

Altered Expression of MicroRNAs Following Chronic Allograft Dysfunction with Interstitial Fibrosis and Tubular Atrophy

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ABSTRACT

Chronic allograft dysfunction (CAD) remains the major cause of renal transplant loss and characterized by interstitial fibrosis and tubular atrophy (IFTA). MicroRNAs (miRNAs) are implicated in many biological processes as well as innate and adaptive immune responses. We aimed to investigate whether CAD with IFTA is associated with differential expression of miR-142-5p, miR-142-3p and miR-211 within biopsy and peripheral blood mononuclear cell (PBMC) samples and whether expression of miRNAs are diagnostic for CAD with IFTA and predicts renal allograft function.

In this study, biopsy and PBMC samples of 16 CAD with IFTA and 17 normal allografts (NA) were collected. Using Taqman MicroRNA Assays the expression levels of miR-142-5p, miR-142-3p and miR-211 were determined in two groups.

Our results showed that miR-142-5p and miR-142-3p were significantly ($p < 0.0001$) up-regulated and miR-211 was significantly ($p < 0.0001$) down-regulated in renal allograft tissues of CAD with IFTA compared with NA recipients. Moreover, miR-142-3p and miR-211 were significantly ($p < 0.0001$) up-regulated and down-regulated respectively in PBMC samples of CAD with IFTA. According to the ROC curve analysis, miR-142-5p in biopsy samples, but miR-142-3p and miR-211 both in biopsy and PBMC samples could be used as a diagnostic biomarker of CAD with IFTA and a prediction factor of allograft function. In this study, miRNAs were differentially expressed in the kidney allograft biopsy and simultaneously in PBMC samples of patients with CAD with IFTA.

We suggest that the expression of miRNAs in PBMC might be used for monitoring the post transplantation and also as potential non-invasive biomarkers of kidney graft function and CAD with IFTA.

Keywords: Biomarker; Chronic allograft dysfunction; IFTA; MicroRNA; Renal transplantation

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INTRODUCTION

Renal transplantation is considered as the most effective treatment for patients with end-stage renal disease.¹ Despite recent advances in immunosuppression therapy, chronic allograft dysfunction (CAD) remains the major cause of renal transplant loss.² Histologically, CAD is characterized by interstitial fibrosis and tubular atrophy (IFTA).³

The diagnosis of chronic renal allograft dysfunction depends upon clinical findings and biopsy histopathology. Renal biopsy remains as a gold standard for diagnosis of renal allograft status.^{4,5} Also, renal biopsy is a risky and invasive procedure that sampling error and variability of reports among pathologists are additional concerns.^{6,7} Therefore, noninvasive, accurate, sensitive and specific biomarkers for monitoring of graft function and tailoring immunosuppressive therapy are critically needed.⁸

MicroRNAs (miRNA) are small, comprising of about 19-25 nucleotides, evolutionary conserved, non-coding RNA molecules that regulate gene expression by inhibiting translation or degradation of their target messenger RNAs.⁹ In addition, microRNAs implicated in many biological processes such as cellular proliferation, differentiation, organ development, oncogenesis, apoptosis as well as important functions in the immune system.¹⁰⁻¹² Moreover, miRNAs are correlated with many human diseases and conditions, including cancer, heart diseases or in pregnancy that is a physiological state.¹³ It has been proposed that miRNAs are potential diagnostic biomarkers of multiple diseases because of their stability, ease of detection and high reliability.¹⁴ In the human renal transplantation, miRNAs have been found to be differentially expressed in tissues, blood or urine of human renal allograft recipients with acute rejection,^{5,15,16} fibrosis,¹⁷⁻¹⁹ chronic antibody mediated rejection,²⁰ and operationally tolerant.²¹ To our knowledge, until now the investigation of miRNA expression in PBMC samples of CAD with IFTA patients has not been evaluated yet.

We aimed to investigate the expression levels of miR-142-5p, miR-142-3p and miR-211 following CAD due to fibrosis insult to determine the predictive/diagnostic value of these miRNAs in renal transplant recipients with CAD IFTA. To the best of

our knowledge, this is the first study which investigated the expression levels of miR-142-5p, miR-142-3p and miR-211 simultaneously in biopsy and PBMC samples of patients diagnosed with IFTA. Since the miRNAs regulate mRNAs, we aimed to investigate whether intragraft and PBMC expression of miRNAs are associated with CAD characterized by IFTA. Moreover, we examined whether miRNAs expression in tissue and PBMC samples distinguish CAD with IFTA from normal allograft (NA) recipients and whether miRNAs are the potential predictive/diagnostic biomarkers of CAD with IFTA and allograft function.

MATERIALS AND METHODS

Patients and Sample Collection

The study was approved by Tehran University of Medical Sciences Ethical Committee and each patient signed written informed consent. We studied 36 renal allograft biopsy samples from renal-transplant recipients who had undergone a clinically-indicated kidney allograft biopsy or protocol follow biopsy (procedure for early diagnosis of rejection or allograft maintenance to prevent rejection by pathological examination of allograft biopsy) at Labbafinejad Medical Center. The peripheral blood of these patients was collected at the time of their biopsy. Biopsy-proven CAD with IFTA (Figure 1) was diagnosed from renal biopsy findings scored according to the 2009 update Banff classification criteria²². Renal transplant recipients were categorized in two groups; CAD with IFTA patients (Grade II and Grade III) and NA without evidence of rejection based on histopathological evidence of biopsies. Histological examination of biopsy samples was performed by a pathologist who was blinded to the result of molecular studies. Clinical and demographic characteristic information of patients have been shown in table 1.

In this study, biopsy and PBMC samples of 16 CAD with IFTA renal allograft recipients and 17 NA recipients were collected. The inclusion criteria were patients undergoing their first transplantation without history of CAD with IFTA during 5 years and signing of the inform consent forms. The exclusion criteria were patients with BK virus infection, histopathological evidence of calcineurin inhibitor nephrotoxicity, urinary tract obstruction and patients undergoing second transplantation.

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Table 1. Demographic and clinical characteristics of subjects

Topics	Normal allograft	CAD with IFTA
Number of patients	17	16
Male (n;%)	11	12
Female (n;%)	6	4
Age (years; min-max)	52 (34-67)	47 (25-57)
Types of allografts		
Deceased	2	5
Living	15	11
Types of donors		
Related	2	0
Unrelated	15	16
Recipient CMV* (pos)	1	3
CMV disease	0	0
Donor CMV(pos)	3	2
Donor HCV(pos)	0	0
Donor HBS Ag (pos)	0	0
Donor HBC Ab (pos)	2	0
Donor HIV(pos)	0	0
Blood creatinine level (mg/dl)	1.23±0.20	3.44±1.30
Date of biopsy (months post-transplant)	14.11±3.87	56.37±22.97

* cytomegalovirus

Renal allograft biopsy samples were immediately dipped in RNAlater solution (Ambion) to protect the RNA from degradation until RNA extraction according to the manufacturer's instructions.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of the same patients using standard ficoll density gradient centrifugation. Then, Cellular pellets were placed in RNAlater solution until RNA extraction according to manufacturer's instructions.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from biopsy and PBMC samples using the mirVana miRNA isolation kit (Ambion) then quantified with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies). Total RNA (10 ng/μl) was reverse transcribed into complementary DNA (cDNA) using miRNA-specific stem loop primers for individual miRNAs provided by Taqman MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) and reagents supplied by Taqman MicroRNA Reverse Transcription Kit (Applied biosystems, Foster City, CA, USA).

Real-time qPCR

The expression levels of individual miRNAs were measured using Taqman® MicroRNA Assays (Applied biosystems, Foster City, CA, USA) using specific

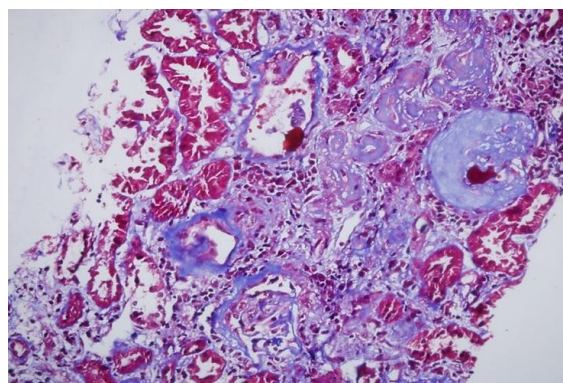


Figure 1. Interstitial fibrosis and tubular atrophy (IFTA) which previously known as chronic allograft nephropathy. This figure is an example of Grade III based on Banff IFTA grade which is characterized by a severe interstitial fibrosis. In this trichrome stain blue colored areas are fibrotic regions (collagen deposition). Grade II are defined as moderate interstitial fibrosis and tubular atrophy.

RESULTS

primer and probes for miR-142-5p (assay ID: 002248), miR-142-3p (assay ID: 000464) and miR-211 (assay ID: 000514), in biopsy and PBMC samples of CAD with IFTA and NA recipients. RNU44 (Assay ID: 001006) was used as an endogenous control for data normalization. For each miRNA-specific assay, we prepared a reaction pre-mix containing sufficient TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and primer/probe mix provided by Tagman miRNA Assays for all reactions. Real-time PCR was carried out in triplicate on a StepOnePlus Real-Time PCR System (Applied Biosystems).

Data Analysis

The data were expressed as median and inter-quartile range (IQR) values. The expression fold change (FC) was calculated using the $\Delta\Delta C_t$ method.²³ In summary, relative expression = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (\Delta C_t \text{ of CAD}) - (\Delta C_t \text{ of NA})$. Non-parametric Mann-Whitney test was performed to determine the differential expression of miRNAs. Also, receiver operating characteristic (ROC) curve was used in order to evaluate the diagnostic/predictive value of miRNAs. Optimal cutoff points were found based on Youden J Statistic (Youden J Statistic = Sensitivity + Specificity - 1). All analyses were performed by SPSS software (version 21, IBM Corporation, New York, USA). A *p* value less than 0.05 was considered as statistically significant.

Altered Intra-graft Expression of miR-142-5p, miR-142-3p and miR-211 Is Associated with CAD with IFTA

Biopsy samples were classified as CAD with IFTA (N=16) or NA (N=17) based on the histological reports according to the 2009 update Banff classification criteria.²² The expression of miR-142-5p, miR-142-3p and miR-211 were determined in biopsy samples of patients with CAD with IFTA compared with NA using Taqman® MicroRNA Assays (Applied biosystems, Foster City, CA, USA). We found miR-142-5p (FC=3.11, *p*<0.0001), miR-142-3p (FC=13.74, *p*<0.0001) and miR-211 (0.05, *p*<0.0001) were significantly expressed in biopsy samples of CAD with IFTA compared with normal allografts (Figure 2 and Table 2).

The ROC curve analysis of miRNA levels was performed in order to determine the cutoff points with the highest sensitivity and specificity to discriminate CAD with IFTA patients from normal allograft recipients. ROC curve analysis showed, CAD with IFTA could be distinguished with a high degree of accuracy together with the highest sensitivity and specificity using intragraft levels of miR-142-5p (AUC=1.00, CI_{95%}=[1.00–1.00]; *p*<0.0001), miR-142-3p (AUC=1.00, CI_{95%}=[1.00–1.00]; *p*<0.0001) and miR-211 (AUC=1.00, CI_{95%}=[1.00–1.00]; *p*<0.0001) (Figure 4 and Table 3). The line have been overlapped

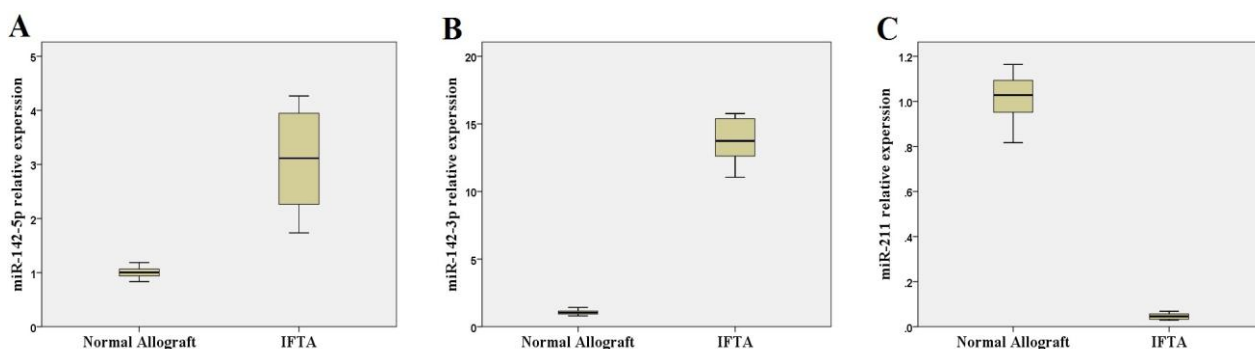


Figure 2. The expression levels of miR-142-5p, miR-142-3p and miR-211 in renal allograft tissue samples of CAD with IFTA patients compared with normal allograft recipients. Taqman MicroRNA Assays RT-qPCR results revealed that (A) miR-142-5p (*p*<0.0001) and (B) miR-142-3p (*p*<0.0001), were significantly upregulated. (C) miR-211 (*p*<0.0001) was significantly downregulated in the biopsy samples of CAD with IFTA patients compared with normal allograft recipients.

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Table 2. The expression of miRNAs in biopsy and PBMC samples

Sample	miRNAs	Normal allograft Fold change median (IQR) ^a	CAD with IFTA Fold change median (IQR)	P value
Biopsy	miR-142-5p	1.00 (0.94-1.07)	3.11 (2.26-4.09)	<0.0001*
	miR-142-3p	1.04 (0.92-1.18)	13.74 (12.61-15.40)	<0.0001*
	miR-211	1.03 (0.95-1.10)	0.05 (0.03-0.06)	<0.0001*
PBMC	miR-142-5p	1.04 (0.92-1.13)	1.14 (0.98-1.24)	0.171
	miR-142-3p	1.02 (0.89-1.20)	2.94 (2.35-4.11)	<0.0001*
	miR-211	1.03 (0.85-1.12)	0.43 (0.39-0.53)	<0.0001*

^a Inter-quartile range

* Significant values

on vertical and horizontal axis of ROC figure, because sensitivity was 1 and 1-specificity was zero for miRNAs in the grafts.

Expression of miRNAs in PBMC Samples

In order to identify miRNAs as diagnostic biomarker for noninvasive diagnosis of CAD with IFTA, we measured the expression levels of miR-142-5p, miR-142-3p and miR-211 by Taqman MicroRNA Assays in PBMC samples of CAD with IFTA and normal allograft recipients. The expression levels of miR-142-3p (FC=2.94, $p<0.0001$) and miR-211 (FC=0.43, $p<0.0001$) were statistically significant in PBMC samples from CAD with IFTA compared with normal allograft recipients (Figure 3B, 3C and Table 2) As well as biopsy samples. However, the expression levels

of miR-142-5p did not meet statistical significance (FC=1.14, $p=0.171$) in PBMC samples from CAD with IFTA compared with normal allograft recipients (figure 3A and Table 2).

ROC curve analysis revealed that CAD with IFTA could be distinguished using expression levels of miR-142-3p (AUC=0.99, $CI_{95\%}=[0.97-1.00]$; $p<0.0001$) and miR-211 (AUC=1.00, $CI_{95\%}=[1.00-1.00]$; $p<0.0001$) in PBMC samples. However, despite biopsies, the expression levels of miR-142-5p in PBMC samples could not well distinguished CAD with IFTA patients from normal allografts. So, CAD with IFTA patients have been distinguished from normal allograft recipients using ROC curve analysis of miR-142-3p and miR-211 expression levels in PBMC samples (Figure 4 and Table 3).

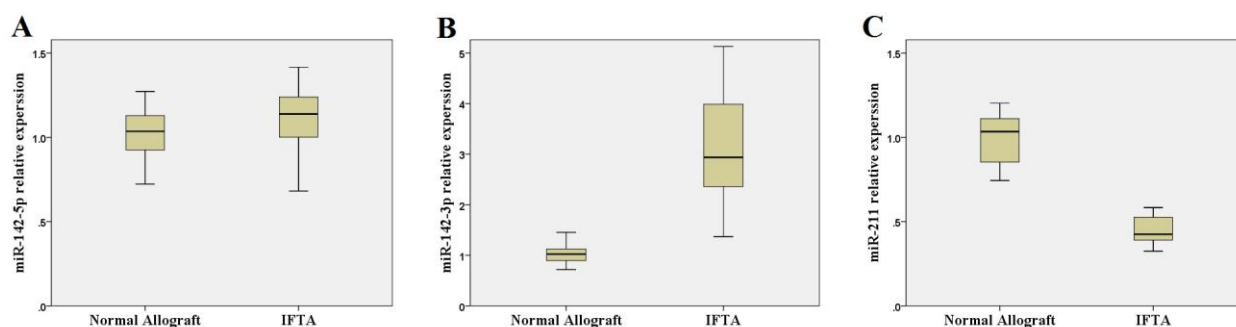


Figure 3. The expression levels of miR-142-5p, miR-142-3p and miR-211 in PBMC samples of CAD with IFTA patients compared with normal allograft recipients. Taqman MicroRNA Assays RT-qPCR results revealed that (A) miR-142-5p did not meet statistical significant ($p=0.171$). (B) miR-142-3p was significantly upregulated ($p<0.0001$) and (C) miR-211 ($p<0.0001$) was significantly downregulated in the PBMC samples of CAD with IFTA patients compared with normal allograft recipients.

Table 3. ROC curve analysis of miRNAs in biopsy and PBMC samples

Sample	miRNAs	Cutoff point	Sensitivity	1 - Specificity	AUC ^a (95% CI ^b)	P value
Biopsy	miR-142-5p	1.45	1.00	0	1.00 (1.00-1.00)	<0.0001*
	miR-142-3p	6.29	1.00	0	1.00 (1.00-1.00)	<0.0001*
	miR-211	0.44	1.00	0	1.00 (1.00-1.00)	<0.0001*
PBMC	miR-142-5p	1.17	0.50	0.11	0.64 (0.44-0.83)	0.171
	miR-142-3p	1.54	0.93	0	0.99 (0.97-1.00)	<0.0001*
	miR-211	0.66	1.00	0	1.00 (1.00-1.00)	<0.0001*

^a Area under the curve^b Confidence interval

* Significant values

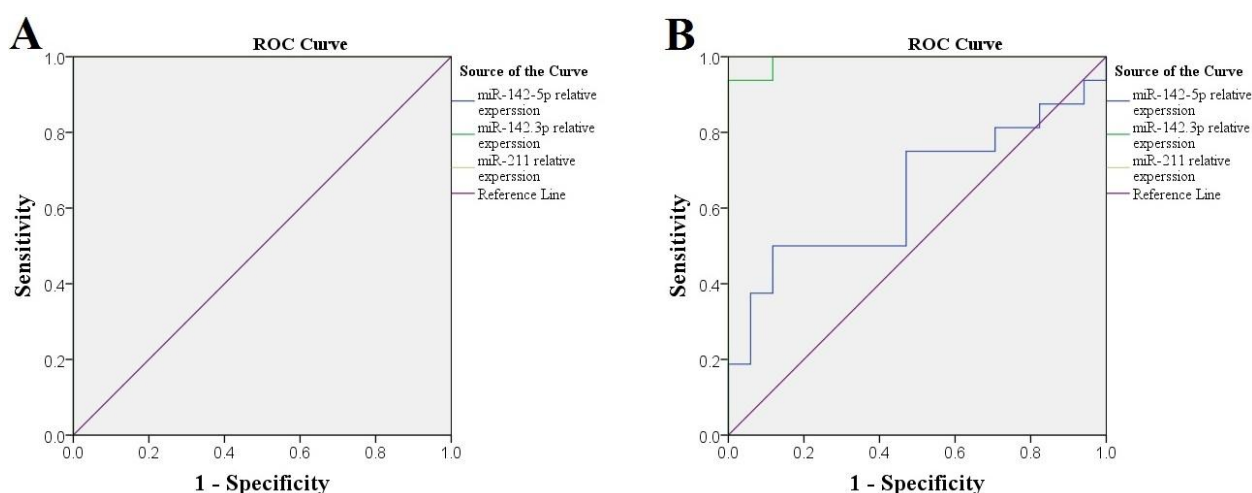


Figure 4. The ROC curve analysis for discriminative ability of 3 miRNA between IFTA patients and normal allografts. A) ROC curve for 3 miRNA to separate CAD with IFTA from normal allografts in biopsy samples. B) ROC curve for 3 miRNA to separate CAD with IFTA from normal allografts in PBMC samples.

DISCUSSION

Currently it is common to evaluate the graft function mainly based on histological evidence of the biopsy taken, when physiological parameters indicate probable renal dysfunction.²⁴ Recent progress in miRNA research improved our understanding about many diseases and their possible implications as diagnostic biomarkers. The expression of miRNAs have been associated with cancer and other diseases and the potential predictive value of these biomarkers have been reported.^{25,26}

The primary aim of this study was to determine whether expression of miR-142-5p, miR-142-3p and miR-211 in biopsy and PBMC samples is associated with the diagnosis of CAD with IFTA and predict renal allograft function. Our result showed that expression of miRNAs in Biopsy and PBMC samples is associated with CAD with IFTA. The second aim of this study

was to determine the predictive/diagnostic value of these miRNAs in renal transplant recipients with CAD with IFTA. We have found that miR-142-5p, miR-142-3p and miR-211 were statistically significant expressed in allograft tissues following CAD with IFTA compared with NA. ROC curve analysis was performed to determine the possibility of these miRNAs as predictive biomarkers. We found CAD with IFTA and renal allograft function could be predicted with a high level of precision using intragraft levels of miR-142-5p, miR-142-3p and miR-211. Moreover, miR-142-3p and miR-211 were differentially expressed in PBMC samples of CAD with IFTA patients and could be distinguished CAD with IFTA patients from NA recipients with high sensitivity and specificity. To our knowledge, for the first time we showed significant expression of miR-142-3p and miR-211 in PBMC samples of CAD with IFTA patients. Therefore, we propose that these miRNAs can be used as potential

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non-invasive diagnostic biomarker of CAD with IFTA in renal transplant recipients instead or along with the risky diagnostic procedures such as biopsy.

Acute rejection is the most important risk factor for graft fibrosis.²⁷ It has been identified that the occurrence of subclinical inflammation is associated with IFTA, even when it is below the Banff classification criteria for diagnosis of acute rejection.²⁸ Recently, miRNAs have been identified and validated in CAD with IFTA.¹⁷⁻¹⁹ However, the mechanism of alterations in miRNAs expression in IFTA remains unclear. Interestingly, Scian and his colleagues¹⁹ have reported similar directional expression changes of five miRNAs (let-7, miR-30c, miR-204, miR-223 and miR-142-3p) with Anglicheau et al. study which they found differential expression of miRNAs in acute rejection.¹⁵ Moreover, in our previous study we found that miR-142-5p and miR-142-3p were differentially expressed in allograft tissues from acute T-cell mediated rejection patients.⁵ Therefore, we suggest a similarity in the underlying biological processes of the two pathologies.

It has been shown that miR-142 is specific for hematopoietic cell lineage.²⁹ In addition, miR-142-3p regulates the production of cAMP and then implicated in the regulation of macrophages and regulatory T cells. It has also been shown that FOXP3 leading to transcriptional repression of miR-142-3p.³⁰ So, the increased expression levels of miR-142-3p in tissue samples of renal allografts support this idea that regulatory T cells lose their ability to suppress immunological processes occurring within the kidney.

Differential expression of miR-142-3p and miR-211 in allograft tissues and paired urines of CAD with IFTA previously reported by Scian and his colleagues which their study included only deceased donor.¹⁹ Also, Ben-Dov and colleagues have reported up-regulation of miR-142-5p and miR-142-3p in biopsy samples of CAD with IFTA which their study was included both living and deceased donor.¹⁷ Our study included both living and deceased donors which indicated similar directional expression changes in miR-142-5p, miR-142-3p and miR-211 with Scian et al. and Ben-Dov et al. studies.

Because of the stability of miRNAs in the body fluids, miRNAs currently explored for their potential as diagnostic biomarkers in renal transplantation. The ideal biomarker must be accessible using non-invasive methods, accurate and inexpensive to detect, highly

sensitive and specific to the disease of interest, and rapid changes upon the development of disease.³¹ Circulating miRNAs have characteristics of these criteria. In addition, miR-142-3p and miR-211 were differentially expressed in PBMC samples together with high sensitivity and specificity for CAD with IFTA, this they can be considered as ideal biomarkers for CAD with IFTA.

Increased levels of miR-142-5p, miR-142-3p and miR-211 have been found in allograft tissues from CAD with IFTA patients.^{17,19} Also, miR-142-5p and miR-142-3p previously have been reported statistically significant expressed in biopsy samples of acute rejection.¹⁵ Recently, high expression of miR-142-5p and miR-142-3p has been shown in PBMC samples of chronic antibody-mediated rejection and operationally tolerant patients respectively.^{20,21} Also, in our previous study miR-142-5p and miR-142-3p were upregulated in biopsy samples and miR-142-3p was increased in PBMC samples of acute T-cell mediated rejection patients.⁵ In this study, we have found miR-142-5p, miR-142-3p and miR-211 were differentially expressed in allograft tissues and miR-142-3p and miR-211 were statistically significant expressed in PBMC samples following CAD with IFTA which could be distinguished CAD with IFTA from NA with high sensitivity and specificity which could be considered as biomarkers. These findings support the hypothesis that miRNAs involved in CAD with IFTA, acute rejection, chronic antibody-mediated rejection, allograft tolerance are overlapping. Therefore, we suggest that the final panel of predictive/diagnostic biomarkers should be a set of markers instead of unique markers to discriminate CAD with IFTA from other renal allograft complications.

In summary, we have found that the expression levels of miR-142-5p, miR-142-3p and miR-211 in renal allograft tissues can accurately distinguish CAD with IFTA patients from NA recipients. Moreover, we suggest that measurement of miR-142-3p and miR-211 expression levels in PBMC samples of CAD with IFTA patients may serve as a non-invasive diagnostic biomarker for CAD with IFTA. Since miRNAs may have important functions in the pathogenesis of CAD with IFTA, we suggest that differentially expressed miRNAs in grafts and their targets are good candidates for further investigations to understand the CAD with IFTA mechanism and managements of renal allograft recipients.

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