

Preliminary Communication

Circulating microRNA expression is reduced in chronic kidney disease

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Abstract

Background. MicroRNAs (miRNAs) are important regulators of gene expression, which have roles in renal development and disease. They exist in biological fluids including blood and urine and may have signalling roles and potential as disease biomarkers.

Methods. We measured the levels of miRNAs in patients with different stages of chronic kidney failure including those receiving maintenance haemodialysis treatment.

Results. In patients with severe chronic renal failure, circulating levels of total and specific miRNAs are reduced in comparison to patients with mild renal impairment or normal renal function. A strong correlation exists between detected circulating miRNAs and estimated glomerular filtration rate, and less strong correlations with other features of chronic kidney disease, such as anaemia and hyperparathyroidism.

Conclusion. These findings have important implications for the use of circulating miRNAs as biomarkers in individuals with renal impairment and for the pathogenesis of uraemia.

Keywords: biomarker; chronic kidney disease; exosomes; microRNA; renal failure

Introduction

MicroRNAs (miRNAs) are non-coding RNA oligonucleotides ~22 nucleotides in length that function as important negative regulators of gene expression. They act by decreasing the stability and/or translation of specific messenger RNA molecules [1]. Currently, >1000 miRNAs have been identified in humans (miRbase release 13.0), though more recent studies have suggested lower numbers of functional mammalian miRNAs [2]. The expression of many miRNAs is tissue specific and their dysregulation has been associated with various diseases including many cancers [3, 4], heart disease [5] and kidney diseases (for reviews, see [6] and [7]). In particular, altered miRNA expression has been found in polycystic kidney disease [8, 9], renal cell carcinoma [10–13] and allograft rejection [14].

miRNAs appear to have roles in many physiological, developmental and pathological processes. Evidence for the particular importance of miRNAs in the kidney has been obtained using mouse models with a podocyte-specific loss of Dicer. Dicer is a key enzyme involved in the production of mature miRNAs and its ablation in podocytes led to proteinuria, foot process effacement and glomerular basement membrane abnormalities [15–17]. Furthermore, in a mouse model with targeted Dicer deletion in renal proximal tubules, while the mice had normal renal function and histology, they were resistant to renal ischaemia–reperfusion injury, showing significantly better renal function, less tissue damage and improved survival compared with their wild-type counterparts [18].

In recent years, it has been found that miRNAs previously identified in specific tissues can also be detected in many extracellular fluids including plasma and serum [19, 20], saliva [21], urine [22], amniotic fluid, breast milk, seminal fluid and tears [23]. Many of the miRNAs found in these fluids have been found to be potential biomarkers for a range of diseases. In particular, some miRNAs identified in plasma and serum have been identified as diagnostic biomarkers of particular cancers [19, 20, 24–26] and circulating miR-208a has been identified as a potential biomarker for the early detection of myocardial infarction [27]. To date, no circulating miRNA has been identified as a biomarker of particular kidney diseases. To our knowledge, there is no published literature regarding the relationship between the level of circulating miRNAs and renal function.

The potential use of miRNAs as circulating biomarkers and a potential signalling role has been highlighted by the observations of their relative stability in blood. This stability of RNA molecules in plasma and serum is believed to be due, in part, to their containment in cell-derived microvesicles known as exosomes and protection from circulating ribonucleases. Exosomes are released from most cell types under various physiological and pathological conditions. They are then taken up by surrounding cells and play a key role in cell-to-cell communication [28, 29]. Many bodily fluids have been shown to contain exosomes including plasma [30], urine [31] and saliva [32]. Tumour-derived

exosomes found in plasma are increasingly being identified as diagnostic biomarkers for cancers [33–36]. Proteins and nucleic acids contained in urinary exosomes have also been reported to be useful as biomarkers of renal disease [37–40].

Animal studies have found that systemically delivered antisense and small interfering RNA oligonucleotides are targeted to the kidney and liver and undergo clearance by the renal, hepatic and gastrointestinal systems [41–43]. Furthermore, an increase in circulating microvesicles has been observed in patients with vascular dysfunction [44] and chronic renal failure [45]. Given these potential influences on the levels of circulating miRNAs in kidney disease, the interest in the physiological functions of circulating miRNAs and their potential utility as biomarkers we wished to examine the effects of kidney function on the levels of circulating miRNAs. We found a surprising and striking reduction in the levels of circulating miRNAs in patients with severe chronic kidney disease (CKD).

Materials and methods

Patient samples

Seventy-five patients with normal kidney function, mild to severe CKD and end-stage renal disease (ESRD) receiving haemodialysis treatment at our institution between 2009 and 2010 were studied. Samples were obtained at the Flinders Medical Centre and the Repatriation General Hospital. Ethical approval for this study was obtained from the Southern Adelaide Health and Flinders University Clinical Human Research Ethics Committee. The clinical details of the patients are summarized in Table 1. All patients gave informed written consent. Blood samples were obtained by venepuncture or directly from dialysis needles prior to haemodialysis treatment into ethylenediaminetetraacetic acid-containing tubes and then centrifuged at 3000 g for 15 min at room temperature. For the patients receiving dialysis treatments, samples were collected immediately pre-dialysis from fistula needles prior to machine connection or any heparin exposure. The patients will therefore have had heparin exposure at their previous haemodialysis session 48–72 h prior to sample collection. There is potential for interference of polymerase chain reaction (PCR)-based assays by heparin anticoagulation and indeed, we have observed post-dialysis interference with such assays that could be abrogated by heparanase treatment. Consistent with the absence of heparin, this treatment did not affect the determination of miRNA abundance in the pre-dialysis samples. Plasma was removed and stored at -80°C . Estimated glomerular filtration rate (eGFR) was calculated from the serum creatinine using the Modification of Diet in Renal Disease formula [46], the pre-dialysis creatinine being used for patients receiving regular haemodialysis. Other routine laboratory measurements including haemoglobin, calcium, phosphate, albumin and parathyroid hormone (PTH) were determined.

RNA extraction and quantification

RNA was extracted from 1.5 mL of plasma using Trizol LS (Invitrogen, Newcastle, Australia) according to the manufacturer's instructions with the addition of 1 μL glycogen (Roche, Branchburg, NJ) prior to precipitation overnight at -20°C with isopropanol. The RNA pellet was resuspended in 30 μL RNase-free H_2O .

RNA was extracted from 7 mL of urine according to [22]. In brief, 7 mL of urine was stored at -80°C with 6 M guanidinium thiocyanate, 0.025 M sodium acetate, 0.25% *N*-lauroylsarcosine and 0.5 M HEPES pH 7 in a final volume of 10 mL and stored at -80°C until RNA extraction. RNA was extracted with equal volumes of phenol and chloroform. The top aqueous layer was then mixed with a 1.5 volume of 100% (vol/vol) ethanol and loaded onto the miRNeasy kit spin columns (Qiagen, Hilden, Germany). Further preparation of the RNA sample was conducted according to the manufacturer's instructions. RNA was eluted from the column in a volume of 30 μL RNase-free H_2O and stored at -80°C .

The Agilent 2100 Bioanalyzer Small RNA kit was used according to the manufacturer's instructions to quantify RNAs in the 6–150 nucleotide size range.

Quantitative real-time PCR

miRNA expression was assessed by relative quantitative real-time PCR (qPCR) using TaqMan miRNA assays (Applied Biosystems, Foster City, CA). Complementary DNA was synthesized from total RNA extracted from the equivalent of 200 μL of plasma using TaqMan miRNA-specific primers and the TaqMan miRNA reverse transcription kit (Applied Biosystems). qPCR was performed using the Corbett Rotor-gene 2000 (Corbett Research, Sydney, Australia). Each PCR was performed in triplicate and contained 1 μL of reverse transcription product, $1\times$ Taqman Universal PCR mastermix No AmpErase UNG and 0.5 μL of primer and hydrolysis probe mix of the TaqMan miRNA assay (assay IDs: hsa-miR-16: 000319, hsa-miR-21: 000397, hsa-miR-155: 000479, hsa-miR-210: 000512, hsa-miR-638: 001582; Applied Biosystems). The 10 μL reactions were incubated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Data were generated and analysed using Corbett Rotor-gene software version 5.0.61 (Corbett Research). The raw cycle threshold (Ct) value was used to assess the amount of an individual miRNA extracted from the equivalent of 200 μL of patient plasma. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample) and are plotted as $40-\text{Ct}$ values such that increased y-axis values reflect increased nucleic acid concentration.

Exosome analysis

Plasma was collected from patients with normal renal function and ESRD patients. An initial volume of $2\times 240\text{-}\mu\text{L}$ was removed from each sample to establish endogenous circulating exosome levels for each patient. To examine the stability of exogenous exosomes, exosomes were isolated from culture medium of HT29 colorectal cancer cells (grown in Dulbecco's modified Eagle's medium with 10% exosome free fetal bovine serum) and added to 0.6 mL of plasma. Samples were maintained at 37°C and aliquots of 240 μL were taken at 0, 3 and 24 h. Exosomes were isolated by ultracentrifugation using the method of Thery *et al.* [47] and quantified with western blot analysis using an antibody specific for CD63 (BD Pharmingen™) [30, 48].

Statistical analysis

All statistical analyses were done using PASW Statistics 17 (Somers, NY). Spearman's rho correlation coefficient was used to assess correlations between miRNA expression and disease severity. The Mann–Whitney *U*-test was used to assess the difference between miRNA expression between patient groups. Results were considered statistically significant at $P \leq 0.05$. For box and whisker plots, the box represents the upper and lower quartiles, whereas the whiskers represent values 1.5 interquartile ranges (IQRs) from the end of the box, $^{\circ}$ = outliers (values between 1.5 and 3 IQRs from the end of box) are represented by $^{\circ}$ and extreme outliers (values >3 IQR's from the end of box) are represented by $*$.

Results

Total circulating small RNA level is decreased in patients with impaired kidney function

The total level of small RNAs (18–25 nucleotides) present in 50 μL of plasma of the patients shown in Table 1 was measured with the Agilent 2100 Bioanalyzer. A significant correlation was observed between total small RNA level and kidney function (eGFR) shown in Figure 1a ($P \leq 0.0001$, $r = 0.553$; Spearman rho). Furthermore, when patients were divided into disease stages (normal, Stage 3 CKD, Stage 4 CKD and ESRD), a significant difference was observed in total small RNA concentration in plasma between normal/Stage 3 and Stage 4/ESRD ($P \leq 0.0001$; Mann–Whitney *U*-test) with the normal/Stage 3 group displaying >3 -fold more total circulating miRNAs compared to the Stage 4/ESRD group, shown in Figure 1b.

Given the significant difference in the level of circulating small RNAs, we wished to examine whether there were differences in the plasma abundance of miRNAs. The level of miRNAs in the plasma samples was then assessed using

Table 1. Summary of the patient details^a

| | Control (eGFR >60 mL/min) | Stage 3 CKD (eGFR 30–59 mL/min) | Stage 4 CKD (eGFR <30 mL/min) | ESRD (receiving haemodialysis) |
|---|------------------------------|------------------------------------|----------------------------------|-----------------------------------|
| Number of patients | 22 | 15 | 18 | 20 |
| Sex | | | | |
| Male | 17 | 12 | 11 | 10 |
| Female | 5 | 3 | 7 | 10 |
| Age (years) | | | | |
| Median | 63 | 66 | 76 | 69 |
| Range | 38–83 | 42–81 | 56–87 | 29–88 |
| Serum creatinine (mean, $\mu\text{mol/L}$) | 84.3 (± 14.42) | 127.0 (± 28.12) | 301.4 (± 124.61) | 567.9 (± 178.78) |
| eGFR (mean, mL/min) | 74.8 (± 10.63) | 48.6 (± 8.90) | 18.5 (± 6.24) | 8.5 (± 3.62) |
| Haemoglobin (mean, g/L) | 146.4 (± 8.30) | 136.5 (± 15.69) | 111.9 (± 10.30) | 115.7 (± 12.56) |
| Calcium (mean, mM/L) | 2.34 (± 0.09) | 2.34 (± 0.08) | 2.28 (± 0.09) | 2.29 (± 0.17) |
| Phosphate (mean, mM/L) | 1.07 (± 0.14) | 1.07 (± 0.17) | 1.33 (± 0.31) | 1.57 (± 0.45) |
| Albumin (mean, g/L) | 39.2 (± 3.89) | 37.5 (± 4.89) | 36.4 (± 3.71) | 35.0 (± 5.36) |
| PTH (mean, pM/L) | N/A | 5.9 (± 2.24) | 16.5 (± 6.39) | 29.6 (± 29.60) |

^aGender, age, creatinine, eGFR, haemoglobin, calcium, phosphate, albumin and PTH of the 75 patients are shown. Values are means \pm SD.

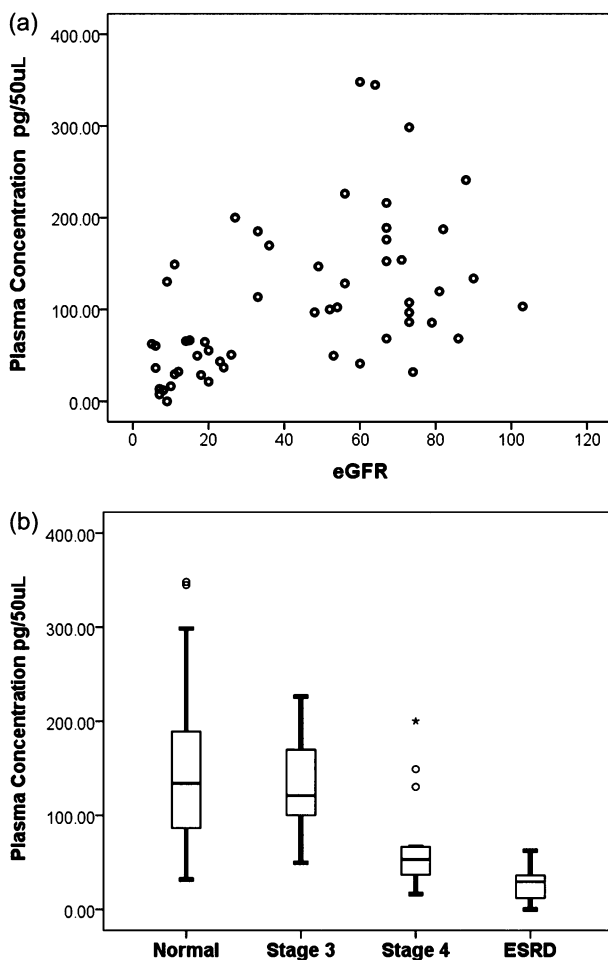


Fig. 1. Total miRNA concentration in the plasma of normal, Stage 3 CKD, Stage 4 CKD and ESRD patients. Total miRNA concentration (pg/ μL) was measured using the Agilent 2100 Bioanalyzer small RNA chip. **(a)** Concentration of miRNAs declines with kidney function (eGFR) ($P \leq 0.0001$; $r = 0.553$; Spearman's rho). **(b)** Stage 4 CKD patients and ESRD patients have significantly lower concentrations of total miRNA compared to Stage 3 CKD and normal patients ($P \leq 0.0001$; Mann–Whitney U -test).

qPCR for five different miRNAs: miR-16, miR-21, miR-155, miR-210 and miR-638. These miRNAs were selected for study as previous work in our laboratory has shown these five miRNAs to be detectable in plasma. Also, miR-16 and miR-638 have previously been used as controls for miRNA expression studies in plasma [49–51].

In keeping with the plasma levels of small RNAs, all five miRNAs showed a significant inverse correlation between abundance (Ct value) and kidney function (eGFR) (miR-16: $P \leq 0.0001$; $r = -0.647$, miR-21: $P = 0.004$; $r = -0.347$, miR-155: $P \leq 0.0001$; $r = -0.481$, miR-210: $P \leq 0.0001$; $r = -0.723$, miR-638: $P \leq 0.0001$; $r = -0.452$) indicating that these miRNAs are present at a higher concentration in patients with normal kidney function (Figure 2).

The level of expression of these five miRNAs was also assessed between different patient groups (normal, Stage 3 CKD, Stage 4 CKD and ESRD) (Figure 3). The Ct value for each miRNA was used as an indication of miRNA abundance and therefore, a one-cycle decrease in the Ct value represents a 2-fold increase in abundance. No significant difference was observed between the normal cohort and Stage 3 CKD for any of the five miRNAs. However, significant differences were observed between normal and Stage 4 CKD for miR-210 (three cycles: 8-fold, $P \leq 0.0001$), miR-16 (three cycles: 8-fold, $P = 0.024$) and miR-155 (two cycles: 4-fold, $P = 0.002$). Highly significant differences were observed between the normal/Stage 3 CKD and ESRD cohorts (2-fold miR-638, 4-fold miR-21 and miR-155, 64-fold miR-210 and 512-fold miR-16; $P \leq 0.01$). Furthermore, significant differences were also observed between Stage 4 CKD and ESRD patients for miR-210 (three cycles: 8-fold, $P = 0.003$) and miR-16 (seven cycles: 128-fold, $P \leq 0.0001$). The correlation between miRNA abundance and eGFR remains significant when the patients undergoing haemodialysis are excluded from the analysis, with the exception of miR-21 and miR-155 levels.

Further correlations between miRNA level and other laboratory measurements

In view of the strong association of eGFR determined from serum creatinine with miRNA levels, we sought to examine

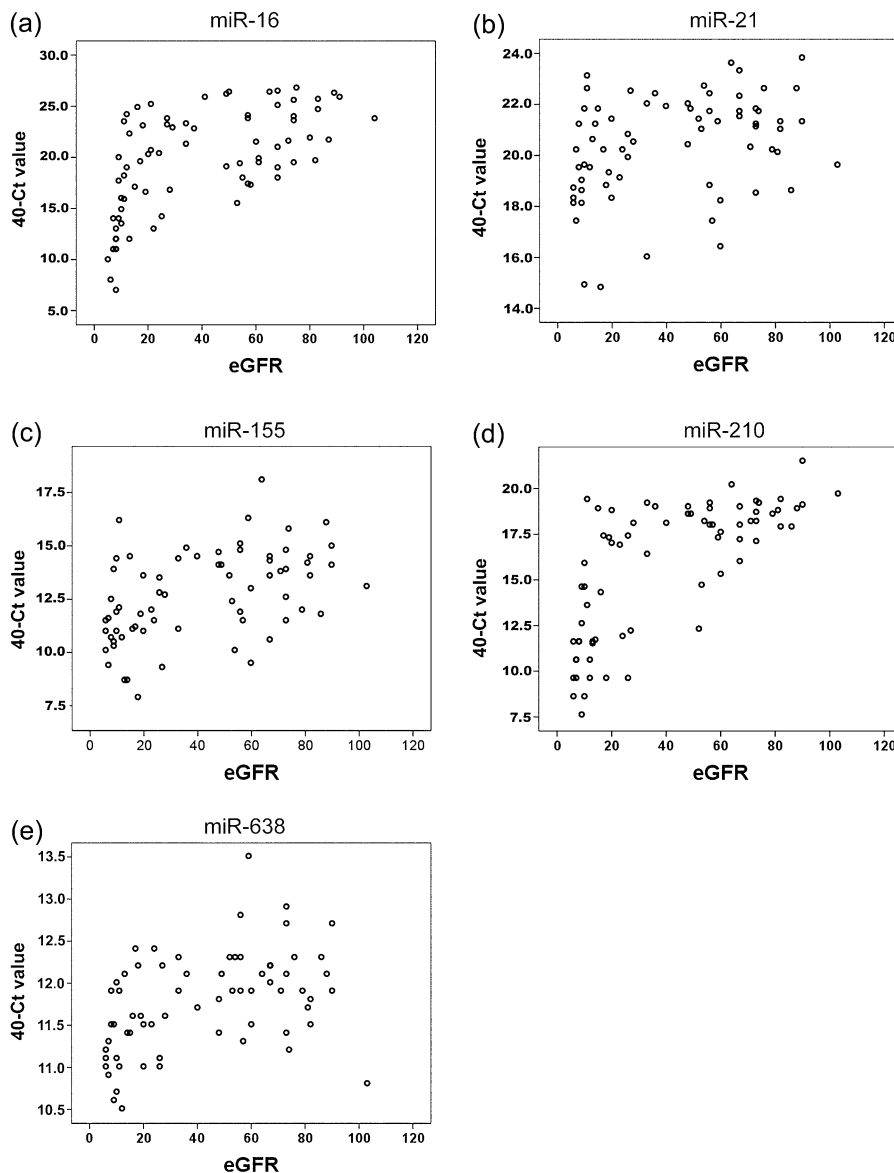


Fig. 2. Correlation between the level of specific circulating miRNAs and kidney function. The level of five individual miRNAs (miR-16, miR-21, miR-155, miR-210 and miR-638) was assessed using qPCR. A significant inverse correlation was found between the Ct value (cycle threshold) and kidney function (eGFR) indicating a lower level of miRNAs in patients with impaired kidney function. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample) and are plotted as 40-Ct values such that increased y-axis values reflect increased nucleic acid concentration (a) miR-16: $P \leq 0.0001$; $r = -0.647$, (b) miR-21: $P = 0.004$; $r = -0.347$, (c) miR-155: $P \leq 0.0001$; $r = -0.481$, (d) miR-210: $P \leq 0.0001$; $r = -0.723$, (e) miR-638: $P \leq 0.0001$; $r = -0.452$.

whether other features associated with renal failure showed a more or less powerful relationship with miRNA abundance. Other laboratory features of the 75 patients including haemoglobin, calcium, albumin, phosphate and PTH levels were analysed with respect to total small RNA level and five individual miRNAs (Table 2). A significant correlation was observed between haemoglobin levels and the concentration of small RNAs in plasma ($P = 0.003$, $r = 0.413$) and a significant inverse correlation was observed between haemoglobin levels and Ct values for miR-16 ($P = 0.002$, $r = -0.368$); miR-21 ($P = 0.027$, $r = -0.271$); miR-155 ($P = 0.002$, $r = -0.366$); miR-210 ($P \leq 0.0001$, $r = -0.454$) and miR-638 ($P = 0.004$, $r = -0.347$), indicating that lower haemoglobin levels in patients with CKD

correlate with lower levels of miRNAs. Also, a significant association was found between miR-21, miR-155 and miR-638 Ct values and PTH level ($P = 0.002$, $r = 0.490$; $P = 0.003$, $r = 0.476$ and $P = 0.023$; $r = 0.379$, respectively). The concentration of small RNAs in plasma was also significantly inversely correlated with phosphate ($P = 0.026$, $r = -0.316$) and Ct values of miR-16, miR-155, miR-210 and miR-638 were also highly correlated with phosphate levels ($P \leq 0.0001$, $r = 0.451$; $P = 0.003$, $r = 0.366$; $P \leq 0.0001$, $r = 0.432$ and $P \leq 0.0001$, $r = 0.439$, respectively). The Ct value of miR-638 was also significantly inversely correlated with the level of albumin ($P = 0.013$, $r = -0.303$). Of all the clinical features analysed, however, the most powerful correlations were found between miRNA

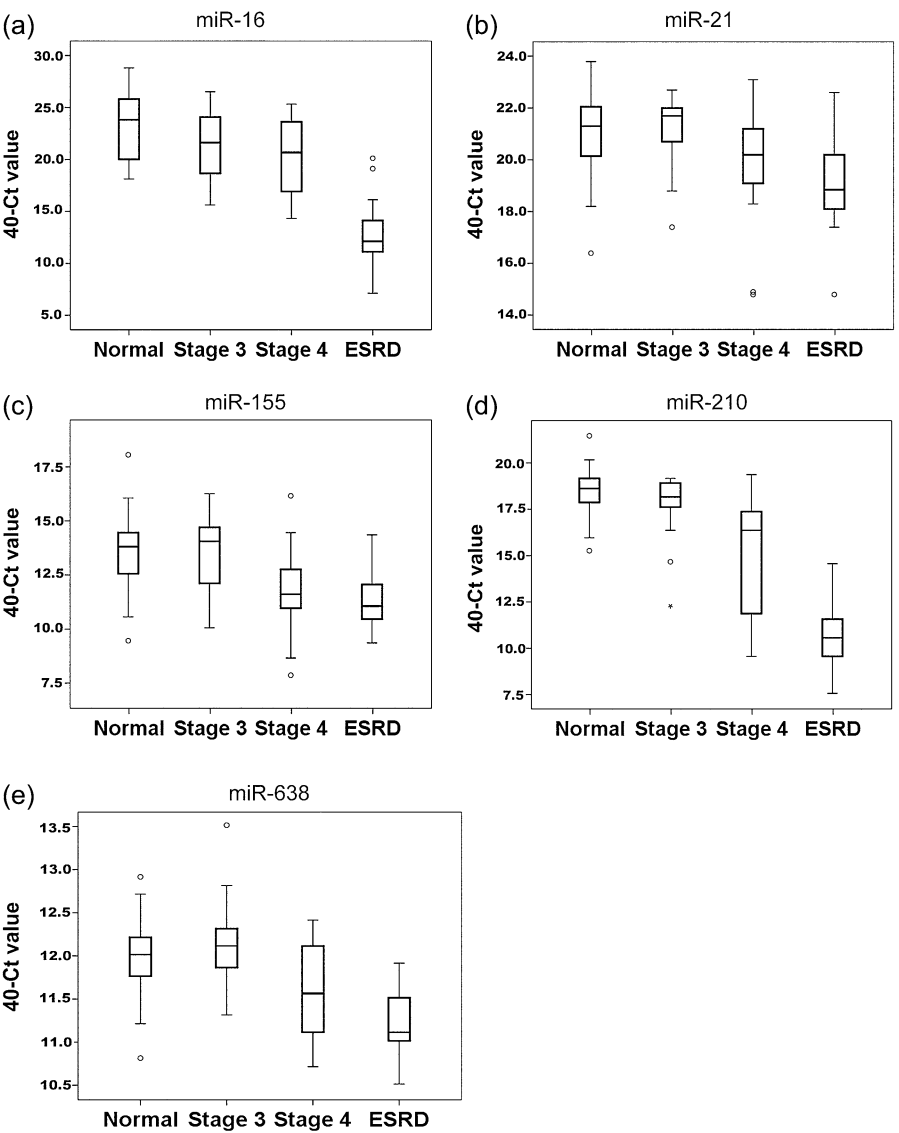


Fig. 3. Level of five individual miRNAs in the plasma of normal, Stage 3 CKD, Stage 4 CKD and ESRD patients. The level of five individual miRNAs (miR-16, miR-21, miR-155, miR-210 and miR-638) was assessed using qPCR. Significant differences were observed between normal and Stage 4 CKD for miR-210 ($P \leq 0.0001$), miR-16 ($P = 0.024$) and miR-155 ($P = 0.002$). Highly significant differences were observed between the normal/Stage 3 CKD and ESRD cohorts ($P \leq 0.01$). Significant differences were also observed between Stage 4 CKD and ESRD patients for miR-210 ($P = 0.003$), miR-16 ($P \leq 0.0001$) and miR-638 ($P = 0.036$). Each sample was assayed in triplicate.

Table 2. Summary of the correlation (r) and significance (P) of associations between miRNA abundance in plasma and laboratory measurements^a

| | Plasma concentration | | miR-16 | | miR-21 | | miR-155 | | miR-210 | | miR-638 | |
|-------------|----------------------|--------|---------------|--------|--------|--------|---------------|--------|---------------|--------|---------------|--------|
| | P | r | P | r | P | r | P | r | P | r | P | r |
| eGFR | ≤ 0.0001 | 0.553 | ≤ 0.0001 | -0.647 | 0.004 | -0.347 | ≤ 0.0001 | -0.481 | ≤ 0.0001 | -0.723 | ≤ 0.0001 | -0.452 |
| Creatinine | ≤ 0.0001 | -0.588 | ≤ 0.0001 | 0.638 | 0.008 | 0.313 | ≤ 0.0001 | 0.488 | ≤ 0.0001 | 0.714 | ≤ 0.0001 | 0.435 |
| Haemoglobin | 0.003 | 0.413 | 0.002 | -0.368 | 0.027 | -0.271 | 0.002 | -0.366 | ≤ 0.0001 | -0.454 | 0.004 | -0.347 |
| Calcium | 0.189 | 0.189 | 0.327 | -0.120 | 0.968 | 0.006 | 0.092 | -0.209 | 0.088 | -0.207 | 0.271 | -0.139 |
| Phosphate | 0.026 | -0.316 | ≤ 0.0001 | 0.451 | 0.111 | 0.2 | 0.003 | 0.366 | ≤ 0.0001 | 0.432 | ≤ 0.0001 | 0.439 |
| Albumin | 0.043 | 0.285 | 0.655 | -0.054 | 0.697 | -0.048 | 0.891 | 0.002 | 0.556 | -0.072 | 0.013 | -0.303 |
| PTH | 0.221 | -0.244 | 0.259 | 0.185 | 0.002 | 0.49 | 0.003 | 0.476 | 0.207 | 0.207 | 0.023 | 0.379 |

^aCorrelations between the concentration of small RNAs in plasma, and the Ct values of miR-16, miR-21, miR-155, miR-210 and miR-638 with laboratory measurements of eGFR, creatinine, haemoglobin, calcium, phosphate, albumin and PTH.

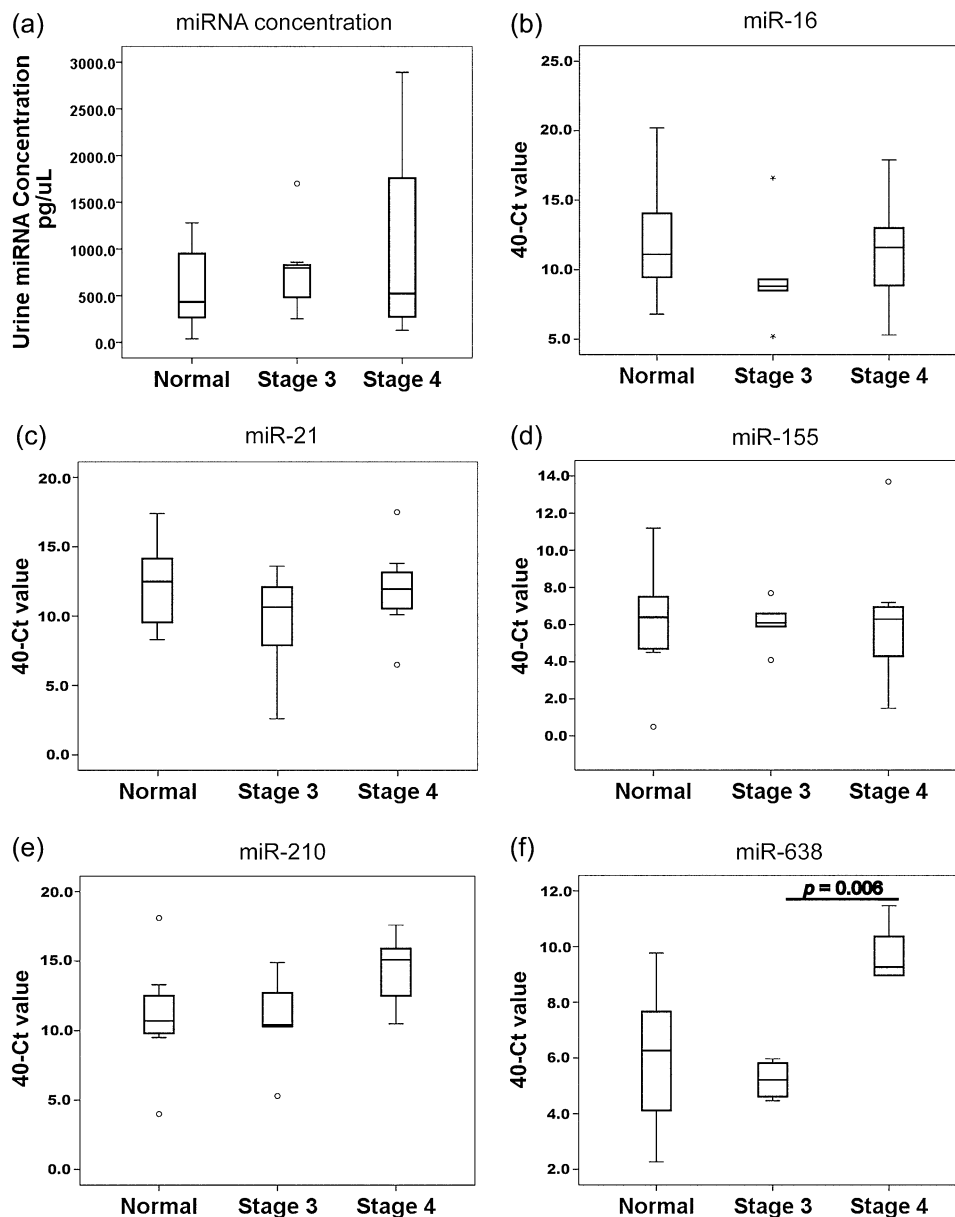


Fig. 4 miRNA concentration in the urine of normal, Stage 3 CKD and Stage 4 CKD patients ($n = 9, 8$ and 5 , respectively). Concentration was assessed using Agilent 2100 Bioanalyzer (a), and the Ct value from the qPCR of five miRNAs: miR-16 (b), miR-21 (c), miR-155 (d), miR-210 (e) and miR-638 (f). No association between patient groups was observed, except for miR-638 which shows a significant difference between normal/Stage 3 CKD and Stage 4 CKD patients ($P = 0.006$; Mann–Whitney U -test). Each sample was assayed in triplicate.

abundance and eGFR with the exception of miR-21, which was more highly correlated with PTH than with eGFR ($P = 0.002$, $r = -0.490$ and $P = 0.004$, $r = -0.347$, respectively). No association was seen between calcium levels and levels of circulating miRNAs.

Excretion of miRNAs in the urine of CKD patients

In order to determine whether the decrease in circulating miRNAs in CKD patients was associated with an increase in miRNAs in excreted urine, we determined the concentration of small RNAs using the Agilent 2100 bioanalyzer in the urine of 22 patients (9 normal, 8 Stage 3 CKD and 5 Stage 4 CKD). No difference between the patient groups was observed (see Figure 4a).

We then assessed the level of individual miRNAs in 19 patients (7 normal, 5 Stage 3 CKD and 7 Stage 4 CKD) using qPCR. Again, for most of the miRNAs (miR-16, miR-21, miR-155 and miR-210), no association was observed between urinary miRNA level and kidney function (Figure 4b–e). The only exception to this was miR-638, which showed a significant increase in the urine of patients with Stage 4 CKD compared to normal and Stage 3 CKD patients ($P = 0.006$; Mann–Whitney U -test) (Figure 4f).

The level of miRNA degradation in vitro is increased in plasma from patients with severe CKD

There is evidence that the level of circulating RNases may be substantially increased in patients with impaired kidney

function [52–57]. We sought to determine whether increased degradation of circulating miRNAs or of circulating exosomes in the plasma of patients with severe CKD could account for the reduced level of total miRNA observed in the plasma of these patients. We examined the stability of exosomes purified from cell culture and exposed to normal and uraemic serum for varying time periods. We assessed exosome abundance by purification followed by CD63 immunoblotting. We also studied the levels of circulating exosomes in individuals with and without renal failure. However, there was significant variation in exosome abundance and no consistent differences in exosome abundance or stability in uraemic versus normal serum (see Supplementary figure 1).

Consistent with previous work on miRNAs, levels remain stable when plasma is subjected to prolonged room temperature incubation [20], we saw little miRNA degradation in plasma from individuals with normal renal function (Figure 5). However, in contrast, in patients with severe kidney disease, the rate of miRNA degradation was increased. Figure 5 shows that the rate of degradation increased with reduced kidney function for miR-210, miR-16 and miR-21 >10 h. The rate of *ex vivo* degradation of miR-210, miR-16 and miR-21 was higher in ESRD patient plasma compared to plasma from individuals with normal renal function ($P = 0.024$, 0.024 and $P = 0.04$, respectively). For Stage 4 CKD patients, the rate of degradation of miR-210 was also higher compared to that from individuals with normal renal function ($P = 0.024$).

Discussion

This study shows that the concentration of circulating miRNAs is reduced in patients with impaired kidney function. This was true for patients with ESRD receiving haemodialysis treatment and for those with Stage 4 CKD. It was seen both for the total amounts of circulating small RNA measured with the Agilent 2100 bioanalyzer (which uses a microfluidic and fluorescent-labelling method) and for five specific miRNAs that were assayed by qPCR. Although the abundance of all of the five miRNAs analysed in this study showed a positive correlation with kidney function, it is possible that other circulating miRNAs may not be associated with kidney function in this way.

The correlation of miRNA concentration and eGFR was better than with any other clinical feature analysed (calcium, phosphate, albumin, haemoglobin and PTH), with the exception of miR-21 which showed a higher correlation with PTH concentration. This tends to suggest that the association of miRNA level with these other features that occur in severe kidney failure (anaemia, hyperparathyroidism, hyperphosphataemia and hypoalbuminaemia) is indirect and mediated primarily by an effect of impaired glomerular filtration rate.

These results combined with the data on urine excretion of miRNAs suggest that the kidneys are not involved in the physiological clearance of circulating miRNAs. However, the mechanism for the reduced levels of circulating miRNAs in reduced kidney function is unclear. In renal failure, there is a marked accumulation of low-molecular weight

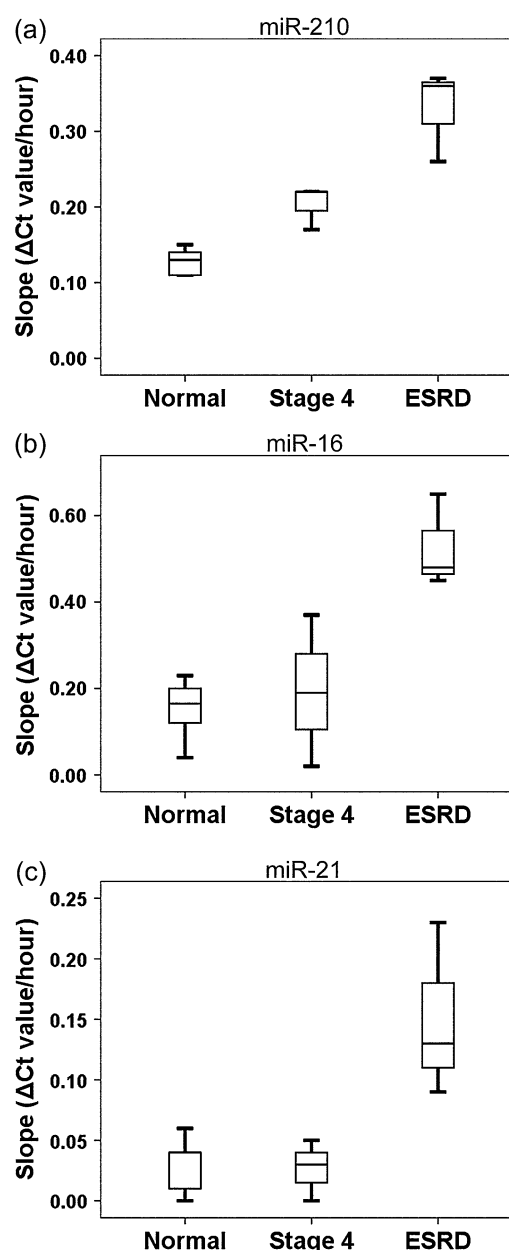


Fig. 5. The rate of *ex vivo* microRNA degradation of miR-210, miR-16 and miR-21 was higher in ESRD patient plasma compared to plasma from individuals with normal renal function ($P = 0.024$, 0.024 and $P = 0.04$, respectively). For Stage 4 CKD patients, the rate of degradation was also higher for miR-210 compared to samples from individuals with normal renal function ($P = 0.024$). Normal $n = 12$, Stage 4 CKD $n = 6$, ESRD $n = 6$. Slope was calculated as a ratio of decline in Ct value >10 h. Three time points were used at 0, 5 and 10 h.

proteins, such as RNases [52–57]. It is therefore attractive to suggest that enhanced levels of these enzymes lead to increased degradation of circulating miRNA. The very recent demonstration that many circulating miRNAs exist bound to Argonaute 2-containing protein complexes [58] or to high-density lipoproteins [59] rather than within vesicles suggests that reduced exosomal protection of miRNAs [20] is unlikely to be responsible for the reduced levels of circulating miRNAs in kidney failure.

Whatever the explanation for the reduced levels of circulating miRNAs in renal failure, it will be important to consider kidney function when interpreting studies seeking to utilize circulating miRNAs as biomarkers of renal and other diseases. The reduced levels of circulating miRNAs may also be relevant to the pathogenesis of proteinuria and uraemia. Further work is required to examine whether the levels of all circulating miRNAs and indeed, other RNAs are reduced in patients with severe kidney disease and to examine the role of reduced circulating miRNAs in uraemia pathogenesis.

Acknowledgements. This research was supported by a Faculty of Health Sciences Seeding Grant from Flinders University, the Flinders Renal Unit Research Fund and the Flinders Medical Centre Clinicians Special Purpose Fund. We thank Kathy Hill, Research Nurse, for her excellent contribution and the patients for their generous involvement in this study.

Conflict of interest statement. None declared.

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Received for publication: 18.11.10; Accepted in revised form: 14.7.11

Nephrol Dial Transplant (2011) 26: 3802–3805

doi: 10.1093/ndt/gfr503

Advance Access publication 12 September 2011

Short Communication

FTY720 combined with tacrolimus in *de novo* renal transplantation: 1-year, multicenter, open-label randomized study

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Abstract

Background. FTY720 (fingolimod), a novel immunomodulator, has demonstrated potential for prevention of acute rejection in combination with cyclosporine.

Methods. This study evaluated FTY720 2.5 mg versus mycophenolate mofetil (MMF) in a combination regimen with standard tacrolimus and corticosteroids in *de novo* renal transplant recipients for the composite efficacy within 6 months of transplantation.

Results. Incidence of treated biopsy-proven acute rejection was 22.9% with FTY720 and 18.5% with MMF. Increased incidence of macular oedema, transient decrease in heart rate and low rate of infections were seen in the FTY720 arm.

Conclusion. FTY720 combined with tacrolimus and steroids did not show a significant therapeutic advantage over MMF for the prevention of acute rejection in *de novo* renal transplant recipients.

Keywords: FTY720; mycophenolate mofetil; renal transplantation; sphingosine 1-phosphate receptor; tacrolimus

Introduction

FTY720 (fingolimod) is a novel immunomodulator and representative of sphingosine 1-phosphate receptor