

Upregulated Expression of Circulating MicroRNAs in Kidney Transplant Recipients With Interstitial Fibrosis and Tubular Atrophy

Sepideh Zununi Vahed,^{1,2} Ahmad Poursadegh Zonouzi,³
Hossein Ghanbarian,⁴ Moteza Ghojzadeh,⁵ Nasser Samadi,²
Mohammadreza Ardalan¹

¹Kidney Research Center,
Tabriz University of Medical
Sciences, Tabriz, Iran

²School of Advanced
Biomedical Sciences, Tabriz
University of Medical Sciences,
Tabriz, Iran

³Biotechnology Research
Center, Tabriz University of
Medical Sciences, Tabriz, Iran

⁴School of Advanced
Technologies in Medicine,
Shahid Beheshti University of
Medical Sciences, Tehran, Iran

⁵Liver and Gastrointestinal
Disease Research Center,
Tabriz University of Medical
Sciences, Tabriz, Iran

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Introduction. The discovery of circulating microRNAs (miRNAs), as potential noninvasive diagnostic biomarkers, has opened new avenues of research for identifying transplant patients with chronic allograft dysfunction. The present study aimed to investigate the expression levels of 4 immune-related miRNAs, miR-21, miR-31, miR-142-3p, and miR-155, in plasma samples of kidney allograft recipients.

Materials and Methods. The plasma expression levels of the miRNAs were evaluated by quantitative real-time polymerase chain reaction in 53 kidney recipients with long-term stable allograft function (n = 27), biopsy-proven interstitial fibrosis and tubular atrophy (n = 26), and healthy controls (n = 15). The possible correlation between clinical parameters and the circulating miRNAs and the receiver-operating characteristic analysis were performed.

Results. Significantly upregulated expressions of miR-21 ($P = .02$), miR-142-3p ($P = .048$), and miR-155 ($P = .005$) were observed in plasma samples of recipients with interstitial fibrosis and tubular atrophy in comparison to the stable allograft function and healthy control groups. Expression level of the miR-21 in plasma was correlated with creatinine ($r = -0.432$, $P = .03$) and estimated glomerular filtration rate ($r = 0.423$, $P = .031$). Multivariable analysis indicated that miR-21, miR-142-3p, and miR-155 in plasma samples could discriminate almost most of the patients with interstitial fibrosis and tubular atrophy (area under curve, 0.802; sensitivity, 81%; specificity, 92%).

Conclusions. Our data suggested that altered expression of miR-21, miR-142-3p, and miR-155 in plasma samples might be associated with kidney allograft dysfunction and could be used for graft monitoring in kidney transplantation.

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INTRODUCTION

Kidney transplantation is the preferred therapy for life-threatening kidney failure, which has been increasingly successful with the advent of advanced immunosuppressive drugs and donor-recipient

matching procedures.^{1,2} However, posttransplant complications such as chronic allograft dysfunction remain unsolved issues that are associated with poor allograft survival.^{3,4} It has been suggested that activation of different components of the

immune responses and the presence of persistent inflammation accelerate the possibility of chronic allograft dysfunction and end-stage kidney disease progression.⁵⁻⁷ Therefore, immune monitoring of the kidney allograft has great potential benefits to improve long-term allograft function.

During the past decades, the discovery of microRNAs (miRNAs) has revolutionized different aspects of transplant immunobiology. MicroRNAs are short noncoding endogenous RNAs that regulate gene expression posttranscription through binding to specific mRNA targets and repressing their translation or inducing their degradation.⁸ The tissue and cell-specific distribution characteristics of miRNAs and their relatively stable existence in serum and plasma make them attractive noninvasive diagnostic biomarkers for early diagnosis of various diseases.⁹ By regulating cell growth, proliferation, differentiation, and death, miRNAs play pivotal roles in immune responses, innate immunity, inflammation and kidney development, physiology, homeostasis, and diseases.¹⁰ These observations underscore the importance of circulating miRNA signatures as possible diagnostic and prognostic biomarkers in monitoring of kidney transplants.

Altered expression of miRNAs have previously been reported in posttransplant episodes of human kidney allograft recipients with acute rejection,¹¹⁻¹⁴ antibody-mediated chronic rejection,¹² operational tolerance,¹⁵ and chronic allograft dysfunction.¹⁶⁻²¹ In present study, we focused on miR-21, miR-31, miR-142-3p, and miR-155, since they are specific to immune cell lineages.^{11,12} Meanwhile they are deregulated in different diseases. We aimed to investigate whether circulating miR-21, miR-31, miR-142-3p, and miR-155 in kidney recipient's plasma are associated with interstitial fibrosis and tubular atrophy (IFTA) and have potential benefits in monitoring of immune responses in kidney transplant recipients.

MATERIALS AND METHODS

Participants

Fifty-three kidney transplant recipients of living donors (41 men and 12 women) were enrolled from the Department of Kidney Transplantation of Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran. This cross-sectional study was performed between April 2014 and August 2015. Patients with the following criteria

were included in the study: age range between 21 and 60 years and transplantation at least 3 years earlier. The exclusion criteria for the were diabetes mellitus; serologic positivity for human immunodeficiency virus, hepatitis B virus, hepatitis C virus, and cytomegalovirus; severe bacterial infection; history of cancer; obstruction in the kidney allograft system; inflammatory diseases; severe organ failure; and autoimmune disorders. Renal biopsies were performed in 26 kidney transplant recipients with graft dysfunction. Biopsy samples of recipients with impaired kidney allograft function were examined histologically for rejection, viral nephropathy, interstitial involvement, and IFTA. Accordingly, based on the graft function²² and the histological studies, recipients were divided into 2 major groups of recipients with stable graft function (SGF; n = 27, 19 men and 8 women), and those with impaired kidney allograft function (n = 26, 22 men and 4 women). Recipients with SGF were defined by sustained estimated glomerular filtration rate (GFR) values less than 40 mL/min/1.73 m², without a history of acute rejection or delayed graft function. We allocated the SGF group based on their clinical condition without any knowledge about their histology. Recipients with continued decrease in GFR and histological evidence of IFTA were defined as the case group. Moreover, healthy volunteer individuals were also recruited as controls (n = 15). Immunosuppression regimen in the studied population consisted of triple therapies of mycophenolate mofetil, cyclosporine, and prednisone. The study protocol was approved by the Ethics and Human Rights Committee of Tabriz University of Medical Sciences, Tabriz, Iran (code: TBZMED.REC.1394.931) and written informed consent was obtained from all participants.

RNA Extraction and MicroRNAs Reverses Transcription

The fasting blood sample (2 mL) were collected in ethylenediaminetetraacetic acid-containing vacutainers and transferred into a microtube. The samples were centrifuged at 1000 × g at 4°C for 10 minutes. Then, plasma was transferred into another microtube without touching the leukocyte layer and recentrifuged at 1000 × g at 4°C for 10 minutes. Plasma was collected carefully and aliquoted in RNase-free tubes and stored at -80°C until future use. All plasma samples were completely thawed

on ice and centrifuged at $3000 \times g$ at 4°C for 5 minutes to remove the remaining cell debris. Then, 200 μL of plasma sample was transferred to a new microtube, and circulating RNA were isolated from all specimens using miRCURY RNA Isolation kit, Biofluid, (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. The quality and quantity of extracted RNA were confirmed by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted RNAs were reverse-transcribed using the miRCURY LNA Universal RT cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark), following the manufacturer's recommended protocols. To avoid inhibition of the RT-reaction in plasma samples, we assayed the optimum plasma miRNA input that could be used in all samples since RNA yields of some samples were very low. Based on the results, 4- μL RNA was reverse transcribed in a 20- μL reaction volume at 42°C for 60 minutes, followed by an inactivation reaction at 95°C for 5 minutes.

Quantitative real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed in triplicate using specific locked nucleic acids primers miR-21-5p (assay ID, 204230), miR-31-5p (assay ID, 204236), miR-142-3p (assay ID, 204291), miR-155-5p (assay ID, 204308), and miR-191-5p (assay ID, 204306), based on the protocol of the SYBR Green master mix kits (Exiqon, Vedbaek, Denmark) by the iCycler iQ system (Bio-Rad). The amplification was performed at 95°C for 30 seconds, followed by 40 cycles at 94°C for 5 seconds, 60°C for 60 seconds. The miR-191-5p was used as internal control. After the completion of the polymerase chain reaction cycling, melting curves were generated at 95°C to verify specificity. The real-time polymerase chain reaction efficiency was tested on each primer pairs using the 10-fold serial dilution method (10^{-1} - 10^{-5} dilutions) to generate standard curves.

Statistical Analysis

The data were shown as mean \pm standard deviation for normally distributed values and median (range) for nonparametric values. Statistical significance between groups were analyzed by the Student *t* test and the 1-way analysis of variance, followed by the Tukey Post Hoc test, when the

variables between groups were normally distributed. The Mann-Whitney U test and the Kruskal-Wallis test were used for nonparametric values. To assess the correlation between plasma levels of studied circulating miRNAs and clinicopathological parameters, we used the Spearman rank order correlation test. The receiver operating characteristic (ROC) analysis was applied to diagnose the recipients with biopsy-proven IFTA from recipients with SGF. Moreover, a logistic regression was conducted with desired miRNAs and the predicted probabilities were used as a test variable in ROC curve analysis to test their combined effect. The sensitivity and specificity of the miRNAs were calculated using the online Centre for Evidence-Based Medicine, Toronto (<http://ktclearinghouse.ca/cebmd/practise/ca/calculators/statscalc>) and the MedCalc software (version 15.11.4). Statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences, version 17.0, IBM Corp, New York, NY, USA). *P* values less than .05 were considered significant.

RESULTS

Participants

Demographic and clinical characteristics of the patients are presented in Table 1.

Expression of Circulating MicroRNAs

As shown in Figure 1A, elevated levels of miR-21 (1.77-fold, $P = .02$), miR-142-3p (1.2-fold, $P = .048$), and miR-155 (1.03-fold, $P = .005$) were observed in the IFTA group when compared to the SGF controls. Increased levels of these miRNAs in the IFTA group were further confirmed when their expression levels were compared to the healthy controls (Table 2). Increased expression levels of circulating miR-21 was significant in the patients with IFTA grade I (1.98-fold, $P = .01$) but not in the patients with grade IFTA III (1.43-fold, $P = .16$; Figure 1B). Elevated levels of miR-142-3p were not significant in IFTA grades (Figure 1C). Levels of miR-155 was significant in patients with severe IFTA (2.46-fold change, $P = .01$). In the most of plasma samples, miR-31 was not detectable (cycle threshold > 40). Significant internal correlation was observed between miR-21 and miR-155 change in cycle threshold ($r = -0.416$, $P = .002$), but not miR-142 and miR-21 ($r = -0.080$, $P = .57$) nor miR-155 ($r = 0.096$, $P = .49$) in any of the plasma samples.

Table 1. Demographic and Baseline Clinical Data of Kidney Allograft Recipients With Interstitial Fibrosis and Tubular Atrophy (IFTA) and Stable Graft Function (SGF) Compared to Controls*

Characteristics	IFTA Group	SGF Group	Healthy Controls	P
Number of patients	26	27	15	...
Sex				
Male	21	19	10	
Female	5	8	5	...
Age, y	38 ± 12.0	40 ± 8.0	35 ± 5.0	.55
Body weight, kg	72 ± 15.1	74 ± 10.7	65 ± 9	.07
Delayed graft function	3	0
Acute rejection	2	0
Multiple transplants	4	1
Time posttransplantation, y	8.6 (3 to 14)	9.2 (3 to 23)69
Serum creatinine, mg/dL	3 (1.6 to 8.9)	1.3 (0.6 to 2)	1 (0.6 to 1.2)	< .001
Estimated glomerular filtration rate, mL/min/1.73 m ²	19 (6.8 to 40)	79.7 (41 to 108)	87 (73 to 117)	< .001
IFTA grade (I/III)	16/10
Type of kidney donor				
Related	3	1	...	
Unrelated	23	26
Donor sex				
Male	22	20	...	
Female	4	7
Donor age, y	27.4 ± 5.6	25.0 ± 5.281

*Values are mean standard deviation, median (range), and frequency.

Table 2. Plasma MicroRNAs Expression of Kidney Allograft Recipients With Interstitial Fibrosis and Tubular Atrophy (IFTA) and Stable Graft Function (SGF) Compared to Controls*

MicroRNA	SGF group		IFTA group	
	RQ	P	RQ	P
miR-21	1.04	0.976	1.59	0.027
miR-142-3p	0.24	0.105	0.2	0.082
miR-155	1.57	0.07	37.5	0.033

*RQ indicates relative quantification (fold change).

MicroRNAs Expression and Clinical Parameters

Plasma level of miR-21 was negatively correlated with serum creatinine ($r = -0.432$, $P = .03$) and it was positively correlated with estimated GFR ($r = 0.423$, $P = .03$; Figures 2A and 2B). Serum creatinine and GFR were not correlated with miR-142-3p or miR-155 (Figures 2C to 2F). Donor age, recipient age, and posttransplantation time did not have any correlation with the plasma levels of miR-21, miR-142-3p, and miR-155 (Figure 3 A-I).

Receiver Operating Characteristic Curve Analysis

To evaluate diagnostic values of each miRNA for distinguishing IFTA individuals from otherwise healthy kidney recipients, ROC curve analysis was calculated using the change in cycle threshold value.

The area under curve (AUC) value was 0.740 (95% confidence interval [CI], 0.612 to 0.931; $P < .001$) for miR-21, with a 56% sensitivity and 96% specificity (Figure 4A). Based on Figure 4B, miR-142-3p showed a sensitivity of 50% and specificity of 87% (AUC, 0.580; 95% CI, 0.430 to 0.794). Analysis on miR-155 revealed the AUC value of 0.570 (95% CI, 0.419 to 0.784, $P = .03$) with an 81% sensitivity and 47% specificity (Figure 4C). Multivariable ROC curve analysis showed that combined miRNAs had a higher discriminating value than any of miRNAs alone in plasma samples (AUC, 0.802; 95% CI, 0.650 to 0.935; 81% sensitivity and 92% specificity; Figure 4D).

DISCUSSION

Numerous genetic, epigenetic, pharmacogenetic, and immunological factors are involved in graft failure after transplantation.²³⁻²⁵ A line of evidence specifies a strong correlation between miRNA expression pattern and the status of kidney allografts; therefore, applying these biomarkers in monitoring of graft function in transplant recipients can be helpful.^{11,26-28}

With regards to the upregulated miRNAs, miR-21 is a distinctive miRNA that involves in different signaling pathways including cell

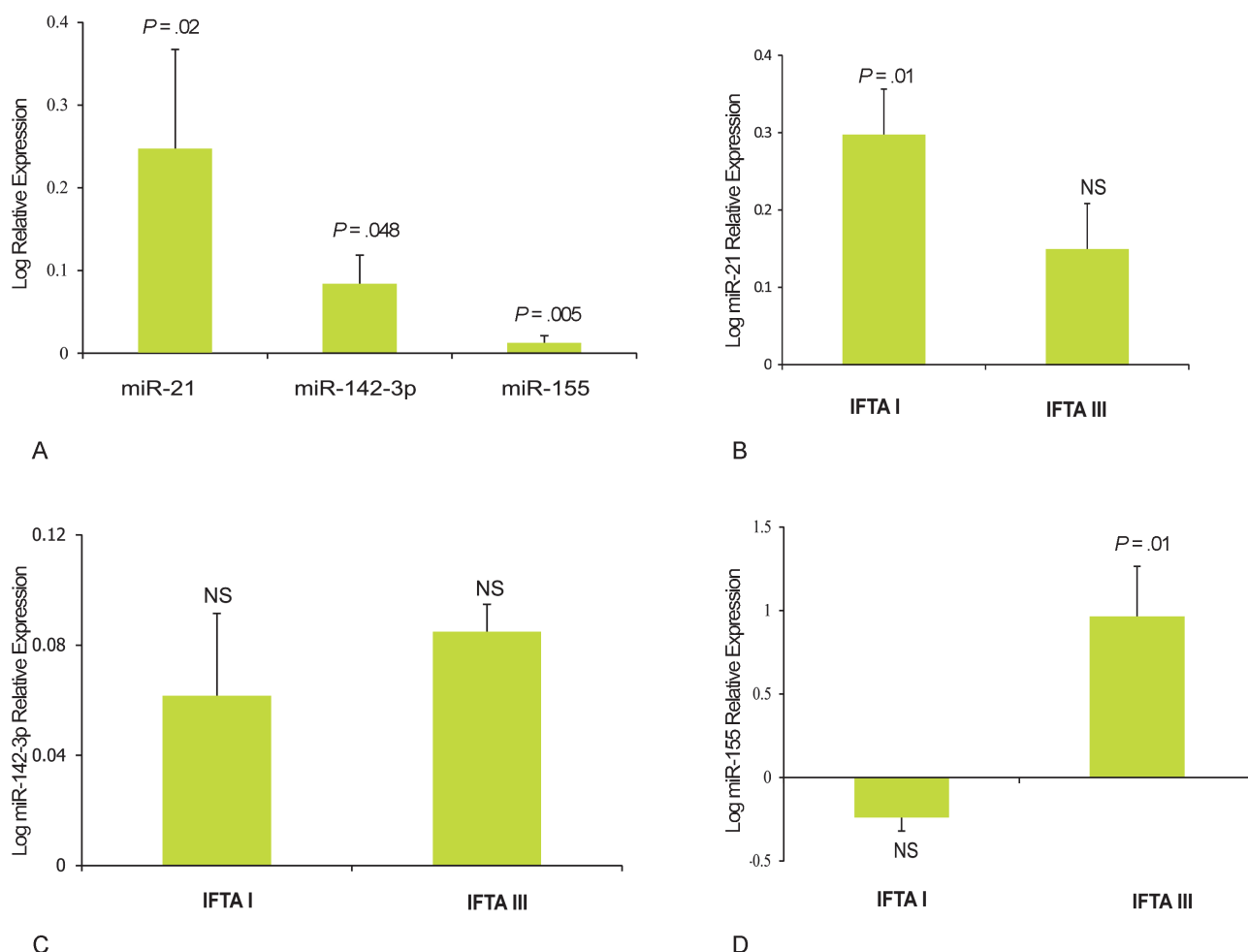


Figure 1. Real-time polymerase chain reaction results. A, Differentially expressed circulating miRNAs in the interstitial fibrosis and tubular atrophy (IFTA) group when compared to SGF group. B to D, Expression of miR-21, miR-142-3p, and miR-155 levels in plasma samples of patients diagnosed as IFTA with different grades IFTA. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression (fold change) between sample groups. Relative expression is indicated as log (relative expression). MicroR-191 was used as normalizing endogenous control.

function, proliferation and apoptosis.²⁹ MicroR-21 has a dynamic role in pro-inflammatory or immunosuppressive states, and also acts as an important mediator between the balance and transition of both states.³⁰ Dysregulated miR-21 leads to cellular transformation, tissue fibrosis and appearance of different kidney diseases. Induction of miR-21 by inflammatory stimuli cells was reported in both animal models and clinical studies.^{18,19,30,31} In the present study, increased expression level of circulating miR-21 was observed in IFTA group and in patients with severe IFTA grades when compared to both control groups (SGF and healthy groups) that indicates active immune responses and damaged kidney tissue and active fibrosis (Figure 1).

Forkhead box P3 (FoxP3) is a master transcription

factor involved in regulatory T (Treg) cell function and development, and also the maintenance of cyclic AMP (cAMP). Cyclic AMP regulates diverse cellular functions.³² Increased levels of Foxp3 have been correlated with the presence of interstitial inflammation, IFTA and tubulitis.³³ MicroR-31 is known to target FoxP3 mRNA and negatively regulates its expression in Treg cells. Given the established role of miR-31, we expected to observe its deregulated expression in plasma samples of kidney recipients diagnosed as IFTA. However, circulating miR-31 in plasma samples was not detectable.

MicroR-142-3p is mainly expressed in peripheral blood mononuclear cells and can control the functions of CD4+ T and CD4+ CD25+ Treg cells.^{32,34} It is reported that Foxp3 downregulates miR-142-

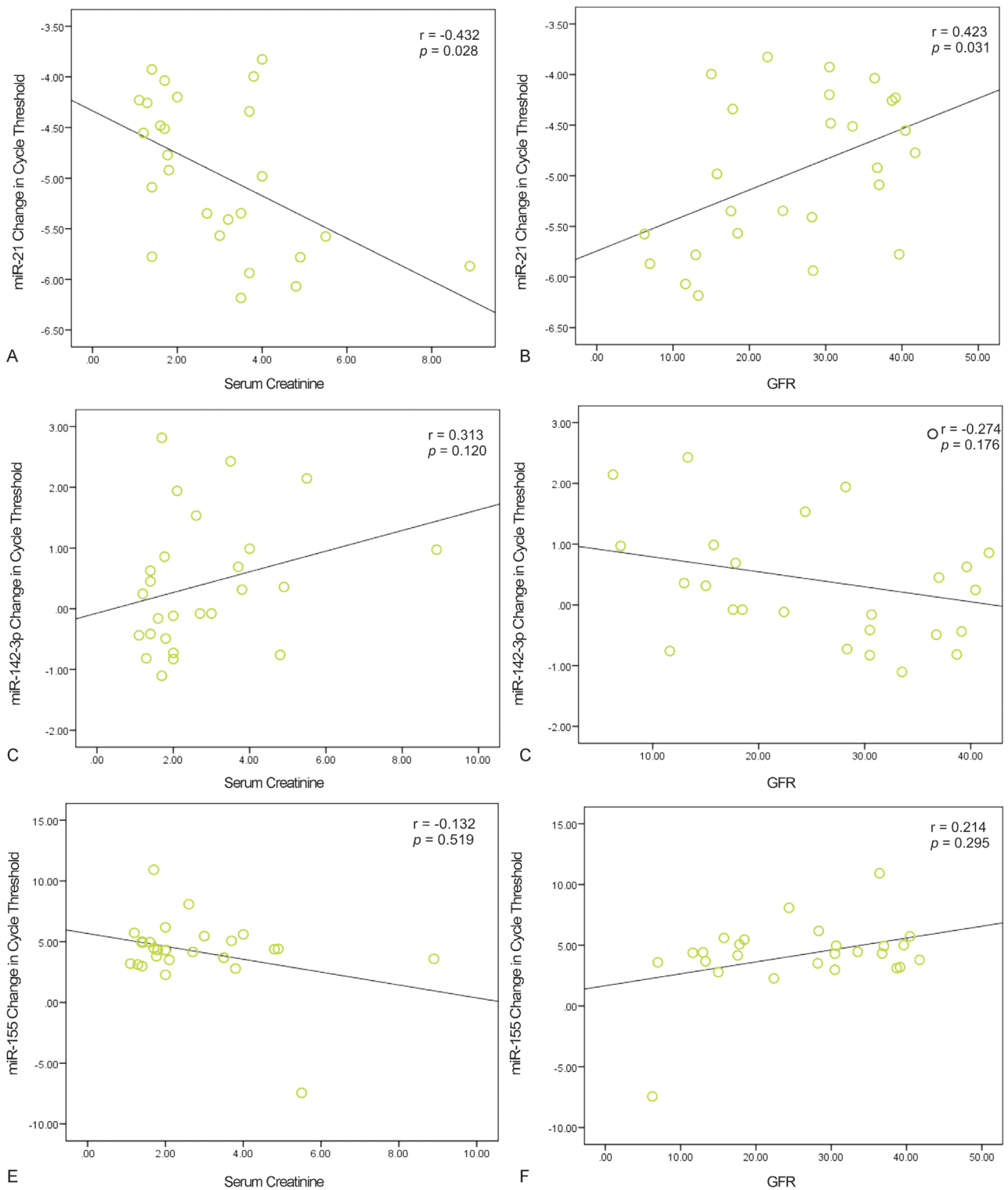


Figure 2. The correlation between the levels of circulating microRNAs (change in cycle threshold) and clinical parameters and serum creatinine and estimated glomerular filtration rate (GFR) in the interstitial fibrosis and tubular atrophy group.

3p to maintain the AC9-cAMP pathway active in Treg cells.³² Moreover, miR-142 is involved in chronic antibody-mediated rejection and fibrosis.

Upregulation of miR-142-5p and miR-142-3p have been reported in kidney biopsies and peripheral blood mononuclear cells samples of recipients with

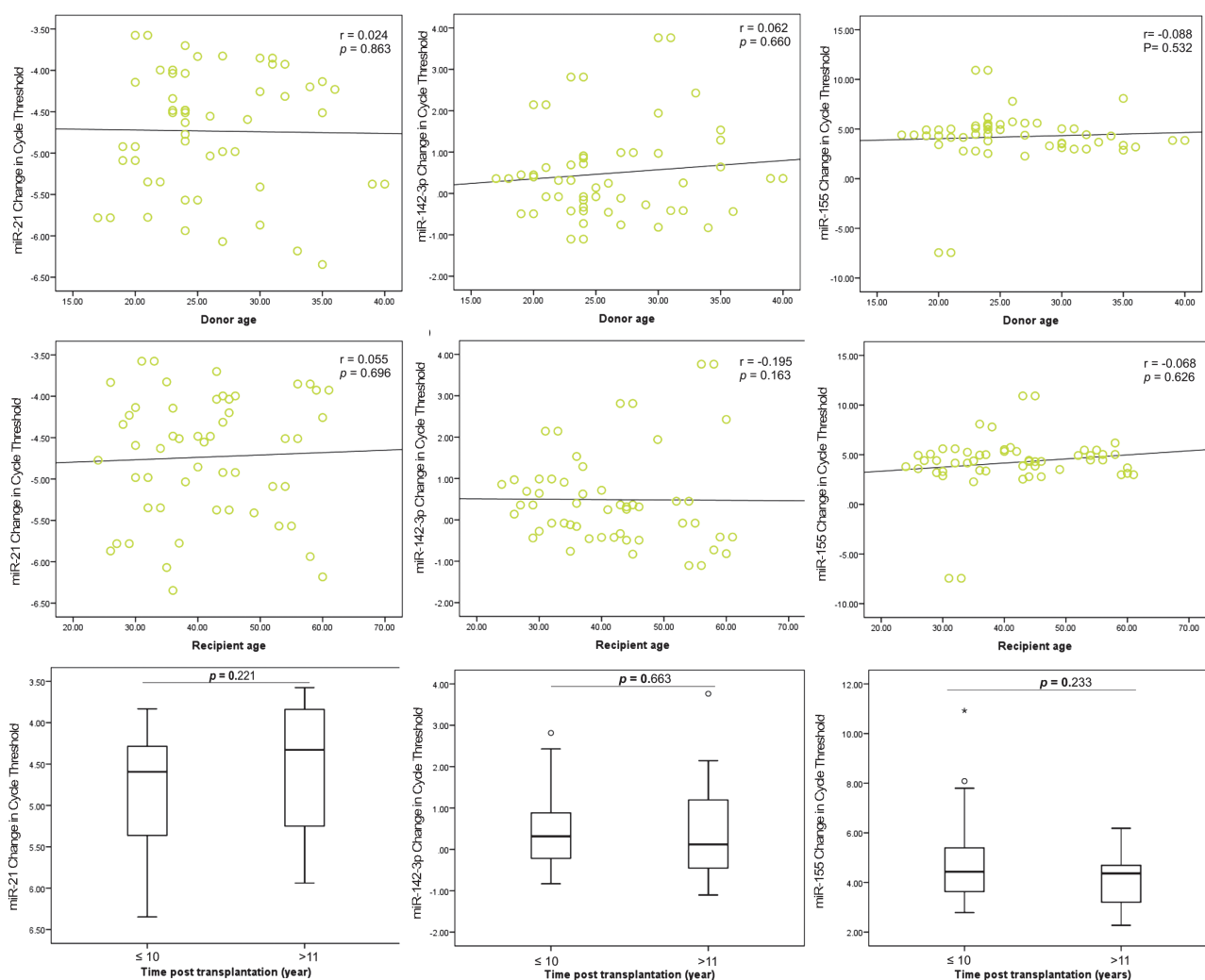


Figure 3. The correlation between the levels of circulating microRNAs (change in cycle threshold) and demographic parameters.

coronary artery disease.^{18,20 21} Moreover, Scian and colleagues have reported differential expression of miR-142-3p in biopsy samples and urinary cells of kidney allografts with IFTA.²⁰ Increased levels of miR-142 has also been observed in peripheral blood mononuclear cells samples of operationally tolerant and chronic antibody-mediated rejection.¹⁵ In this study, increased levels of circulating miR-142-3p (Figure 1) in IFTA individuals may decline the ability of Treg cells to suppress immune related processes.

MicroR-155 is associated with the inflammatory response in multiple immune cell lineages. It plays key roles in adaptive immunity and T-cell-mediated antibody response in regulating T helper cell differentiation. Huang and colleagues reported the upregulation of miR-155 in the diabetic nephropathy patients that are contributed

to inflammation-mediated glomerular endothelial injury.³⁵ Overexpressed miR-155 was also seen in acute rejection biopsy samples compared to normal allograft biopsies.¹¹ Consistent with these studies, we showed that circulating miR-155 was higher in plasma samples of patients with IFTA compared with SGF recipients; its fold change was even higher in IFTA group when compared to healthy controls (Table 2, Figure 1C) that indicated active inflammation.

The possibility of applying these miRNAs as diagnostic markers was evaluated using ROC curve analysis. The plasma expression levels of miR-21, miR-142-3p, and miR-155 alone could not properly distinguished all IFTA recipients from normal allografts significantly (Figure 4A and B). However, the combined miRNAs had high discriminating value in plasma samples with an

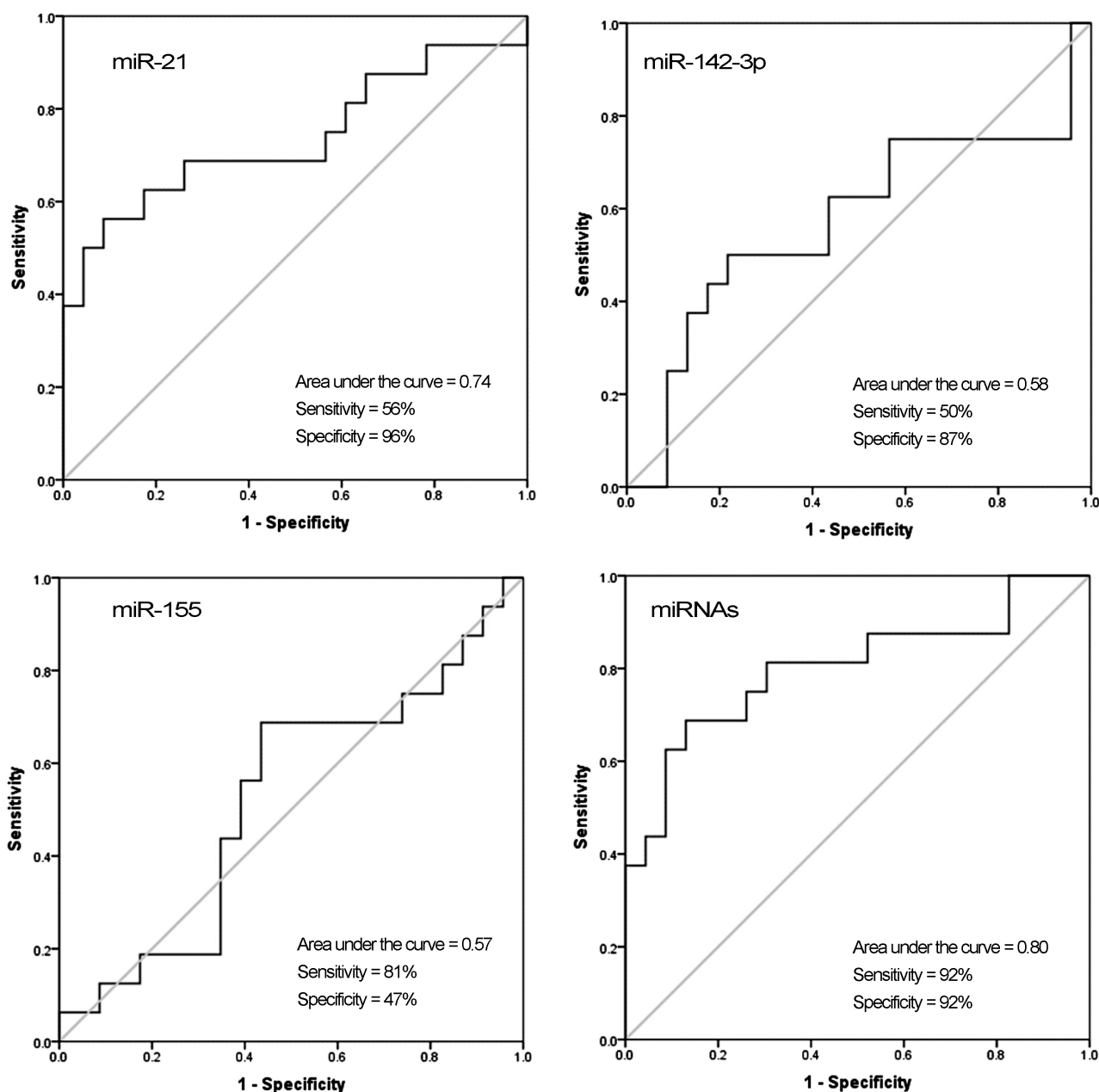


Figure 4. The receiver operating characteristic curve analysis of the diagnostic power of microRNAs in distinguishing the interstitial fibrosis and tubular atrophy, based on real-time polymerase chain reaction data.

AUC of 0.802 (Figure 4D).

Some limitations existed in the present study. The limited number of patients was due to the strict criteria used for the selection of patients. Moreover, limitation of kidney tissue study in majority of patients for an obvious reason, patients were disputed with biopsy. Thus, to recognize reliable diagnostic and prognostic biomarkers for graft immune monitoring after transplantation on programmed timelines, additional studies with large

prospective cohorts and more validated miRNAs are required to interpret them into clinical routine.

CONCLUSIONS

The expression levels of immune-related miRNAs such as miR-21, miR-142-3p, and miR-155 can be used for immune response monitoring in plasma samples of kidney transplant recipients. They can accurately distinguish almost most of IFTA cases from otherwise healthy recipients. Therefore,

their expression levels may serve as non-invasive diagnostic biomarkers in kidney transplantation beside other validated markers.

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CONFLICT OF INTEREST

None declared.

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Correspondence to:
Mohammadreza Ardalani, MD
Kidney Research Center, Tabriz University of Medical Sciences,
Tabriz, Iran
Tel: +98 914 116 8518
Fax: +98 411 336 6579
E-mail: ardalani34@yahoo.com

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