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The urine microRNA profile may help monitor post-transplant renal graft function

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Abstract

Non-invasive, cost-effective biomarkers that allow accurate monitoring of graft function are needed in kidney transplantation. Since microRNAs (miRNAs) have emerged as promising disease biomarkers we sought to establish an miRNA signature in urinary cell pellets comparing kidney transplant patients diagnosed with chronic allograft dysfunction (CAD) with interstitial fibrosis and tubular atrophy and those recipients with normal graft function. Overall, we evaluated 191 samples from 125 deceased donor primary kidney transplant recipients in the discovery, initial validation and the longitudinal validation studies for non-invasive monitoring of graft function. Of 1,733 mature miRNAs studied using microarrays, 22 were found to be differentially expressed between groups. Ontology and pathway analyses showed inflammation as the principal biological function associated with these miRNAs. Twelve selected miRNAs were longitudinally evaluated

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article. The supporting information consists of:

1. **Supplemental Figure 1.** Merging of the mRNA-miRNA co-expression and protein-protein interaction networks from Figure 4.
2. Supplemental information about comparison of selected mRNA and miRNAs between urinary cell pellets and urinary exosomes and detailed methodological methods for miRNA microarray reactions and data analysis.

Supplementary information is available at Kidney International's website

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the journal Kidney International

in urine samples of an independent set of 66 patients, at two time-points post-kidney transplant. A subset of these miRNAs was found to be differentially expressed between groups early post-kidney transplant before histological allograft injury was evident. Thus, a panel of urine miRNAs was identified as potential biomarkers for monitoring graft function and anticipating progression to CAD in kidney transplant patients.

Keywords

urinary cell pellets; microRNA; chronic allograft dysfunction; biomarkers

INTRODUCTION

A major obstacle in the management of kidney transplant recipients is the lack of specific biomarkers for continuous monitoring of graft function post-kidney transplantation (KT). The current gold standard is the histological evaluation of biopsies. Additional markers such as serum creatinine (Cr), estimated glomerular filtration rate (eGFR) and/or proteinuria (1-9) are routinely used to monitor graft function. Unfortunately, these currently available methods are either ineffective, inaccurate or invasive (5-9) and suffer from limitations in predicting outcomes (10-16).

Non-invasive, cost-effective biomarkers that allow frequent and accurate monitoring of graft function are needed in KT (14-16). Recently, microRNAs (miRNAs) have emerged as promising biomarkers for a variety of different pathologies (17, 18). Often found in association with exosomes, miRNAs are now shown to be stably expressed in serum, plasma, urine, saliva, and other body fluids. Lower complexity than mRNAs, no post processing modification, tissue specific expression, and amplifiable signals make miRNAs in the urine ideal candidates as non-invasive biomarkers of kidney disease. As a biofluid, the urine allows repeated and non-invasive collection, and its molecular composition highly reflects intra-renal events (19-21). We and others have published the utility of assessing mRNAs levels in urinary pellet for the evaluation of acute cellular rejection (ACR) (22, 23), BK virus nephropathy (24) and chronic allograft dysfunction (CAD) with interstitial fibrosis and tubular atrophy (IF/TA) (25, 26). However, so far, there have been only been few studies reported evaluating global miRNA expression changes associated with ACR or CAD with IF/TA in kidney allografts (27-30). Thus, the use of miRNA profiles as non-invasive biomarkers for monitoring graft function might have potential for non-invasively monitoring graft function and deserves further exploration.

We recently reported a miRNA profile of allograft tissue using microarrays, where miR-142-3p, miR-204, and miR-211 were differentially expressed between patients with histological diagnosis CAD with IF/TA when compared to patients with normal histology and functioning allografts (normal allograft function: NAF), in both allograft tissue biopsies and paired urinary cell pellet samples (30). Similar IF/TA-like expression changes were also detected in urinary cell pellets of patients with stable graft function, but that later developed CAD. This preliminary report suggested that miRNAs could be used to non-invasively monitor graft function. Detection of individual miRNAs (first identified in tissue samples) in

urinary cell pellets using reverse transcriptase-quantitative PCR (RT-QPCR), also suggested the feasibility of generating a miRNA signatures from urinary cell pellet samples.

Based on our initial encouraging results, we now expand our CAD with IF/TA tissue miRNA signature by establishing a miRNA signature in urinary cell pellets using microarrays, and prospectively evaluating a combined panel of tissue/urine differentially expressed miRNAs. In order to validate the initially identified biomarkers and to establish a global miRNA signature in urine samples, we employed a well-designed methodological approach to integrate the transcriptional profiles of tissue biopsies and urinary cell pellet samples from patients with and without biopsy-proven CAD with IF/TA. A selected panel of 12 combined (tissue and urinary cell pellet) differentially expressed miRNA markers were tested in an independent cohort of kidney transplant recipients at two time points post-KT to assess their utility for the monitoring of graft function.

RESULTS

Use of Urinary Cell Pellets to Monitor Graft Function

As first step in our study, we aimed to evaluate the utility of urinary cells pellets *versus* urinary exosomes as targets for evaluating kidney allograft using mRNA/miRNA measurements. After evaluating the expression of mRNAs representing specific regions of the kidney, such as the nephron and the collecting duct in both urinary cell pellets and urinary exosomes, we observed expression of all the evaluated mRNA in both sample types. Even when the level of expression of the studied genes was lower in urinary exosomes, they were comparable between sample types (Supplemental information. *mRNA/miRNA detection in urine samples: exosomes versus sediments*). Similar findings were observed for the tested miRNAs. Thus, this preliminary data was used as a *proof of principle* to support our hypothesis that urinary cell pellets represent an appropriate source of mRNAs/miRNAs for evaluating kidney function, warranting cross-sectional and prospective miRNA studies in our patient cohorts. Moreover, technical issues associated with isolation of urinary exosomes (e.g., ultracentrifugation, RNA concentration) limit the utility of potential new biomarkers to be readily adaptable in the clinical setting.

Identification of MiRNA Signatures in Urinary Cell Pellet

The overall study design is shown in the Figure 1. Demographic and clinical patient data can be found in Table 1. Urinary cell pellets from patients with histological diagnosed CAD with IF/TA and patients with NAF were selected for the initial discovery phase. These patients included the same cohort of enrolled cases for the evaluation and establishment of the global miRNA signature in allograft tissue recently reported (30) and an additional set to increase the sample size. From this analysis, 22 miRNAs were identified as significantly differentially expressed (FDR = 15%, and ≥ 2-fold change) between CAD with IF/TA and NAF samples (Figure 2, and Table 2). Core analysis was performed to interpret the data set in the context of biological processes, pathways and molecular networks. The top scored network (score = 33) showed connective tissue disorders, inflammatory disease, and inflammatory response as the associated network functions. Moreover, inflammatory

response was identified as one of the top functions associated with these differentially expressed miRNAs (p=7.03E-18-1.59E-11).

Validation of MiRNA Array Results Using an Independent Case-Control Group

An set of 5 miRNAs were initially selected for independent validation using RT-QPCR, including two miRNAs differentially expressed in urinary cell pellets (miR-125b, miR-203) and 3 miRNAs that were previously identified in tissue samples and correlated with paired urine samples (miR-142-3p, miR-204, miR-211) (30). Additional criteria for selection of the panel included: array fold change, statistical significance and *in silico* mRNA target prediction. The initial validation was performed using an independent set of urinary cell pellets (IF/TA= 7 and NAF=10). Differential expression of all 5 miRNAs was confirmed between NAF and CAD with IF/TA patients (Figure 3). The ΔΔCt method was used to calculate the relative expression (fold change) between sample groups. This signature was then expanded (based on criteria described in *Methods*) and further validated in a larger (N=66), longitudinal independent study, to evaluate utility of the markers for monitoring graft function and progression to CAD.

MicroRNA-mRNAs Interaction Network

We performed an integrative analysis of mRNA and miRNA expression profiles and miRNA target predictions from three different algorithms (PITA, TargetScan, and miRanda) through MAGIA (31, 32). The evaluation of mRNA in urinary cells has been a common approach during the last years for evaluating native and allograft kidneys (22-26, 33-35). However, most of the urine samples have total RNA without the required concentration, and/or quality and integrity for microarray analysis. Since our previously published data showed that miRNAs identified in tissues could also be detected in urinary cell pellets (30), these finding supported an integrative analysis using the new miRNA expression data together with our previously published data (25, 36).

Using MAGIA (miRNA and genes integrated analysis) we identified a large network of correlated mRNA-miRNA pairs. Results were mapped using Cytoscape (37). A filtered network corresponding to the 5 miRNA selected for initial independent validation was extracted from the results (Figure 4A). To identify annotated protein interactions, genes identified within this network were queried using STRING (http://string-db.org/) and mapped using Cytoscape (Figure 4B). Eighty-three genes identified in the mRNA-miRNA network were found to have documented protein-protein interactions with at least one other gene from the network. A merging of the two networks can be found in Supplemental Figure 1.

Biological Processes and Molecular Functions

Toppgene (http://toppgene.cchmc.org) was used to identify biological processes overrepresented by the 83 genes identified above. Top biological processes included regulation of apoptosis (p=1.87E-07), cell activation (p=1.86E-04), immune system process (p=4.66E-04), protein phosphorylation (p=1.01E-03) and activation of JAK2 kinase activity (p=3.15E-03).

Distinctive MiRNA Profiles Early Post-KT in Urinary Samples from Patients with Good vs. Poor Graft Function at 24-months Post-transplantation

As a preliminary analysis and to justify the prospective evaluation of the selected markers in a larger cohort of samples, we tested differences in urinary cells miRNA profiles using microarrays at 3 months post-KT. Total RNA from urinary cells from a set of 20 patients (N = 10, stable good function at 24 months post-KT, N = 10, poor function at 24 months post-KT) were evaluated. From this analysis a total of 48 miRNAs were differentially expressed between groups (p <0.001, and ≥ 2-fold change) (Figure 5A) justifying further validation in the independent patient set with longitudinal samples using only selected markers. Moreover, from the analysis of differentially expressed miRNAs early post-KT and the 22 miRNAs identified as associated with urinary cells from patients with CAD with IF/TA, 5 miRNAs were identified as common between the signatures (Figure 5B). These common miRNAs corresponded to miR-200b, miR-375, miR-423-5p, miR-193b, and miR-345.

Prospective Evaluation of MiRNA Expression

We then tested the expression of the selected miRNAs in urinary cell pellets of kidney transplant recipients (N = 66) collected between 3 months and 24 months post-KT. The resulting miRNA panel, included a total of 12 markers (3 miRNAs differentially expressed between tissues (30) and 9 miRNAs from the urinary cell pellet signature (including 3 miRNAs statistically differentially expressed at both 3 months post-KT and CAD with IF/TA signatures)). MiRNAs were selected for validation as described in *Methods*. We aimed to evaluate the significance of the miRNAs in anticipating the histological damage in the kidney graft that associates to CAD with IF/TA. First, we classified patients depending on graft function at 24-months post-KT (≥40mL/min/1.73m2 or <40mL/min/1.73m2) (38) and presence or absence of histological findings associated with IF/TA in the last protocol biopsy (biopsy collection mean time 13.6±3.5 months post-KT) (defined as TA [ct ≥ 1] and IF [ci ≥ 1] involving more than 25% of the cortical area) (39) as ‘graft with good function (N=41) *versus* grafts with poor function (N=25)’. MiRNA expression was evaluated both in a cross-sectional and longitudinal manner.

The analysis of samples from both groups at the first time-point early post-KT (mean time collection: 3.73±1.30 months post-KT), showed miR-99a (p=0.029), miR-140-3p (p=0.041), miR-200b (p=0.04), miR-200* (p=0.047), and miR-142-3p (p=0.05) as being differentially expressed between the groups. From the longitudinally evaluation of the selected panel of miRNAs, miR-99a (p=0.05), miR-140-3p (p<0.001), miR-200b (p=0.033) and miR-200* (p=0.0018) were also significantly differentially expressed between groups at the second time-point evaluation (mean time collection = 20 ± 4 months post-KT) (Figure 6).

Comparison Analysis between Proteinuria and miR-200b in the Longitudinal Study

We evaluated the utility of an actual marker of graft function (proteinuria) *versus* expression of miR-200b, identified as statistically differentially expressed in the CAD with IF/TA signature, in the early global miRNA signature early post-KT and at the two-time longitudinal analysis between groups. Specifically, proteinuria levels (mg/dL) were evaluated in the same samples collected at two time-points post-KT and used in the longitudinal analysis. Pearson’s correlation was used to evaluate the correlation between

differentially expressed values of miR-200b and proteinuria. From this analysis, we observed that no correlation between proteinuria and miRNAs was observed at the first time-point analysis (r=0.28, p=0.067), while a trend in correlation was observed at the second time-point (r=0.43, p<0.001).

DISCUSSION

Major progress has been made in extending graft and patient survival after KT as a result of the development of improved immunosuppressive drugs and better patient management. Nonetheless, long-term graft outcomes are suboptimal and patients with failed transplants comprise an increasing re transplantation rate (www.unos.org). Development of individualized treatment strategies will require reliable, reproducible, cost-effective, yet non-invasive biomarkers capable of assessing the risk of graft injury.

Recent data suggest the implication of miRNAs in renal development and renal disease pathophysiology (40, 41), including fibrogenesis (42, 43), regulation of innate and adaptive immunity (44, 45), autoimmune diseases (46), and ACR of the renal allograft (27, 29). Two previous studies have identified miRNAs differentially expressed in graft rejection showing their potential as diagnostic biomarkers (23, 28). Our group recently published a miRNA signature in allograft tissue with histological diagnosis of CAD with IF/TA. Furthermore, we demonstrated the utility of selected miRNAs from the tissue signature as biomarkers in paired urine samples (30). A recent study, showed the utility of miRNA for detecting ACR in urine samples (29). However, the use of miRNAs for monitoring post-transplant kidney graft function has not been further yet explored.

An important part of this study includes the evaluation of the merit of urine samples from kidney transplant recipients as a way to non-invasively evaluate graft function using mRNA from different regions of the nephron and the collecting duct. Moreover, we evaluated differential expression of the studied mRNAs in urinary cell pellets *versus* urinary exosomes. Our results showed that urinary cell pellets contains mRNAs that are representative of the nephron and the collecting duct and provide similar information that the one provided by urinary exosomes, for the same mRNAs. However, we recognize that additional evaluations of different contribution of extracellular RNAs from cell-free urine, urinary cells pellets, and urinary exosomes as kidney biomarkers are still needed.

Furthermore, and to the best of our knowledge, herein we present the first miRNA signature generated in urine samples from kidney transplant recipients using microarrays. A total of 1,733 human mature miRNA were tested to establish an initial global signature in urine samples between the different groups (Figure 1). Even when the number of patients used for creating this signature is limited, the strict criteria used for selecting the groups (strict clinical / histological condition), provides a strong signature for biomarker validation. Moreover, urine sample collection from CAD with IF/TA patients is limited by the ability of these patients to produce urine, thus limiting the number of available samples. From this analysis, a total of 22 miRNAs were statistically differentially expressed between the groups (CAD *versus* NAF). Pathway and ontology analyses showed inflammation and fibrosis development patterns associated with the genes targeted for these miRNAs. As a second

step, selected miRNAs were validated using RT-QPCR in an independent set of urine samples.

From our *in silico* results, the miRNA correlation network generated shows numerous mRNA:miRNA pairs identified with several of the mRNAs being correlated to more than one miRNA. When the genes within this network were checked against databases for known protein-protein interactions, we found that several had documented experimental interactions and were functionally related (within the same pathway).

MiR-142-3p is primarily expressed in peripheral blood mononuclear cells (47, 48) and has been implicated in the functional regulation of regulatory T-cells and macrophages (49). MiR-125b has been shown to be expressed in arteriolar renal smooth muscle and juxtaglomerular cells under normal conditions and that its role is to maintain the smooth muscle phenotype of these cells (50). MiR-125b has been also associated with playing a role in inflammation. This miRNA is down-regulated in response to LPS enabling TNF-α translation and resulting in elevated levels of interleukin 6 (*IL6*), C-reactive protein (*CRP*), and monocyte chemo-attractant protein 1 (*MCP1*) (51, 52). Increased levels of miR-125b have also been reported to be necessary in B-cell development (53, 54) and macrophage antigen presentation (55). Studies of miR-203 overexpression, inhibition, and mutagenesis validated posttranscriptional regulation of TNFα and IL24 by miR-203 in cell lines and primary keratinocytes (56). These findings suggest that miR-203 serves to fine-tune cytokine signaling and may dampen skin immune responses by repressing key pro-inflammatory cytokines.

Underlying factorial causes for CAD may in fact be impossible to decipher, when the graft is sampled with established injury on board. As we have shown previously (25, 57), extensive homogeneity of genomic responses are seen at this time. As a consequence, the identified signature may in fact be simply reflecting the current established tissue damage, instead than what initiated such injury. Many of the pathways involved in chronic graft injury might be regulated very early in the course of the damage when the final effects of these alterations are still not evident by histology, suggesting an additional need for early sampling prior to the onset of chronic pathology to identify triggers and early molecular markers for CAD disease progression. Although the study of IF/TA samples is a good starting point, our research focuses on identifying the potential causes leading to IF/TA and establishing potential molecular markers to forecast such events (37).

Following this aim, we evaluated the urinary cell pellet miRNA signature urine samples collected early post-KT and classified depending on patient graft function and histological findings at 24 months after transplantation. From this analysis we obtained early urinary cell pellet signatures differentiating allograft that showed continuous good function from those with decreased or poor function from transplantation. These findings supported the use of miRNAs in urine samples as earlier markers of progression to graft function. Moreover, as we expected, there was overlapping between miRNAs identified as part of the signature that relates with IF/TA and early signatures that associates with progression to CAD.

These encouraging results supported a further prospectively evaluation of the role of 12 selected miRNAs in the development of CAD (defined as grafts with a continued decrease in eGFR and histological evidence of IF/TA). From this longitudinal analysis, we identified a set of miRNAs statistically significantly differentially expressed early post-KT and moreover, a set differentially expressed a both time-points. MiRNA-200b was associated with the initial CAD with IF/TA signature, differentially expressed in the initial independent set of urines from patients classified depending on graft function, and differentially expressed at both time points in the longitudinal evaluation. Recently, miRNA-200b was described as a suppressor of TGFβ1 induced epithelial-to-mesenchymal transition (EMT) via inhibition of ZEB1 and ZEB2 and the extracellular matrix protein fibronectin by directing targeting of its 3'UTR mRNA, independent of pathways directly involved in TGFβ1 signaling (58). It was demonstrated by Xiong *et al*. (59) that the miR-200 family was responsible for protecting tubular epithelial cells from mesenchymal transition by target suppression of zinc finger E-box-binding homeobox (ZEB) proteins ZEB1 and ZEB2, which are E-cadherin transcriptional repressors. The results suggest that down regulation of the miR-200 family initiates the dedifferentiation of renal tubules and progression of renal fibrosis, which might provide important targets for novel therapeutic strategies. In our study, miR-200b and miR-200b*, were down-regulated in samples with CAD with IF/TA and moreover, in samples collected early after transplant from patients whose kidneys showed poor or no function at 24 months post-transplantation.

The analysis of miRNA expression in urine samples and proteinuria showed no correlation early post-KT, indicating the utility of these markers in early monitoring graft function. This finding supports the use of urinary cells miRNA profiles for the identification of patients at risk for accelerated loss of kidney function at early stages, when intervention is more likely to ameliorate outcomes.

We have identified a number of differentially expressed miRNAs in urinary cell pellets of histologically diagnosed CAD with IF/TA patients. RT-QPCR data generated from urine samples show that IF/TA samples segregate apart from NAF samples based solely on the expression levels of the five miRNAs tested (Figure 2). Moreover, when expanding the signature, 4 miRNAs showed to be potential markers of early kidney allograft injury. The longitudinal nature of the study, the sample size and the select group of patients included in the analyses (e.g., all deceased donor primary KT recipients with same immunosuppressant protocol) reinforces the significance of our findings. Further patient follow-up and sample testing is needed to confirm this observation.

METHODS

Enrolled Cohort

A total of 191 samples from 125 kidney transplant recipients of unique deceased donors were included in the study (Figure 1). Patients were enrolled between May 2004 and November 2010. The study was conducted at University of Virginia and Virginia Commonwealth University after Institutional Review Board approval was obtained at both institutions (UVA 14849, VCU#HM11454). Written informed consent was obtained from all patients. No living donors, HIV positive, and/or re-transplant patients were included.

Patients between the ages of 21 and 70 were enrolled in the study. Immunosuppressant protocol consisted of tacrolimus-based therapy, plus mycophenolate mofetil and prednisone. Estimated Glomerular Filtration Rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula (60). Protocol kidney biopsies were performed in all the enrolled patients at 3 and 9-12 months post-transplantation. Blinded centralized histological evaluation was performed by two pathologists using Banff 07 classification (39). Patients that during the follow-up presented causes for late decline in eGFR, (e.g., BK viral nephropathy, original disease recurrence) were not included in the study to avoid potential confounders.

Urine Samples

To generate the initial miRNA signature, urine samples were selected from patients with biopsy proven CAD with IF/TA, (N=10) and from patients with NAF (N=12) (training set). NAF patients were defined as kidney transplant recipients with at least 9 months post-transplant and presenting sustained eGFRs >60mL/min/1.73m^2 since the transplantation date and normal histology to the time of sample collection, no reported delayed graft function (DGF), diagnosis of calcineurin inhibitor nephrotoxicity (CNIT) or ACR episodes. DGF was defined as the need for dialysis within the first 7 days post-KT. A second independent set of urinary cell pellets from CAD with IF/TA (N=7) or NAF (N=10) patients was used for validation of the array results (validation set). Finally, a third set (N=132) was used for evaluating a selected panel of miRNA expression with respect to graft function and histological findings at 24 month post-KT (prospective testing group).

Total RNA Isolation

Urinary cell pellets were collected by centrifugation of the collected urine samples at 3,000 xg for 20 minutes at room temperature. A detailed description of methods is included in the Supplemental Information.

MicroRNA Signature Generation and Data analysis

Total RNA (250 ng) from each specimen was labeled using the FlashTag™ Biotin HSR RNA labeling kit (Genisphere Inc., Hatfield, PA). A detailed description of methods is included in the Supplemental Information.

Identification of Differentially Expressed MiRNAs in Urine Samples Early Post-KT between Patient Groups Using Microarrays

To evaluate the hypothesis that differentially expressed miRNA signatures identified early post-KT can be used for identifying early biomarkers of progression to CAD with IF/TA, urine samples collected at 3 months post-KT from 20 kidney transplant recipients (classified as with good kidney function *vs*. poor graft function following at 24 months post-KT as previously described) were also tested using GeneChip® miRNA v3.0 Array, and scanned on a GeneChip® Scanner 3000 7G.

Selection of MiRNAs for Prospective Validation

From the total of the miRNA that were identified as differentially expressed based on our cut-off criteria, 9 miRNA (41% of the list) were validated in the prospective group of patients. In addition, 3 miRNAs identified as differentially expressed in the recently tissue miRNA signature were also included (30). The total panel of miRNAs used for validation in the prospective set included: miR-125b, miR-203, miR-142-3p, miR-204 (previously described in *Validation of Microarray Results*), and miR-211 (assay ID: 000514), miR-99a (assay ID: 002141), miR106b* (assay ID: 2380), miR-140-3p (assay ID: 002234), miR-185 (assay ID: 000598), miR-200b (assay ID; 002251), miR-200b* (assay ID: 002274) and miR-486-5p (assay ID: 001278). The selection of miRNA for validation was based on statistical significance, folds changes, and described biological function. We then tested the expression of the selected miRNAs in urinary cell pellets of kidney transplant recipients (N = 66) collected between 3 months and 24 months post-KT.

mRNA:miRNA Interaction Network Analysis

A meta-analysis approach was adopted to compare mRNA and miRNA expression signatures using the web based tool MAGIA (MiRNA and Gene Integrative Analysis) (31, 32). Using this tool, an empirical Bayes test (61), as implemented in the *limma* package in the R environment (62), is applied separately to the miRNA and mRNA expression data to identify molecules whose expression changes closely correlate with one another. Simultaneously, for predicted mRNA:miRNA interactions identified based on three prediction algorithms (PITA, TargetScan, and miRanda) the inverse Chi-squared approach is used to combine miRNA and mRNA p-values (31, 32). P-values from this test of over-expressed miRNAs are then combined with those of under-expressed mRNAs and *vice versa*. Network interactions identified by MAGIA were graphed using Cytoscape (37). Biological Processes and molecular functions were identified through the use of ToppGene (http://toppgene.cchmc.org/) and Ingenuity Pathways Analysis (www.ingenuity.com). Functional categories with enrichment (negative binomial) test a p-value <0.05 were considered significant. Biological processes with identical gene lists were considered redundant and manually removed prior to the interpretation of results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACR	acute cellular rejection
CAD	chronic allograft dysfunction
DGF	delayed graft function
eGFR	estimated glomerular filtration rate
IF	interstitial fibrosis

MAGIA	MiRNA and Gene Integrative Analysis
NAF	normal allograft function
RT-QPCR	real-time quantitative-polymerase chain reaction
RMA	Robust Multiarray Analysis
KT	kidney transplant
TA	tubular atrophy

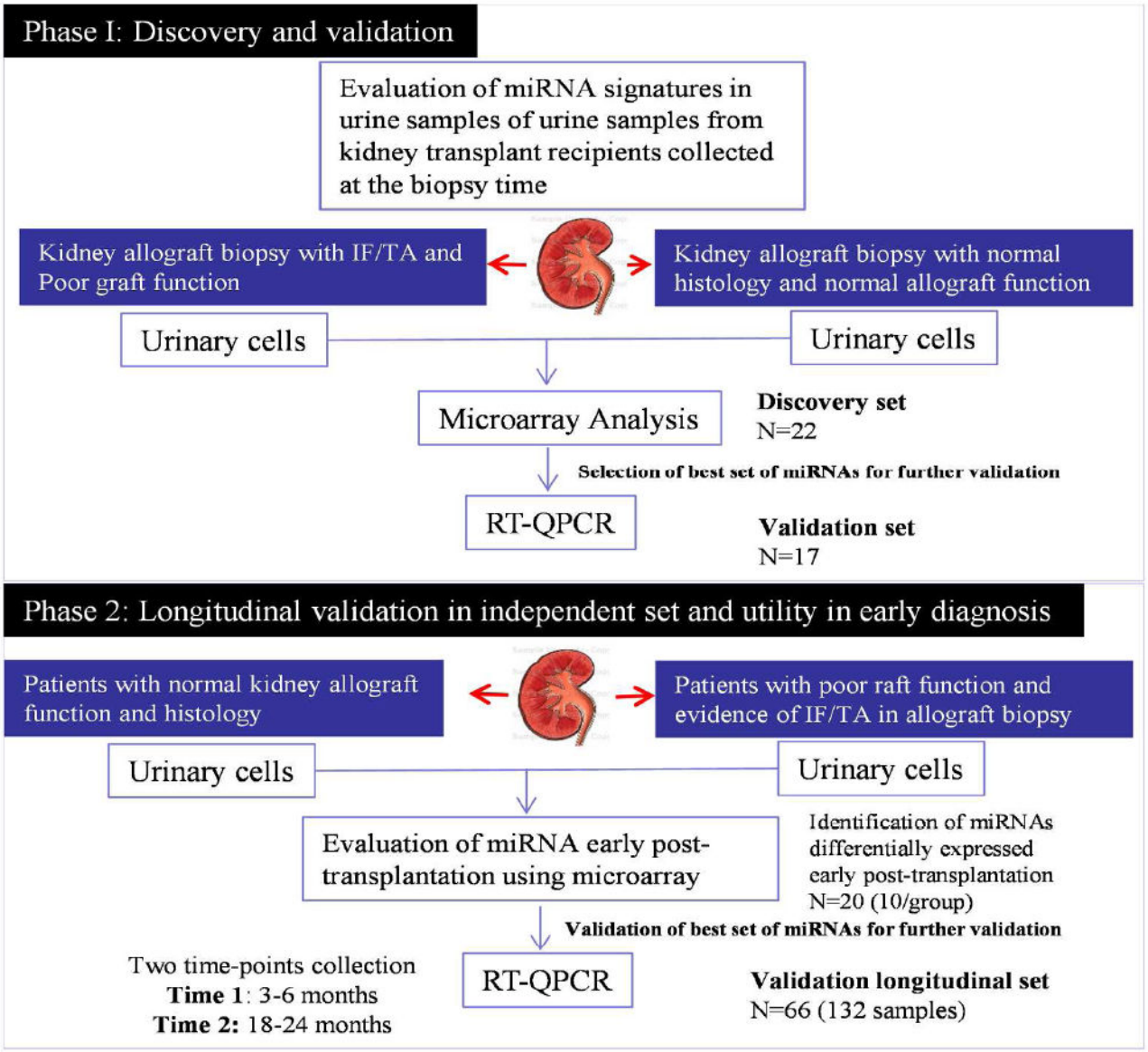

Figure 1. Flow-chart showing the study design

A design using training, validation and longitudinally analyses was used for discovery and validation of miRNAs with potential to detect early allograft injury post KT.

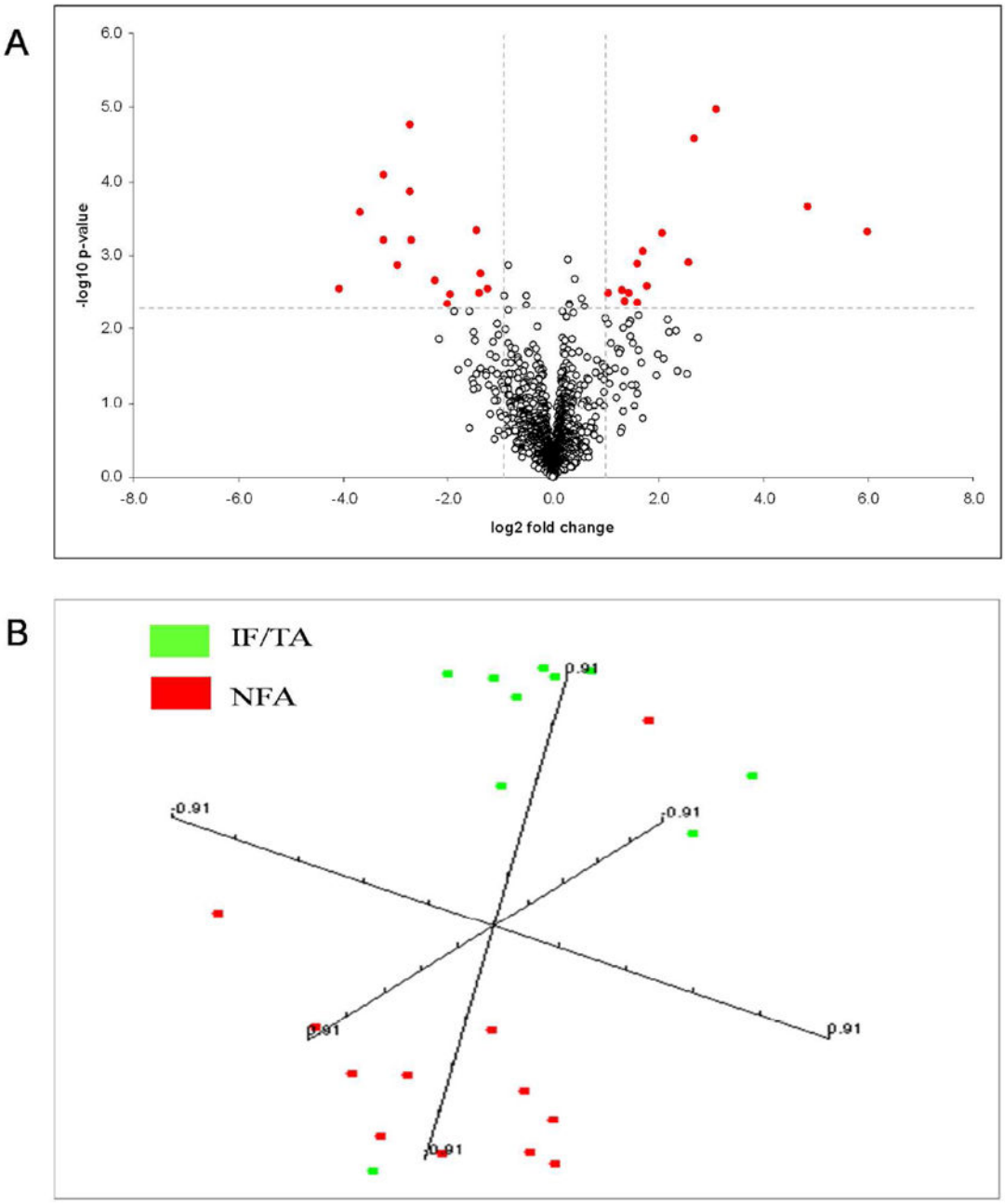

Figure 2.

(A) Volcano plot of miRNA microarray data for NAF and IF/TA samples. The y-axis values show the negative logarithm base 10 of the p-value. The dotted horizontal line on the plot represents the α-level used for this analysis (0.005). The x-axis is shown as the log2-difference in estimated relative expression values. Vertical dotted lines represent the threshold for the $\log_2$-fold change (equivalent to a 2 fold change). Thus, the red dots correspond to miRNAs that show a significant ($p \leq 0.005$) 2-fold or greater change in expression between NAF and IF/TA samples. **B)** Principal component analysis of the

miRNA results using microarrays showing separation of CAD with IF/TA samples from NAF samples using the expression values of the differentially expressed miRNAs.

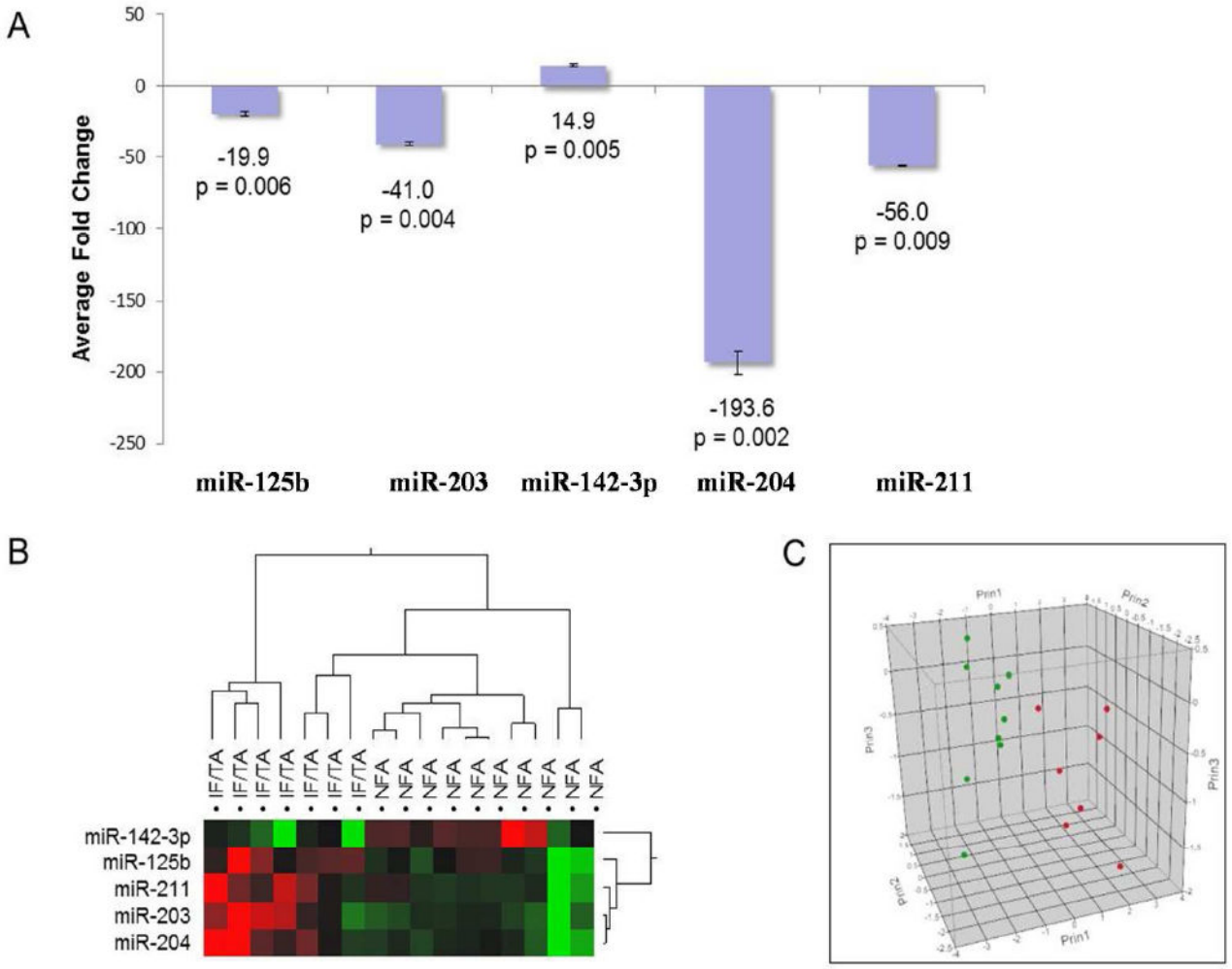

Figure 3.

(A) RT-QPCR validation of the selected miRNAs. Calculated fold changes (CAD with IF/TA *vs*. NAF) and *p*-values are indicated next to each bar. **(B)** Hierarchical clustering using Ward's method of the RT-QPCR data obtained during validation of the array results showing separation of CAD with IF/TA and NAF samples. Higher ΔCt values are colored red; lower values are green. **(C)** Principal component analysis of the RT-QPCR data showing separation of CAD with IF/TA samples from NAF samples using the expression values of the selected miRNAs.

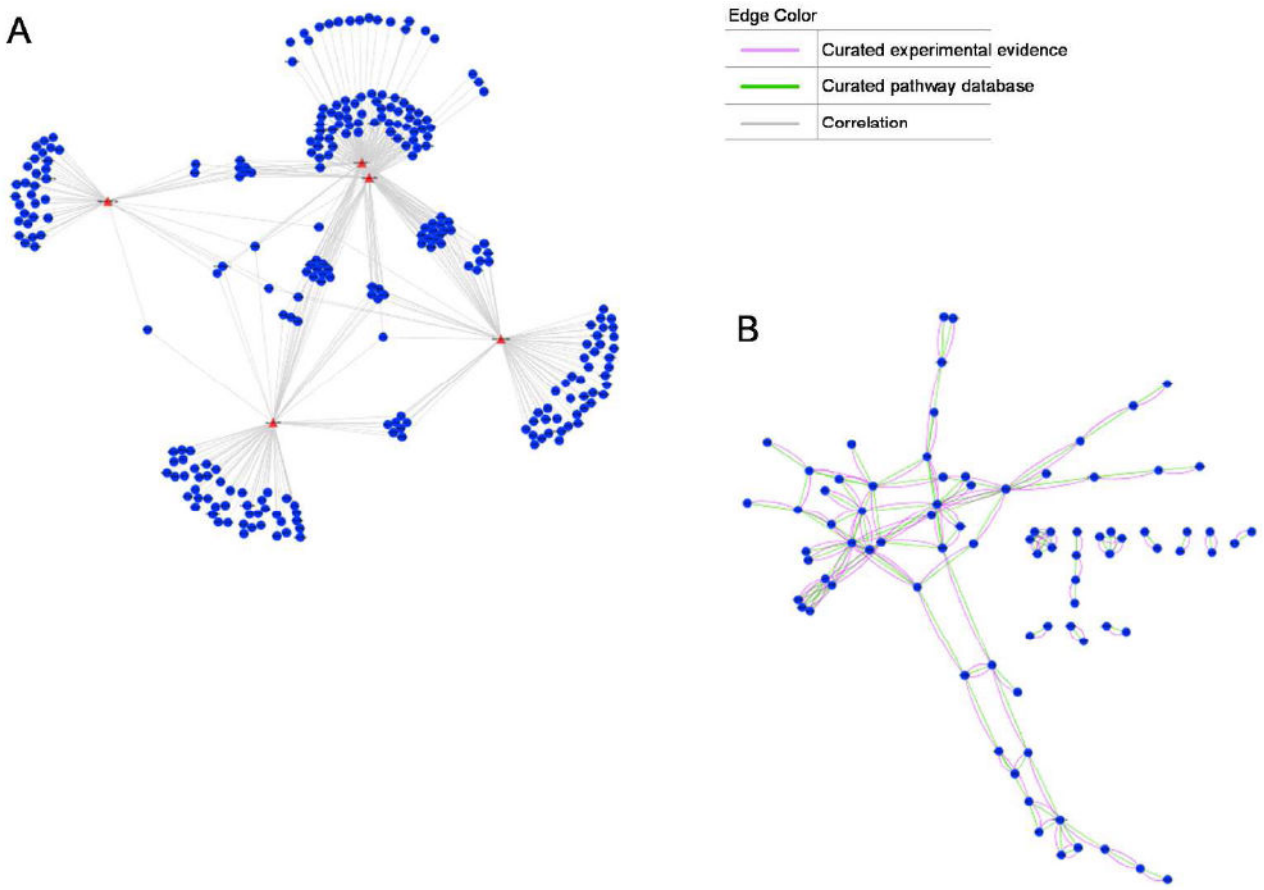

Figure 4.

(A) Filtered network corresponding to the 5 miRNA selected for RT-QPCR validation extracted from the overall MAGIA correlation results. Individual miRNAs might regulate the expression of multiple mRNA targets. In the present study we used an initial set of 5 miRNAs identified from tissue and urinary cell pellet signatures from patients with CAD with IF/TA and performed integrative analyses with our already published gene expression data for the same samples (25, 37, 57), to evaluate the utility of miRNA:miRNA data integration and network identification. These analyses facilitate the identification of pathways that associates with specific miRNAs and have the potential for identifying therapeutic targets. Blue circles represent mRNAs and red triangles represent the 5 miRNAs. The figure insert describes the type of interaction. **(B)** Protein-protein interaction network identified from genes in **(A)**.

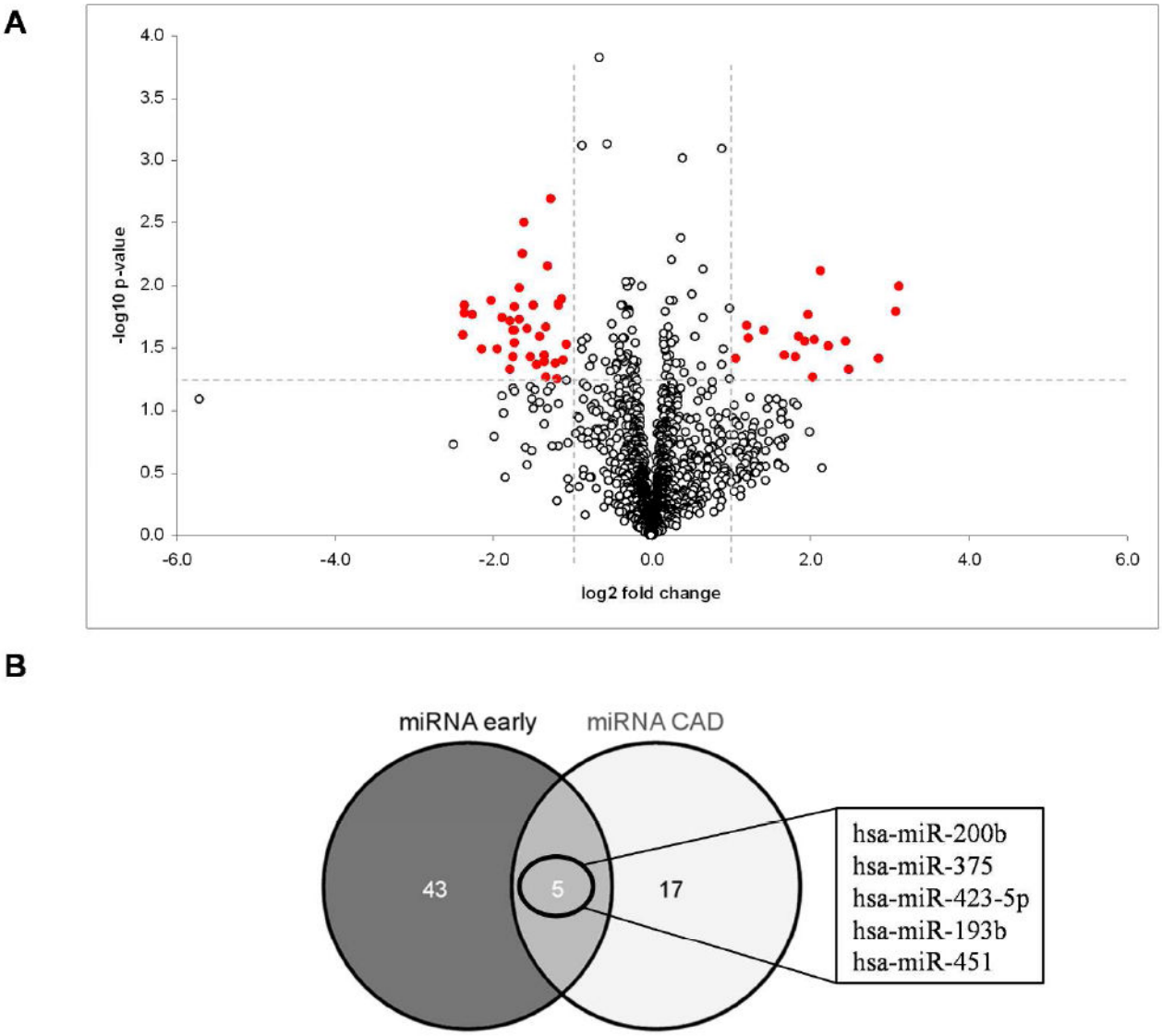

Figure 5.

(A) Volcano plot of miRNA microarray data for urine samples at early post-transplantation. The y-axis values show the negative logarithm base 10 of the p-value. The dotted horizontal line on the plot represents the α-level used for this analysis (0.05). The x-axis is shown as the $\log_2$-difference in estimated relative expression values. Vertical dotted lines represent the threshold for the $\log_2$-fold change (equivalent to a 2 fold change). Thus, the red dots correspond to miRNAs that show a significant ($p \leq 0.05$) 2-fold or greater change in expression between urine samples at 3 months post-KT in patients with stable *versus* poor graft function at 24 months post-KT. **(B)** Venn diagram showing overlapping between miRNAs differentially expressed in the CAD signature versus those differentially expressed early post-KT between urine samples from kidney transplant recipients with good vs. poor function at 24 months post-KT.

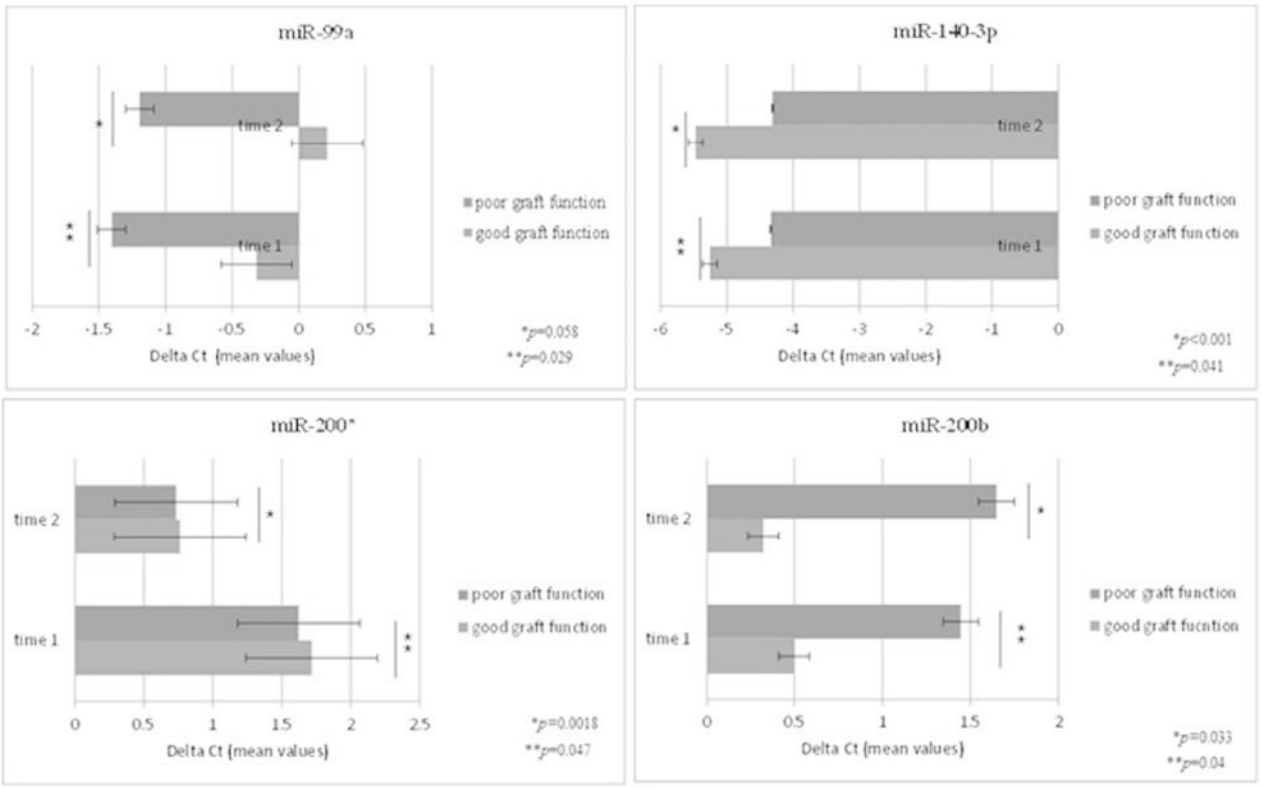

Figure 6.

Bar graphs showing the mean ΔCt ± SD values for miR-99a, miR-140-3p, miR-200*, and miR-200b measured in urinary cell pellets at early post-KT (time 1) and after 18 months post-KT (time 2). Patients were classified according their graft function as patients with good or poor graft function at 24 months post-KT. *P*-values between poor *vs*. good graft function for each miRNA are indicated by asterisks at Time 1 (**) and Time 2 (*).

Table 1

Patient demographics and clinical characteristics by study group

	Training set*		Validation set*		Testing Group**
	IF/TA	**NFA**	**IF/TA**	**NFA**	
Recipient Demographics	avg ± std	avg ± std	avg ± std	avg ± std	avg ± std
Age	42.3 ± 17.6	43.4 ± 14.1	46.1 ± 16.3	41.0 ± 11.2	52.6 ± 12.6
Race (AA/Ca/O)	7/2/1	10/1/1	5/1/1	8/0/2	59/6/1
Gender (M/F)	5/5	6/6	4/3	6/4	34/32
Donor Demographics					
Age	44.0 ± 21.7	41.2 ± 17.2	54.0 ± 12.0	48.7 ± 17.5	40.1 ± 16.8
Race (AA/Ca/O)	5/5/0	5/7/0	4/3/0	2/7/1	21/44/1
Gender (M/F)	5/5	5/7	2/5	3/7	41/25
Transplant					
Donor type (SCD/ECD/DCD)	5/4/1	10/2/0	3/3/1	9/0/1	38/10/18
CIT (min)	875 ± 375	1047 ± 348	884 ± 377	1045 ± 420	1205 ± 387
WIT (min)	29.8 ± 6.6	32.8 ± 10.0	29.7 ± 5.9	33.6 ± 9.8	30.0 ± 6.5
PPP time (min)	640 ± 556	715 ± 402	845 ± 279	900 ± 356	828 ± 359
Last Donor Cr. (mg/dL)	1.09 ± 0.7	0.96 ± 0.4	1.12 ± 0.5	0.97 ± .5	1.1 ± 0.8
DGF	1	0	1	0	23
Acute Rejection	2	0	1	0	5
HLA-A mismatch	0.8 ± 0.9	1.4 ± 0.8	1.1 ± 0.9	1.2 ± 0.9	1.4 ± 0.7
HLA-B mismatch	1.3 ± 1.0	1.8 ± 0.4	1.7 ± 0.8	1.7 ± 0.5	1.7 ± 0.5
HLA-DR mismatch	1.1 ± 0.9	1.3 ± 0.7	1.3 ± 1.0	0.9 ± 0.7	1.2 ± 0.7
HLA Total mismatch	3.3 ± 2.6	4.5 ± 1.1	4.1 ± 2.0	3.8 ± 1.0	4.4 ± 1.2
PRA at Tx (T-cell)	35.0 ± 37.9	64.8 ± 38.3	31.5 ± 35.8	62.1 ± 35.4	45.6 ± 38.3
PRA at Tx (B-cell)	4.7 ± 10.9	23.2 ± 29.2	4.28 ± 10.0	10.4 ± 12.5	17.5 ± 27.8
eGFR at 1mo	57.1 ± 39.1	68.8 ± 20.9	53.4 ± 31.8	59.8 ± 25.6	52.9 ± 22.3
eGFR at 3mo	53.5 ± 37.8	73.7 ± 13.4	43.6 ± 25.1	59.4 ± 27.4	56.7 ± 19.2
eGFR at 6mo	55.4 ± 50.1	75.5 ± 10.19	54.1 ± 44.7	60.8 ± 28.6	57.3 ± 20.9
eGFR at 9mo	45.4 ± 34.9	76.9 ± 15.7	37.3 ± 16.0	63.3 ± 29.0	57.6 ± 21.4
eGFR at 12mo	41.2 ± 21.7	75.6 ± 12.8	28.5 ± 12.7	69.3 ± 16.6	56.6 ± 20.8

	Training set*		Validation set*		Testing Group**
	IF/TA	NFA	IF/TA	NFA	
eGFR at 18 mo	55.2 ± 47.7	77.9 ± 13.3	18.5 ± 16.2	71.0 ± 21.7	56.4 ± 22.7
eGFR at 24 mo (or last known)	17.0 ± 13.8	79.5 ± 31.1	17.9 ± 14.4	71.6 ± 25.0	55.1 ± 25.9

* Cross-sectional evaluation,

** Prospective evaluation (longitudinal study).

CMV: cytomegalovirus; SCD/ECD/DCD: standard criteria donor/extended criteria donor/donation after cardiac death; CIT: cold ischemia time; WIT: warm ischemia time; PPP: pump perfusion preservation; Cr: creatinine; DGF: delayed graft function; PRA: panel reactive antibody; Tx: transplant; eGFR: estimated glomerular filtration rate.

Table 2

List of significantly altered (p<0.01, FDR<15%) miRNAs identified as differentially expressed in urinary cell pellets between subjects with diagnosed CAD with IF/TA and NAF.

microRNA	IFTA Mean (log2)	NAF Mean (log2)	Fold Change	p-value	q-value
hsa-miR-140-3p	9.08	5.62	11.0	4.63E-05	1.83E-02
hsa-miR-106b*	6.48	2.85	12.4	7.13E-05	1.83E-02
hsa-miR-125b	2.86	6.86	-15.9	7.97E-05	1.83E-02
hsa-miR-200b	1.94	5.03	-8.5	1.24E-04	2.14E-02
hsa-miR-200b*	1.77	4.41	-6.3	2.52E-04	2.93E-02
hsa-miR-486-5p	11.59	4.72	116.9	2.56E-04	2.93E-02
hsa-miR-99a	3.03	6.34	-9.9	4.31E-04	4.23E-02
hsa-miR-185	10.13	6.68	10.9	5.05E-04	4.33E-02
hsa-miR-425	8.52	6.48	4.1	1.04E-03	7.51E-02
hsa-miR-92a	10.65	8.68	3.9	1.09E-03	7.51E-02
hsa-miR-513a-5p	0.76	1.12	-1.3	1.61E-03	9.27E-02
hsa-miR-423-5p	6.59	4.28	4.9	1.62E-03	9.27E-02
hsa-miR-23b	7.22	9.17	-3.9	1.75E-03	9.27E-02
hsa-miR-30a*	1.03	3.23	-4.6	2.56E-03	1.11E-01
hsa-miR-193b	3.21	5.89	-6.4	2.66E-03	1.11E-01
hsa-miR-184	1.12	3.28	-4.5	2.84E-03	1.11E-01
hsa-miR-575	1.29	3.09	-3.5	2.93E-03	1.11E-01
hsa-miR-375	1.58	4.51	-7.6	3.11E-03	1.11E-01
hsa-miR-451	8.38	3.54	28.7	3.20E-03	1.11E-01
hsa-miR-203	4.25	9.49	-37.7	3.22E-03	1.11E-01
hsa-let-7f-2*	1.07	0.80	1.2	4.12E-03	1.35E-01
hsa-miR-345	4.60	2.50	4.3	4.64E-03	1.45E-01

*miRNAs in bold were further validated in the longitudinal study.