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DIAGNOSTIC VALUE OF MIRNA-101 AND MIRNA-27 IN ACUTE HEART TRANSPLANT REJECTION

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Objective: to determine the diagnostic value of miRNA-101 and miRNA-27 expression levels for acute heart transplant rejection. **Materials and methods.** The study enrolled 46 heart recipients, among whom were 36 men (78.3%); the average age of recipients was 47.7 = 10.8 (16 to 67) years. Serum microRNA expression levels were measured via quantitative polymerase chain reaction (PCR). Graft rejection was verified through morphological analysis of endomyocardial biopsy specimens. **Results.** The expression levels of miRNA-101 and miRNA-27 in recipients with acute graft rejection are significantly lower than in recipients without rejection ($p = 0.04$ and $p = 0.03$, respectively). When the miRNA-101 expression level is below the determined threshold value, the risk of developing acute graft rejection increases 1.8 times (RR = 1.8 [95% CI 1.13–3.01]). When the miRNA-27 expression level is below the determined threshold value, the risk of developing acute graft rejection increases 1.9 times (RR = 1.9 [95% CI 1.12–3.37]). Simultaneous decrease in the expression levels of miRNA-101 and miRNA-27 below the determined threshold values increases the likelihood of acute graft rejection by 2.0 times (RR = 2.0 [95% CI 1.16–3.36]). **Conclusion.** The serum miRNA-101 and miRNA-27 expression levels are of diagnostic value for acute graft rejection in heart recipients.

Keywords: heart transplantation, miRNA-101, miRNA-27, acute rejection, biomarkers.

Acute transplant rejection is one of the major factors affecting survival in heart recipients. The risk of developing acute heart transplant rejection is higher in the early postoperative period, although acute rejection crises can occur at any time after transplantation. Late rejection crises often occur where there are insufficient doses of immunosuppressive therapy or non-compliance with the prescribed medication regimen, as well as in cases of viral infections leading to activation of the patient's immune system [1, 2].

Endomyocardial biopsy, which is used to verify and control the treatment of acute rejection, is an invasive procedure that is laborious and risky for patients. Biopsy results can be influenced by sampling errors and variability in the assessment of the obtained preparations [3]. In order to improve preclinical diagnostics and reduce the number of repeated invasive diagnostic interventions, there has recently been active development of minimally invasive methods for diagnosing post-transplant complications, which can not only reveal the presence of acute graft rejection, but also control the effectiveness of recipient treatment [4].

To solve this problem, the relationship of various biomarkers, including factors and mediators of inflammation, neoangiogenesis, tissue destruction, thrombus formation, etc., with the risk of cardiovascular diseases and post-transplant complications, is being actively studied all over the world [5–9]. The study of microRNAs (small, non-coding RNAs that repress gene expression) as well as search and validation of new biomarkers for their diagnosis represent a separate subject of research aimed at understanding the pathogenesis of pathological processes in the myocardium. Due to the variety of regulatory functions, miRNAs are a promising group of biomarkers, potentially significant both for diagnosis and for monitoring the progression of post-transplant complications [10–12]. In our previous work, a comparative analysis of miRNA expression in heart recipients found that the expression levels of miRNA-101 and miRNA-27 significantly differed acute graft rejection and without rejection [13].

The objective of this work was to determine the diagnostic accuracy of miRNA-101 and miRNA-27 with respect to acute graft rejection in cardiac recipients.

MATERIALS AND METHODS

The study enrolled 46 patients who, between 2013 and 2016, underwent a heart transplant (HT) surgery at the Shumakov National Medical Research Center of Transplantology and Artificial Organs. Among the pati-

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ents were 36 men (78.3%). The mean age of recipients was 47.7 ± 10.8 (16 to 67) years. Before the HT, 29 (63%) recipients were diagnosed with dilated cardiomyopathy (DCM), 12 (26%) recipients were found to have coronary heart disease (CHD), while 5 (11%) recipients were diagnosed with other conditions. The maximum follow-up period after HT was 2215 days (median 264.5 [32; 785.3]).

All patients with indications for HT underwent a routine examination according to the National Clinical Guidelines '*Heart Transplantation and Mechanical Circulatory Support*' and the patient management protocol at the Shumakov National Medical Research Center of Transplantology and Artificial Organs. After transplantation, routine examinations of the recipient included clinical assessment of the state, general and biochemical blood tests to determine tacrolimus levels, 24-hour blood pressure monitoring (to correct antihypertensive therapy), echocardiographic examination, repeated myocardial biopsies, and annual coronary angiogram. All recipients received a three-component immunosuppressive therapy, including a combination of calcineurin inhibitors (tacrolimus) and cytostatics (mycophenolate mofetil or mycophenolonic acid), as well as varying doses of oral prednisolone, depending on the time after surgery and the frequency of graft rejection episodes and adjuvant medication as indicated [1].

The material for the study of microRNA expression was venous blood plasma (1 to 3 samples from each patient, mean 1.22). Patients' peripheral blood samples were collected in disposable tubes with anticoagulant ethylenediaminetetraacetic acid (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 from 100 μL of blood plasma using Serum Plasma kits (Qiagen, USA) with preliminary addition of 1.6×10^8 copies of synthetic cel-miR-39 microRNA (Qiagen) after plasma incubation with Qiazol phenolic mixture. Cel-miR-39 was used as an internal control for the efficiency of RNA isolation, complementary DNA (cDNA) synthesis, and quantitative polymerase chain reaction (PCR) in real time. The intensity of miRNA expression was calculated using the 2-ΔCT method [14] and was expressed in relative units equivalent to $\log_2 (2^{-\Delta Ct})$, where ΔCt are the working values of the change in the product production cycle relative to the internal control of cel-miR-39 miRNA expression.

Acute cellular rejection (ACR) and antibody-mediated rejection (AMR) were verified by examining endomyocardial biopsy specimens. For histological examination, endomyocardial pieces were fixed in 10% formalin, then washed with water, dehydrated and embedded in paraffin. 3–4 μm thick slices were prepared on a microtome. To diagnose ACR, the slices were stained with hematoxylin and eosin; to diagnose AMR, an immunohistochemical study was used. The degree of ACR and AMR was assessed according to the recommended classifications adopted by the International Society for Heart and Lung Transplantation (ISHLT-2004 and ISHLT-2013).

Statistical data processing

Sensitivity and specificity were measured using ROC analysis. To assess the diagnostic significance of miRNA-101 and miRNA-27, a relative risk index was used. The Youden's index was calculated to determine the threshold level of microRNA expression. The test's sensitivity (Se) is represented by the proportion of true positive cases, while specificity (Sp) is the proportion of true negative cases. The test's diagnostic accuracy (De) is expressed as the percentage ratio of the sum of all true cases to the total number of results obtained. Statistical analysis of results obtained was done using software and standard statistical processing methods of the applied software package Statistica v.13.0 from StatSoftInc. (USA). Obtained data was statistically processed by nonparametric methods: for comparison of dependent samples, the paired Wilcoxon test was calculated, and the Mann–Whitney U test was used to compare the independent variables. The critical level of significance was assumed to be 5%, i.e. the null hypothesis was rejected at $p < 0.05$.

RESULTS AND DISCUSSION

The miRNA-101 and miRNA-27 expression indicators in heart recipients are presented as a median of concentrations [interquartile range] with an indication of the significance of differences, which is due to the distribution of values other than normal.

During the entire follow-up period after transplantation, signs of acute rejection were verified in 27 recipients in 31 endomyocardial biopsy specimens. Among them, ACR (grades R1G–R3G according to ISHLT-2004 classification) was observed in 23 recipients in 24 samples, and AMR in 6 recipients in 6 samples, and mixed acute rejection in 1 sample.

Fig. 1 shows the morphological picture of endomyocardial biopsy specimens of the transplanted heart, in which ACR and AMR signs were detected.

Recipients with and without acute graft rejection did not differ significantly by age ($p = 0.84$), sex ($p = 0.07$), and pre-transplant diagnosis ($p = 0.51$). When we analyzed tacrolimus blood levels in cardiac recipients, no significant differences were found in the group of patients with and without acute rejection – 8.1 [6.7; 10.7] and 9.9 [6.1; 12] ng/mL, respectively ($p = 0.75$).

It was found that the miRNA-101 and miRNA-27 expression levels in recipients with acute graft rejection was significantly lower than in recipients without rejec-

tion (p = 0.04 and p = 0.03, respectively, Fig. 2). The results obtained are consistent with available reports on the inhibitory effect of miRNA-101 [15–17] and miRNA-27 [18, 19] on the development of fibrotic processes in the heart and other organs.

We used the ROC-curve analysis to determine the diagnostic significance of miRNA-101 and miRNA-27 as a marker of acute rejection of transplanted heart (Fig. 3).

Calculations showed that the area under the ROC curve of miRNA-101 and miRNA-27 in heart recipients

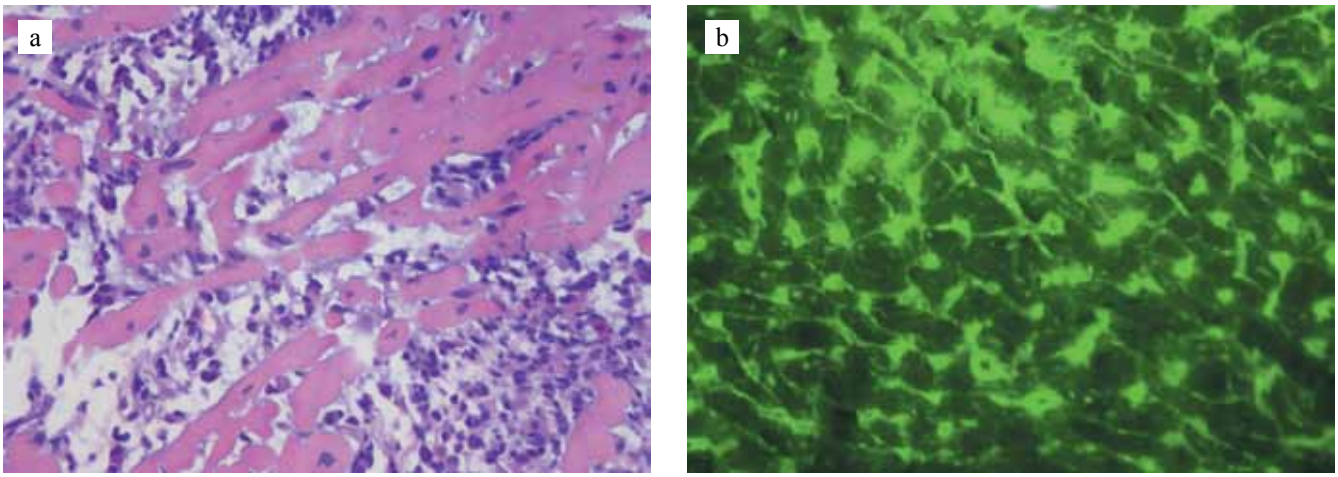

Fig. 1. Study of endomyocardial biopsy specimens. (a) multifocal moderate ACR of a heart transplant (R2G grade according to ISHLT-2004 classification). H&E stain. 400×. (b) fixation of the C4d fragment of the complement in the myocardial capillary wall in AMR. Immunohistochemistry analysis

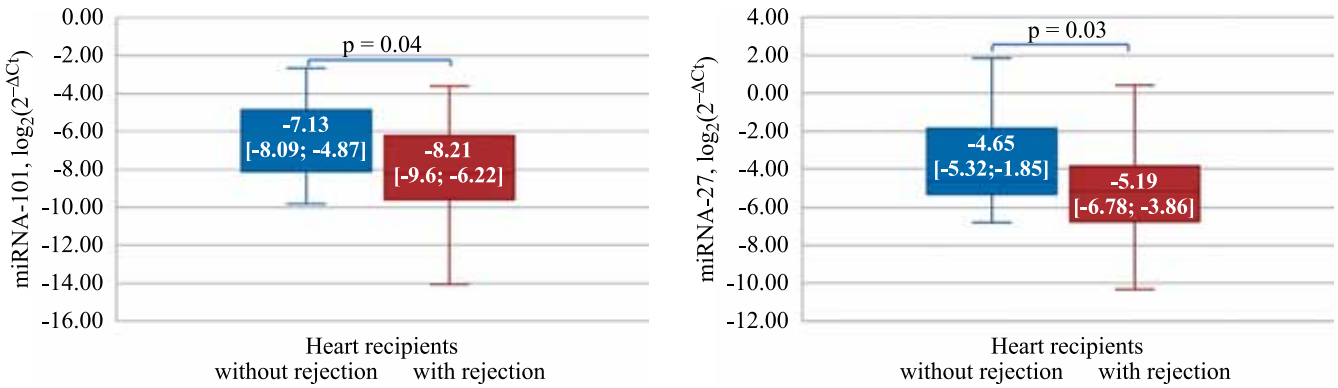

Fig. 2. miRNA-101 and miRNA-27 expression levels in heart recipients with and without graft rejection ($\log_2(2^{-\Delta Ct})$)

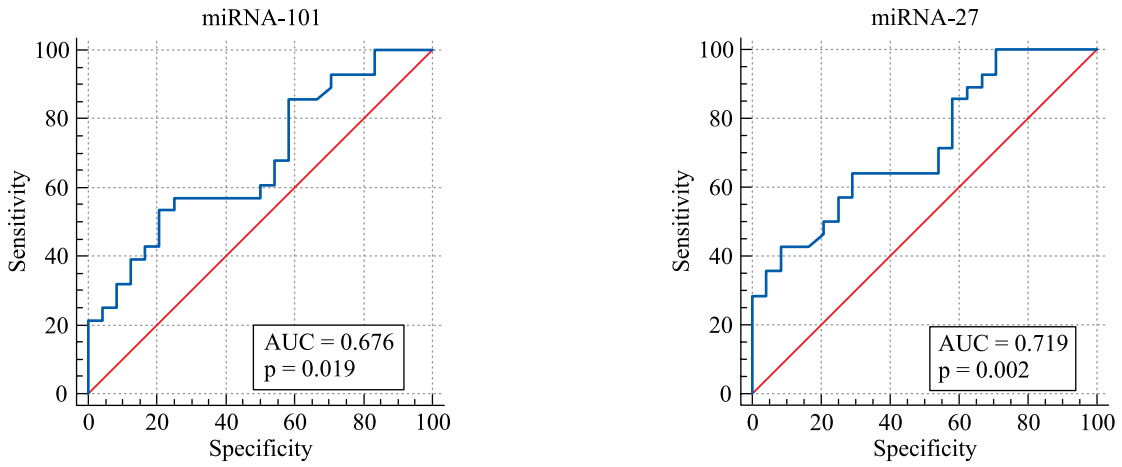

Fig. 3. ROC curves of miRNA-101 and miRNA-27 in heart recipients with acute transplant rejection

with acute graft rejection is 0.676 and 0.719 (p = 0.02 and p = 0.002, respectively).

The threshold expression levels of miRNA-101 and miRNA-27, significant for the diagnosis of acute graft rejection in cardiac recipients, were determined by optimal combination of sensitivity and specificity values corresponding to the highest Youden's index [20].

Fig. 4 shows a graph of sensitivity and specificity dependence on the miRNA-101 and miRNA-27 expression levels in blood plasma in relation to acute graft rejection in cardiac recipients.

The threshold of miRNA-101 expression, which is a significant factor for diagnosis of acute graft rejection in cardiac recipients, was –8.36. When the miRNA-101 expression level is below this threshold, the likelihood of the risk of developing acute graft rejection is 1.8 times higher than in recipients with miRNA-101 expression level above this threshold (sensitivity 53.6%, specificity 79.2%). The diagnostic accuracy of the test was 65.4%. The threshold value of miRNA-27 expression, which is a significant factor for diagnosis of acute graft rejection in cardiac recipients, was –5.07. When the miRNA-27

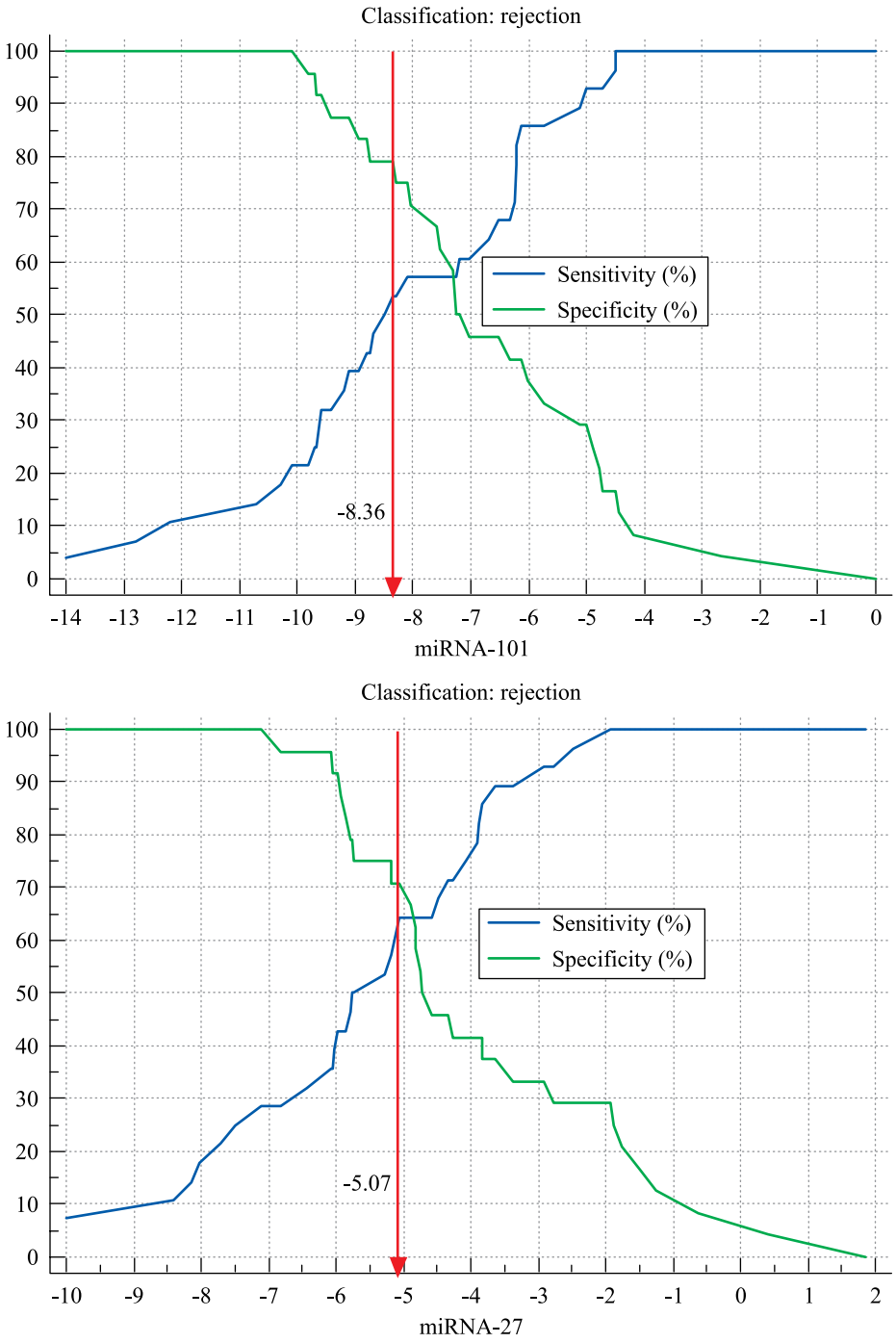

Fig. 4. Diagnostically significant threshold expression levels of miRNA-101 and miRNA-27 in heart recipients with acute transplant rejection

Table

The diagnostic significance of the expression levels of miRNA-101 and miRNA-27 is below the thresholds in heart recipients with acute transplant rejection

miRNA	RR	95% CI	Se	Sp	De
miRNA-101	1.8	[1.132–3.012]	53.6%	79.2%	65.4%
miRNA-27	1.9	[1.122–3.367]	64.3%	70.8%	67.3%
miRNA-101 + miRNA-27	2.0	[1.158–3.364]	60.0%	78.9%	68.2%

expression level is below this threshold, the probability of the risk of developing acute graft rejection is 1.9 times higher than in recipients with miRNA-27 expression level above this threshold (64.3% sensitivity, 70.8% specificity). The test's diagnostic accuracy was 67.3% (Table).

When assessing the diagnostic significance of combined measurement of microRNA-101 and microRNA-27 with respect to acute graft rejection in heart recipients, it was found that when the expression level of these microRNAs is below the thresholds determined, the probability of the risk of developing acute graft rejection is twice as high (60.0% sensitivity, 78.9% specificity). The test's diagnostic accuracy is 68.2%.

CONCLUSION

In heart recipients, the miRNA-101 and miRNA-27 expression levels are of diagnostic significance with respect to acute graft rejection. With microRNA-101 expression level ≤–8.63 units ($\log_2(2^{-\Delta Ct})$), the risk of acute graft rejection in heart recipients is 1.8 times higher than in recipients with microRNA-101 expression level above the threshold. At a microRNA-27 expression level ≤–5.07 arbitrary units ($\log_2(2^{-\Delta Ct})$), the risk of developing acute graft rejection in heart recipients is 1.9 times higher than in recipients with the miRNA-27 expression level above the threshold. When the miRNA-101 and miRNA-27 expression levels below the thresholds simultaneously, the risk of developing acute graft rejection doubles.

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The authors declare no conflict of interest.

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