin-3 level

Galectin-3 plasma levels were measured by enzyme-linked immunosorbent assay (ELISA) with the Human Galectin-3 Platinum ELISA (Bender MedSystems GmbH, Vienna, Austria) in accordance with the instructions attached to the kit.

Statistical analysis

Normally distributed data are reported as mean and standard deviation. Non-normal data are presented as median and interquartile range for continuous variables and number (%) for categorical variables. The Spearman test was applied as appropriate for the calculation of the correlations between two variables. For the independent variables, we used the Mann–Whitney U test. Statistical significance was set at two-tailed $P < 0.05$. The receiver operating characteristic (ROC) curve, the area under the ROC curve (AUC), relative risk (RR) sensitivity (Se), specificity (Sp), diagnostic efficiency (De), positive predictive value (PPV), and negative predictive value (NPV) were utilized to assess the diagnostic value of the identified biomarkers for post-transplant complications. The Youden index was calculated to measure the threshold microRNA expression level [13]. All analyses were carried out using Statistica v. 13.0 (StatSoftInc, Tulsa, OK, USA) or MEDCALC 12.7.5.0 (MedCalc software, Mariakerke, Belgium).

Results

Baseline characteristics of lung transplant recipients, microRNA expression and galectin-3 levels are listed in Table 1.

miRNA expression levels varied widely; therefore, data are presented as median and interquartile range.

The expression levels of miR-27, miR-10, miR-142, miR-339, miR-424, and galectin-3 levels did not correlate with tacrolimus blood levels in lung transplant recipients ($P = 0.91$, $P = 0.18$, $P = 0.91$, $P = 0.37$, $P = 0.33$ and $P = 0.58$, respectively).

During the follow-up, lasted for 1808 (704 +443) days after LTx, in 30 (52.6%) recipients post-transplant complications were detected: 15 cases of infectious complications, 2—acute cellular rejection, 1—infectious and acute cellular rejection association, 12—airway obstruction (including bronchial stenosis of noninfectious genesis); 27 recipients— without any post-transplant complications (the comparison group).

Table 1. Baseline characteristics of lung transplant recipients.

Characteristics	
Number of recipients, *n*	57
Gender, *n* (%)	
Male	34 (59.6%)
Female	23 (40.3%)
Age, years	
Range of variation	10 to 74
Mean ± SD	35 ± 15
Native lung disease, *n* (%)	
Cystic fibrosis	22 (38.6%)
COPD and pulmonary emphysema*	15 (26.3%)
Pulmonary arterial hypertension	9 (15.8%)
Pulmonary fibrosis†	6 (10.5%)
Lymphangioleiomyomatosis	3 (5.3%)
Bronchiectasis	2 (3.5%)
miRNA expression level, relative unit	
miR-27	0.055 [0.022; 0.318]
miR-101	0.038 [0.014; 0.084]
miR-142	0.013 [0.005; 0.024]
miR-339	0.023 [0.004; 0.216]
miR-424	0.013 [0.004; 0.055]
Galectin-3 levels, ng/ml	4.94 [2.03; 17.89]

*Chronic obstructive pulmonary disease.

†Pulmonary fibrosis of various etiologies (idiopathic pulmonary fibrosis; pulmonary fibrosis as a result of exogenous allergic alveolitis; postradiation pulmonary fibrosis).

miRNA levels and airway obstruction after LTx

The expression levels of miR-27, miR-101, miR-142, miR-339, and miR-424 did not differ between recipients with infectious complications and comparison group ($P = 0.39$, $P = 0.24$, $P = 0.39$, $P = 0.21$ and $P = 0.45$, respectively).

miR-339 expression levels were significantly higher in recipients with airway obstruction than those in recipients without any complications ($P = 0.036$, Fig. 2).

For miR-27 ($P = 0.81$), miR-101 ($P = 0.74$), miR-142 ($P = 0.95$), and miR-424 ($P = 0.64$), no such differences were found.

The AUC of miR-339 differed significantly from 0.5 (AUC = 0.69 ± 0.09 [95% CI 0.54 - 0.82; $P = 0.029$] and (Fig. 3).

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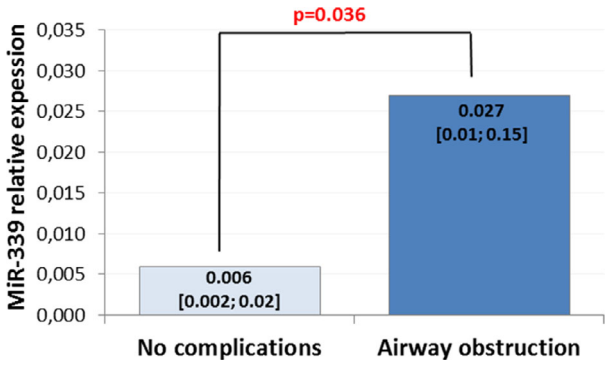

Figure 2 The expression level of miR-339 is higher in lung transplant recipients with airway obstruction compared with lung transplant recipients without any post-transplant complications ($P < 0.05$).

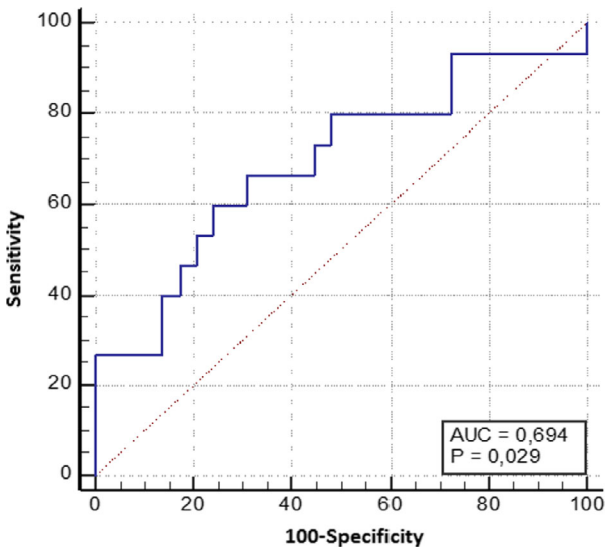

Figure 3 The ROC curve of miR-339 expression in lung transplant recipients with airway obstruction. Receiver operator characteristic curve of risk score for airway obstruction after LTx.

Galectin-3 levels and airway obstruction after LTx

Galectin-3 levels did not differ significantly in recipients with infectious complications and without any complications ($P = 0.86$).

Galectin-3 median level was significantly higher in recipients with airway obstruction in comparison with recipients without ones (17.04 ng/ml vs 4.72 ng/ml, $P = 0.014$, Fig. 4).

AUC for galectin-3 was also different from 0.5 (AUC = 0.74 ± 0.10 [95% CI 0.56–0.89; $P = 0.017$] (Fig. 5).

Diagnostic characteristics of miR-339 and galectin-3

This study revealed significantly higher values of miR-339 expression and galectin-3 levels in recipients with

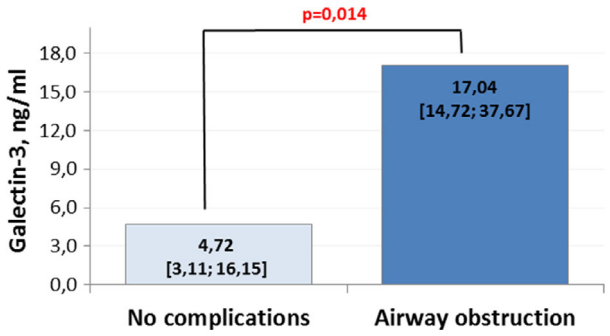

Figure 4 The plasma level of galectin-3 is higher in lung transplant recipients with airway obstruction compared with lung transplant recipients without any post-transplant complications ($P < 0.05$).

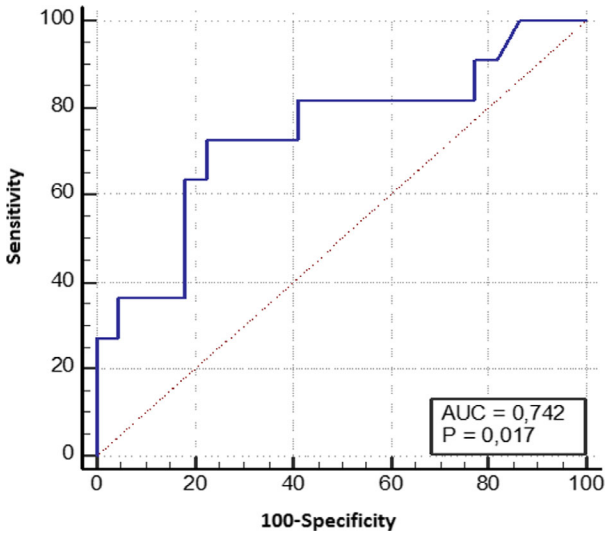

Figure 5 The ROC curve of galectin-3 levels in lung transplant recipients with airway obstruction.

airway obstruction compared with recipients without any complications.

Diagnostically significant thresholds of miR-339 and galectin-3 with respect to development of obstructive processes were set at 0.02 relative units and 11.65 ng/ml, respectively.

The relative risk of detecting obstructive airway changes in recipients with miR-339 expression values above the threshold level was RR = 2.62 ± 0.42 [95% CI 1.14–6.02], $P = 0.02$. The relative risk of developing obstructive bronchial changes with galectin-3 levels above the threshold was RR = 3.62 ± 0.58 [95% CI 1.16–11.24], $P = 0.026$ (Table 2).

A duplex test, including measurement of miR-339 expression and galectin-3 levels, had the best diagnostic characteristics with respect to identifying lung transplant recipients with development of post-transplant airway obstruction (RR = 7.14 ± 0.97 [95% CI 1.05–48.60],

Table 2. Performance of the different classifiers.

Classifier	RR	Se	Sp	De	PPV	NPV
miR-339	2.62	60.0%	75.9%	70.4%	56.3%	78.6%
galectin-3	3.62	72.7%	72.7%	78.8%	57.1%	84.2%
miR-339 + galectin-3	7.14	83.3%	81.8%	82.4%	71.4%	90.0%

De, diagnostic efficiency; NPV, negative predictive value; PPV, positive predictive value; RR, relative risk; Se, sensitivity; Sp, specificity.

$P = 0.04$). Together with this, the sensitivity and specificity of the test were 83.3% and 81.8%, respectively.

Discussion

The outcomes of LTx depend on a wide range of factors. Postoperative complications after LTx significantly reduce the recipient's survival, while early identification and prediction of progressive loss of lung function are a major goal.

Regular monitoring of lung graft function indicators can provide an opportunity for early fine-tuned calibration of immunosuppression or new target treatment, but the invasive diagnostic methods are significantly limited in use [14,15].

Finding new noninvasive diagnostic technologies for early detection of post-transplant complications is widely studied [16]. Noninvasive blood biomarker measurement is a simple screening tool for risk stratification. Having a functional marker would enable preventive measures to be taken at the early stages and reduce the number of invasive diagnostic procedures, which can cause the inflammation and bleeding in lung recipients [17].

miRNAs play a key role in regulating many cellular functions, including epithelial-to-mesenchymal transition, and indicates that there is a need for research the potential role of miRNAs as promising biomarkers [18]. Some of them take part in respiratory and cardiovascular pathological processes and have already been identified as potential biomarkers of fibrogenesis and graft rejection [19].

miRNAs are emerging not only as biomarkers but also as potential therapy. However, the studies that relate to the study of miRNA in lung transplant recipients are few [20–22].

Our study showed the increased miR-339 expression levels in lung recipients with post-transplant obstructive complications. It is suggested that the mechanism of miR-339 action can be associated with increased cell apoptosis induced by oxidative stress through an effect on Nrf2/FOXO3 and MAPK signaling pathways [23,24]. It was shown that miR-339 inhibits proliferation of pulmonary artery smooth muscle cell by targeting fibroblast growth factor signaling [25,26].

Galectin-3 is known as a biomarker of the myocardial fibrosis of cardiac allograft, involved in the inflammatory processes, immune response, fibrosis, and airway structural changes. In present study, higher plasma concentration of galectin-3 was observed in lung recipients with airway obstruction than in patients without them; it was diagnostically significant. This result coincided as data in study by d'Alessandro *et al.* but we did not assess the pathological mechanism of complications; diagnosis was verified by transbronchial biopsy [27]. However, the number of individuals enrolled in this study is relatively small; therefore, our findings need to be replicated in a larger cohort of patients. The diagnostic value was increased when miR-339 and galectin-3 were combined, indicating that this combination can improve the detection rate of airway obstruction in lung recipients after LTx and the relative risk increased to 7 times.

Conclusions

Our study shows that miR-339 and galectin-3 are promising noninvasive biomarkers to detect the lung transplant recipients with high risk of airway obstruction after LTx. Joint detection of miR-339 and galectin-3 can improve the sensitivity and specificity of airway obstruction diagnosis.

Authorship

O.Sh., S.Sh., O.G., participated in the research design, performance of the research, collected data, analyzed data, wrote the manuscript. I.P., M.B., E.Sh., D.V., participated in performance of research, interpretation of results, wrote the manuscript. O.T., S.G. participated in performance of research, contributed important proposals.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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