DOI: 10.15825/1995-1191-2020-1-26-34

MICRORNA EXPRESSION LEVELS IN EARLY AND LONG-TERM PERIOD FOLLOWING HEART TRANSPLANTATION

D.A. Velikiy[1], O.E. Gichkun[1, 2], S.O. Sharapchenko[1], O.P. Shevchenko[1, 2], A.O. Shevchenko[1, 2, 3]

[1] Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation

[2] Sechenov University, Moscow, Russian Federation

[3] Pirogov Medical University, Moscow, Russian Federation

Objective: to conduct comparative analysis of the expression levels of microRNA-101, microRNA-142, microRNA-27, microRNA-339 and microRNA-424 in patients with severe chronic heart failure and in heart recipients in the early and long-term period following heart transplantation and to determine the association with acute transplant rejection. **Materials and methods.** The study included 46 heart recipients, among whom were 36 men (78.3%); the average age of the recipients was 47.7 ± 10.8 (16 to 67) years, and 12 patients with end-stage chronic heart failure, among whom were 8 men (66.7%); the average age of the patients was 46.1 ± 6.4 (37 to 64) years. The control group consisted of 12 healthy individuals, not significantly different by gender and age. microRNA expression levels in blood plasma were determined through quantitative polymerase chain reaction (Q-PCR). Transplant rejection was verified via morphological analysis of endomyocardial biopsy specimens. **Results.** Blood plasma of patients with end-stage chronic heart failure had significantly higher expression rates of microRNA-101, microRNA-27, microRNA-339 and microRNA-424 than in healthy individuals ($p < 0.02$). In the early stages following transplantation, the expression levels of microRNA-101 and microRNA-27 in heart recipients were significantly lower than in patients with severe chronic heart failure ($p < 0.003$). A year or more after transplantation, there were no significant differences in the expression levels of microRNA-101, microRNA-142, and microRNA-339 in heart recipients and in healthy individuals. In recipients with acute rejection, the expression levels of microRNA-101 and microRNA-27 significantly differed from that of recipients without signs of rejection ($p = 0.04$ and $p = 0.03$, respectively). **Conclusion.** The obtained data on changes in the expression levels of microRNA-101 and microRNA-27 in heart recipients with acute transplant rejection suggests possible diagnostic value of these biomarkers in determining the risk of rejection.

Keywords: heart transplantation, microRNA, chronic heart failure, biomarkers, rejection.

Significant advances in surgical techniques and improved immunosuppressive therapy have increased survival and improved quality of life for heart recipients. The most important task in managing patients after transplantation is to prevent graft rejection along with minimizing the dose of immunosuppressive drugs. Development of non-invasive methods for detecting transplant rejection will improve early diagnosis and increase life expectancy by reducing the number of late post-transplant complications [1–3].

In recent years, a number of biomarkers have been shown to be involved in cardiovascular complications in patients with heart failure and in heart transplant recipients. It has been demonstrated that an estimate of concentration of these biomarkers can be used to predict and diagnose heart transplant rejection [4–6]. A separate group consists of microRNAs – small, non-coding RNAs that regulate gene expression. Ability to accurately and quickly determine microRNA content in biological fluids in combination with their tissue and nosological specificity makes these small signaling molecules promising candidates for the role of rejection biomarkers in heart transplant recipients [7–9].

In the present work, we performed a comparative analysis of the expression levels of miRNA-27, miRNA-101, miRNA-142, miRNA-339 and miRNA-424 in heart recipients in early and long-term follow-up after transplantation and determined the relationship with acute graft rejection.

MATERIALS AND METHODS

The study included 46 patients who, in the period from 2013 to 2016, at Shumakov National Medical Research Center of Transplantology and Artificial Organs,

Corresponding author: Dmitriy Velikiy. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Tel. (499) 193-87-62. E-mail: dim_vel@mail.ru

underwent a heart transplant (HT) surgery. Among them were 36 men (78.3%); average age of the recipients was 47.7 ± 10.8 (16 to 67) years. In the study were also 12 patients with severe chronic heart failure (NYHA class III and IV), 8 of them were men (66.7%); average age of the patients was 46.1 ± 6.4 (37 to 64) years. Dilated cardiomyopathy (DCM) was diagnosed in 29 (63%) recipients before HT. Coronary heart disease (CHD) was diagnosed in 12 (26%) recipients before HT, while other complications were found in 5 (11%) recipients. The recipients were observed for a maximum of 2215 days (median 264.5 [32; 785.3]) after HT. The control group consisted of 12 healthy persons, who did not significantly differ by sex and age.

All patients who had indications for HT were routinely examined in accordance with the national clinical recommendations "Heart Transplantation and Mechanical Circulatory Support" and the patient management protocol at Shumakov National Medical Research Center of Transplantology and Artificial Organs. After transplantation, scheduled recipient examinations included: clinical evaluation of the condition, general and biochemical blood tests (including measurement of tacrolimus blood concentrations), 24-hour blood pressure monitoring (to correct antihypertensive therapy), echocardiography, repeated myocardial biopsies, and annual coronary angiography.

All recipients received a three-component immunosuppressive therapy, including a combination of calcineurin inhibitors (tacrolimus) and cytostatic inhibitors (mycophenolate mofetil or mycophenolic acid), as well as varying doses of prednisolone orally (depending on the time after surgery and the frequency of episodes of graft rejection) and adjuvant therapy, if indicated [10].

Acute heart transplant rejection was diagnosed based on histological examinations and humoral and immunohistochemical tests of endomyocardial biopsy specimens.

Venous blood plasma served as the material for studying miRNA expression; in heart recipients, 56 samples were studied at various times after transplantation (1 to 3 samples from each patient, average of 1.22).

Total RNA isolation from peripheral blood plasma

Peripheral blood samples of patients were collected in disposable tubes with an EDTA anticoagulant, centrifuged for 10 minutes at 3000 rpm. Blood plasma was separated from the cell pellet and immediately frozen at –200 °C. RNA was isolated from 100μL blood plasma using Serum Plasma kits (Qiagen, USA) with preliminary addition of 1.6 x10^8 copies of synthetic miRNA cel-miR-39 (Qiagen) after plasma incubation with Qiazol phenolic mixture. Cel-miR-39 was used as an internal control to check RNA isolation efficiency, complementary DNA (cDNA) synthesis and real-time polymerase chain reaction (PCR).

Real-Time quantitative reverse transcription PCR

Total RNA from each sample was converted to cDNA in a reaction mixture (20μL) containing 1xmi Script HiSpec Buffer, 1xmi Script Nucleics Mix, at T = 37 °C for 60 minutes, followed by incubation at 95 °C for 5 minutes, cooling on ice and bringing the sample volume to 200 μL with deionized water. Synthesized cDNA (2 μL) was the matrix in real-time PCR using primers specific for the studied miRNAs: miRNA-27, miRNA-101, miRNA-142, miRNA-339, miRNA-424, Ce_miR-39 (miScript Primer assay, Ce_miR-39_1, Qiagen), and the miScript SYBR Green PCR Kit (Qiagen). PCR reaction conditions: 15 minutes at T = 95 °C followed by 40 cycles of 15 seconds at T = 94 °C, 30 seconds at T = 50 °C and 30 seconds at T = 70 °C in a CFX 96 amplifier (Biorad). MicroRNA expression intensity was calculated using the $2^{-\Delta Ct}$ method [11] and was expressed in relative units equivalent to $\log_2 (2^{-\Delta Ct})$, where ΔCt are the working values of change in the product production cycle relative to the internal control of miRNA (Ce_miR-39) expression.

Statistical data processing

Statistical analysis of results was done using application software package IBM SPSS STATISTICS 20 (IBM SPSS Inc., USA). Statistical processing of obtained data was carried out by nonparametric methods: Wilcoxon signed-rank test was used to compare dependent samples, while the Mann-Whitney U test was used to compare independent variables. The critical significance level was taken to be 5%, i.e. the null hypothesis was rejected at $p < 0.05$.

RESULTS

In patients with end-stage chronic heart failure, as well as in heart transplant recipients, miRNA expression indicators varied over a wide range. They exhibited a nonparametric distribution. In this paper, results are represented by median and interquartile range, expressed in relative units.

Expression levels of miRNA-101, miRNA-142, miRNA-27, miRNA-339 and miRNA-424 in patients with severe chronic heart failure and heart transplant recipients did not significantly differ in men and women ($p = 0.29$, $p = 0.33$, $p = 0.25$, $p = 0.71$ and $p = 0.07$, respectively). MicroRNA expression indicators were not dependent on age.

A comparative analysis of miRNA expression revealed significantly higher expression levels of miRNA-101, miRNA-27, miRNA-339 and miRNA-424 in

the blood plasma of patients with end-stage chronic heart failure compared with the healthy persons (Fig. 1).

The established differences in the expression levels of miRNA-101, miRNA-27, miRNA-339 and miRNA-424 probably reflect the totality of pathological conditions in the myocardium of patients with severe chronic heart failure.

No significant differences in the expression levels of miRNA-101, miRNA-142, miRNA-27, miRNA-339 and miRNA-424 were found in heart transplant recipients depending on the initial diagnosis, serving as an indication for transplantation: DCM or CHD ($p = 0.89$, $p = 0.44$, $p = 0.87$, $p = 0.08$ and $p = 0.52$, respectively).

There was no reliable correlation between the expression level of miRNA-101 ($r = 0.001$, $p = 0.99$), miRNA-142 ($r = 0.004$, $p = 0.98$), miRNA-27 ($r = -0.06$, $p = 0.68$), miRNA-339 ($r = 0.06$, $p = 0.7$) and miRNA-424 ($r = 0.03$, $p = 0.84$) and tacrolimus blood concentrations in heart transplant recipients (Fig. 2).

The results of comparative analysis of miRNA expression indicators in patients with severe heart failure and in heart transplant recipients are presented in Table 1.

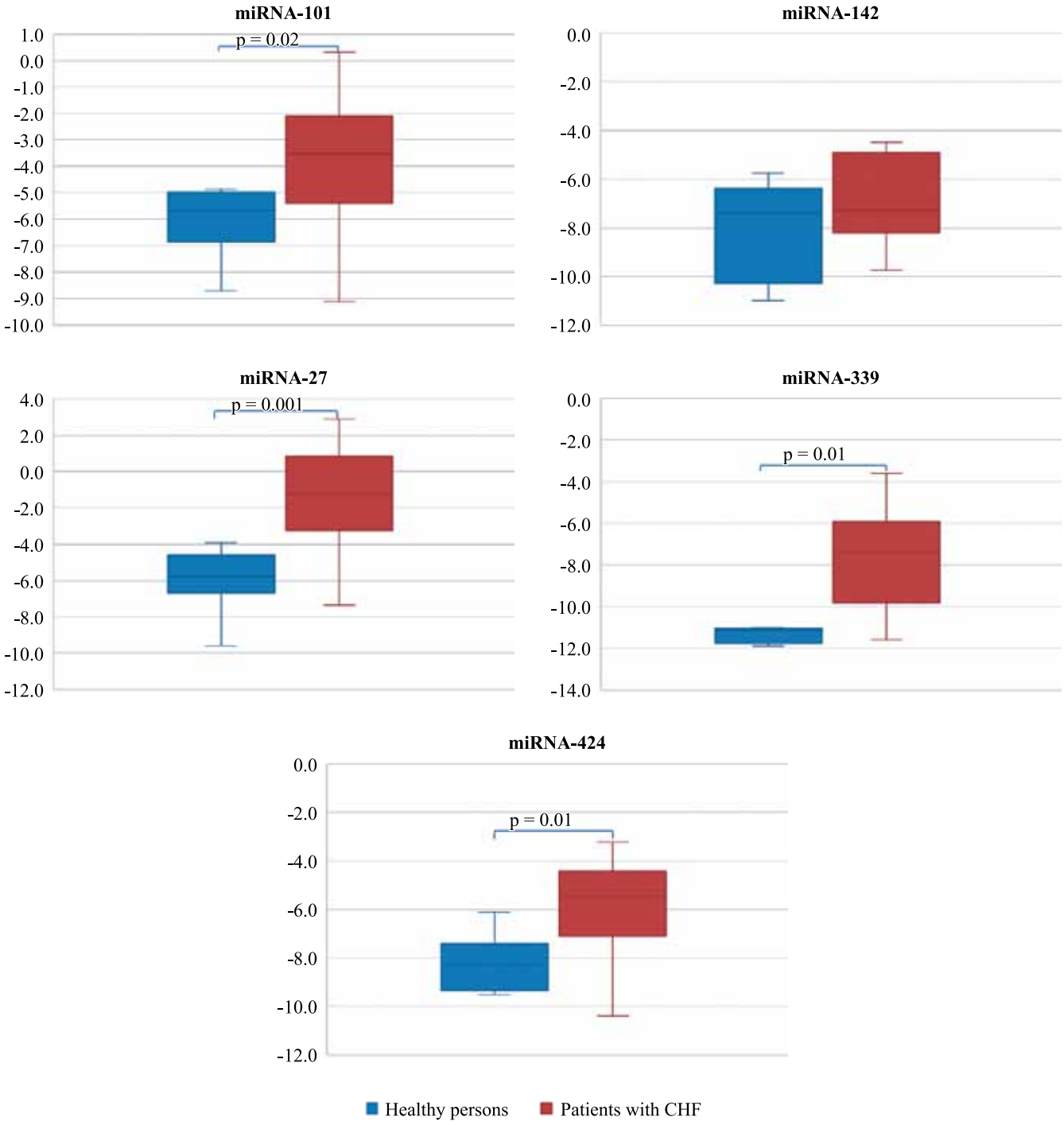

Fig. 1. miRNA expression levels in healthy persons and in patients with severe chronic heart failure, $\log_2(2^{-\Delta Ct})$

Table 1

Comparative analysis of miRNA expression in patients with chronic heart failure and in heart recipients

microRNA, $\log_2(2^{-\Delta Ct})$	CHF patients	Heart recipients	Significance, p
miRNA-101	–3.53 [–5.4; –2.09]	–7.21 [–8.98; –5.1]	**0.0002**
miRNA-142	–7.27 [–8.2; –4.9]	–6.67 [–8.19; –5.5]	0.67
miRNA-27	–1.23 [–3.26; 0.84]	–4.78 [–5.94; –2.88]	**0.01**
miRNA-339	–7.38 [–9.81; –5.9]	–10.13 [–11.59; –9.02]	**0.04**
miRNA-424	–5.46 [–7.11; –4.42]	–7.05 [–8.11; –5.81]	0.52

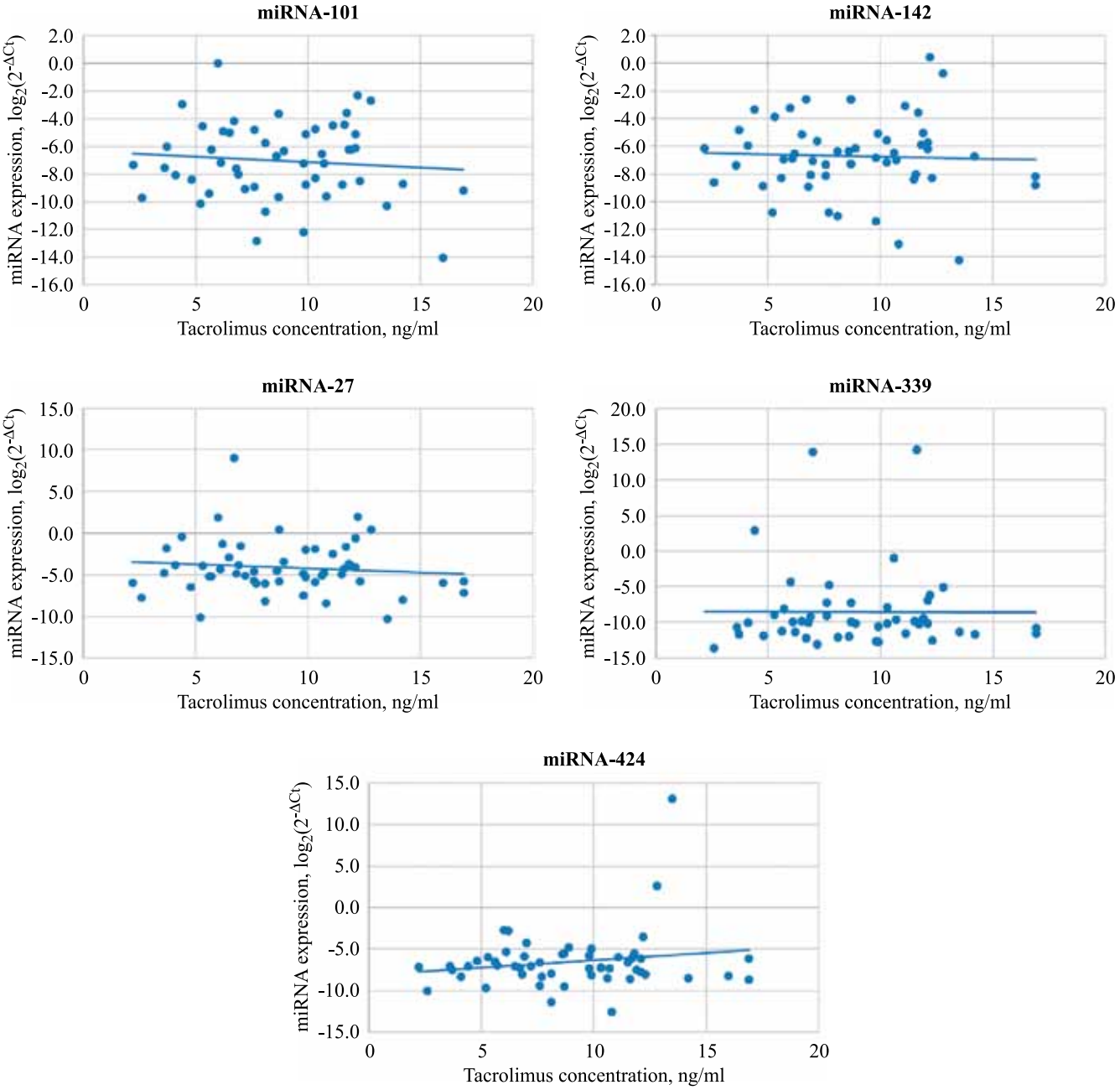

Fig. 2. Analysis of correlation between miRNA expression levels and tacrolimus concentration

Groups of patients with severe chronic heart failure and heart transplant recipients did not significantly differ by gender and age.

The differences in the expression levels of miRNA-101, miRNA-27, miRNA-339 in the groups of potential heart recipients and all patients included in the study after transplantation were significant (p = 0.0002, p = 0.01 and p = 0.04, respectively).

It was found that in the early post-transplant period (median 24 [10; 35] days), the expression levels of miRNA-142, miRNA-339 and miRNA-424 in heart recipients did not differ significantly from patients with severe chronic heart failure, although there was a tendency towards reduction in the levels. Differences in the expression levels of miRNA-101 and miRNA-27 in these groups were significant (p = 0.0001 and p = 0.003, respectively). Changes in the expression profile of miRNA-101 and miRNA-27 in the early post-transplant period may be due to the action of a complex of various factors associated with surgical intervention, including systemic inflammatory response, adaptation of the body to the transplanted organ, and immunosuppressive therapy. There is evidence that these signaling molecules are involved in regulation of myocardial fibrosis through interactions with transcription factor RUNX1 and transforming growth factor-beta receptor (TGFβR1) [12, 13].

Comparative analysis of miRNA expression in heart recipients in the early and long-term follow-up after transplantation is presented in table 2.

In heart recipients, expression levels of miRNA-142, miRNA-339 and miRNA-424 a year or more after transplantation did not significantly differ compared with recipients in the early stages after HT, but there was a tendency towards increase in the levels. Differences in the expression levels of miRNA-101 and miRNA-27 in these groups were significant (p = 0.008 and p = 0.04, respectively).

Fig. 3 shows miRNA expression indicators in heart transplant recipients in the early and long-term post-transplant periods and in healthy persons.

It was found that a year or more after transplantation, the expression levels of miRNA-101, miRNA-142 and miRNA-339 in heart recipients did not significantly differ from those of healthy persons. Differences in the expression levels of miRNA-27 and miRNA-424 in these groups were significant (p = 0.003 and p = 0.01, respectively).

Analysis of the effect of acute graft rejection on expression level of the studied miRNAs revealed the following. During the entire follow-up period after transplantation, signs of acute graft rejection were verified in 27 recipients through 31 endomyocardial biopsy samples. Among them were acute cellular rejection (R1G–R3G according to ISHLT-2004 grading system) was observed in 23 recipients in 24 samples, humoral rejection in 6 recipients in 6 samples and mixed rejection in one sample (Table 3).

Recipients with and without acute graft rejection did not significantly differ by age, gender, and pre-transplant diagnosis. Analysis of tacrolimus blood concentration in heart recipients revealed no significant differences in the group of patients with and without acute graft rejection. The concentration levels were 8.1 [6.7; 10.7] and 9.9 [6.1; 12] ng/ml, respectively (p = 0.75).

Table 4 presents a comparative analysis of miRNA expression in heart recipients with and without acute graft rejection.

Significant differences were found in the expression values of miRNA-101 and miRNA-27 in recipients with acute graft rejection compared with recipients without rejection (p = 0.04 and p = 0.03, respectively). The results confirm the available data on the possible diagnostic role of miRNAs, particularly miRNA-101, in acute heart transplant rejection [14].

Table 2

Comparative analysis of miRNA expression in heart transplant recipients in the early and long-term post-transplant periods

microRNA, $\log_2(2^{-\Delta Ct})$	Post-HT period		Significance, p
	1 month, n = 22	1 year or more, n = 34	
miRNA-101	–8.75 [–9.74; –6.76]	–6.22 [–7.59; –4.78]	**0.008**
miRNA-142	–7.03 [–8.35; –6.01]	–6.52 [–7.39; –5.09]	0.25
miRNA-27	–5.79 [–6.06; –4.61]	–4.08 [–5.07; –1.92]	**0.04**
miRNA-339	–10.61 [–11.73; –9.93]	–9.88 [–11.37; –8.15]	0.83
miRNA-424	–7.13 [–8.15; –6.13]	–6.99 [–8.01;–5.74]	0.45

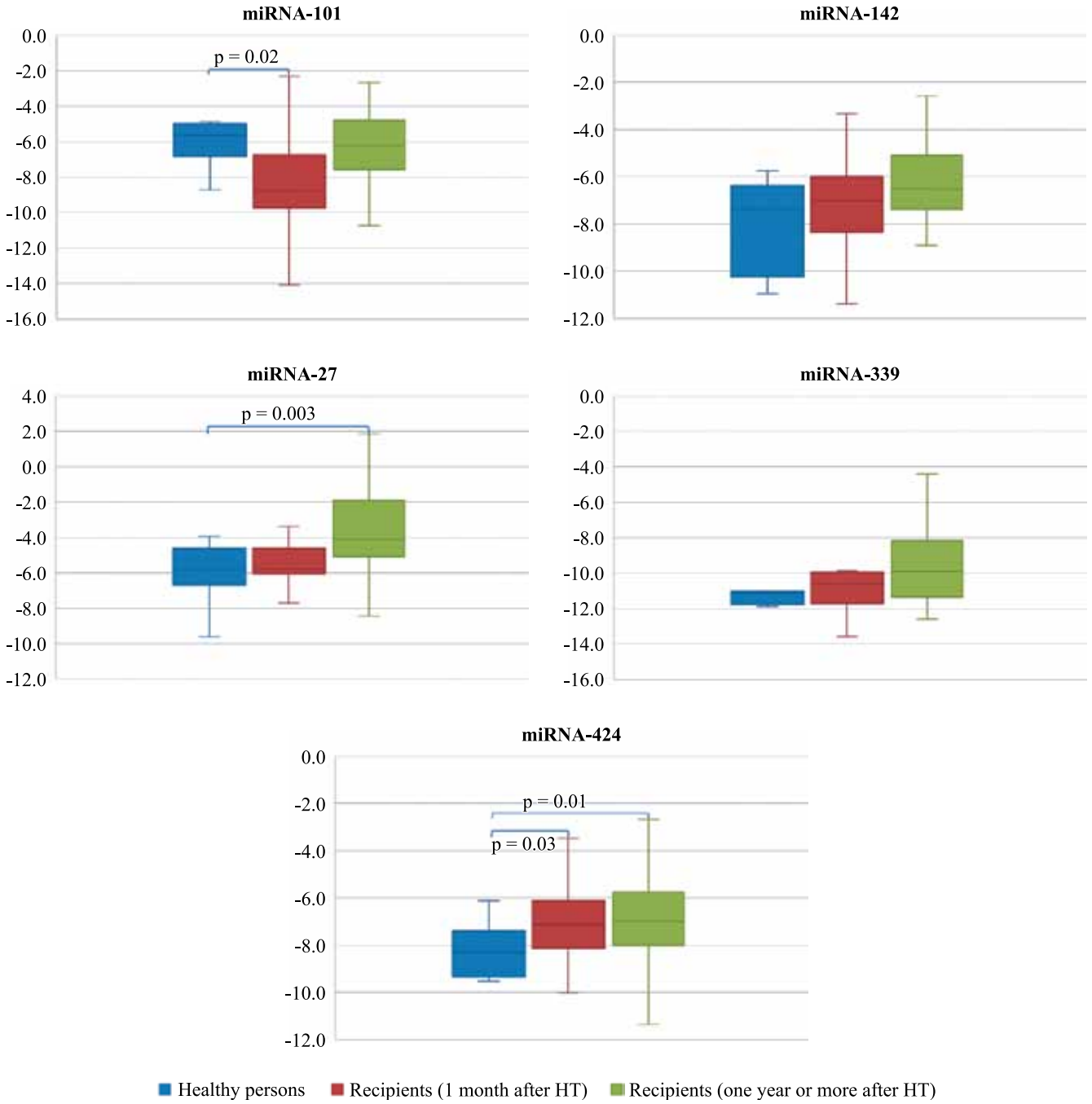

Fig. 3. miRNA expression levels in heart recipients in the early and long-term post-transplant periods, $\log_2(2^{-\Delta Ct})$

Table 3

Characteristics of heart transplant recipients with and without rejection

Parameter	Recipients	
	without rejection	with rejection
Age (years)	48.4 ± 9.9	49 ± 10.4*
Male (n,%)	16 (64%)	27 (87%)
Pre-HT diagnosis (n,%)	DCM – 17 (68%) CHD – 5 (20%) Other – 3 (12%)	DCM – 18 (58%) CHD – 10 (32%) Other – 3 (10%)
Tacrolimus concentration (ng/ml)	9.9 [6.1; 12]	8.1 [6.7; 10.7]*

Note. * – p > 0,05, compared with recipients without rejection.

Table 4

Comparative analysis of microRNA expression in heart transplant recipients with and without rejection

miRNA, $\log_2(2^{-\Delta Ct})$	Recipients		Significance, p
	without rejection	with rejection	
miRNA-101	–7.13 [–8.09; –4.87]	–8.21 [–9.60; –6.22]	**0.04**
miRNA-142	–7.02 [–8.04; –5.73]	–6.36 [–8.72; –5.11]	0.51
miRNA-27	–4.65 [–5.32; –1.85]	–5.19 [–6.78; –3.86]	**0.03**
miRNA-339	–10.04 [–11.2; –8.22]	–10.26 [–11.62; –9.2]	0.81
miRNA-424	–7.04 [–7.47; –5.79]	–7.05 [–8.27; –5.92]	0.98

CONCLUSION

Expression levels of miRNA-101, miRNA-27, miRNA-339 and miRNA-424 in patients with end-stage chronic heart failure – potential heart recipients – are higher than in healthy persons. A year or more following transplantation, the expression levels of miRNA-101, miRNA-142 and miRNA-339 in heart recipients do not differ from that in healthy persons. This may reflect normalization of adaptation processes in the graft.

Differences in the expression levels of miRNA-101 and miRNA-27 were found in recipients with acute graft rejection compared with recipients without it. This may be potentially important for assessing the risk of graft rejection and the possibility of minimizing immunosuppressive therapy. To assess the diagnostic effectiveness of miRNAs, further studies on the expression profile of these biomarkers in heart recipients are needed.

The work was supported by a grant (No. NSh-2598.2020.7) of the President of the Russian Federation for government support of leading research schools in the Russian Federation.

The authors declare no conflict of interest.

REFERENCES

1. *Kransdorf EP, Kobashigawa JA.* Novel molecular approaches to the detection of heart transplant rejection. *Per Med*. 2017 Jul; 14 (4): 293–297.
2. *Crespo-Leiro MG, Barge-Caballero G, Couto-Mallon D.* Noninvasive monitoring of acute and chronic rejection in heart transplantation. *Curr Opin Cardiol*. 2017 Mar 16. doi: 10.1097/HCO.0000000000000400. [Epub ahead of print].
3. *van Gelder T.* Biomarkers in solid organ transplantation. *Br J Clin Pharmacol*. 2017 Dec; 83 (12): 2602–2604.
4. *Velikiy DA, Gichkun OE, Shevchenko AO.* MikroRNK: rol' v razvitii serdechno-sosudistykh zabolevanij, perspektivy' klinicheskogo primeneniya. *Klinicheskaya laboratornaya diagnostika*. 2018; 63 (7): 403–409. [In Russ, English abstract].
5. *Savic-Radojevic A, Pljesa-Ercegovac M, Matic M et al.* Novel biomarkers of heart failure. *Advances In Clinical Chemistry*. 2017; 79: 93–152.
6. *Starling RC, Stehlik J, Baran DA et al.* Multicenter analysis of immune biomarkers and heart transplant outcomes: results of the clinical trials in organ transplantation-05 study. *American Journal of Transplantation*. 2016; 16: 121–136.
7. *Di Francesco A, Fedrigo M, Santovito D, Natarelli L, Castellani C, De Pascale F et al*. MicroRNA signatures in cardiac biopsies and detection of allograft rejection. *J Heart Lung Transplant*. 2018 Nov; 37 (11): 1329–1340.
8. *Shah P, Bristow MR, Port JD.* MicroRNAs in Heart Failure, Cardiac Transplantation, and Myocardial Recovery: Biomarkers with Therapeutic Potential. *Curr Heart Fail Rep*. 2017 Dec; 14 (6): 454–464.
9. *Khush K, Zarafshar S.* Molecular Diagnostic Testing in Cardiac Transplantation. *Curr Cardiol Rep*. 2017 Oct 13; 19 (11): 118.
10. *Gautier SV, Shevchenko AO, Popcov VN*. Pacient s transplantirovannym serdcem: rukovodstvo dlya vrachej po vedeniyu pacientov, perenesshih transplantaciyu serdca. M.: Triada, 2014: 144.
11. *Livak KJ, Schmittgen TD.* Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. *Methods*. 2001 Dec; 25 (4): 402–408.
12. *Zhang XL, An BF, Zhang GC.* MiR-27 alleviates myocardial cell damage induced by hypoxia/reoxygenation via targeting TGFBR1 and inhibiting NF-κB pathway. *Kaohsiung J Med Sci*. 2019 Oct; 35 (10): 607–614.
13. *Li X, Zhang S, Wa M, Liu Z, Hu S.* MicroRNA-101 Protects Against Cardiac Remodeling Following Myocardial Infarction via Downregulation of Runt-Related Transcription Factor 1. *J Am Heart Assoc*. 2019 Dec 3; 8 (23): e013112.
14. *Sukma Dewi I, Hollander Z, Lam KK, McManus JW, Tebbutt SJ, Ng RT et al*. Association of Serum MiR-142-3p and MiR-101-3p Levels with Acute Cellular Rejection after Heart Transplantation. *PLoS One*. 2017; 12 (1): e0170842.

The article was submitted to the journal on 21.11.2019