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ORIGINAL ARTICLE

# MicroRNA-21 in plasma exosome, but not from whole plasma, as a biomarker for the severe interstitial fibrosis and tubular atrophy (IF/TA) in post-renal transplantation

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## Abstract

**Background:** Non-invasive diagnosis of interstitial fibrosis and tubular atrophy (IF/TA), a major cause of chronic allograft dysfunction in post-kidney transplantation (post-KT), is needed.

**Objective:** Several candidates of microRNAs (miRs) in plasma exosome or whole plasma were evaluated for IF/TA biomarker.

**Methods:** Kidney samples from biopsy and plasma were tested for miRs expression.

**Results:** Expression of miR-21, miR-142-3p and miR-221 in renal histology with high fibrosis score (Banff classification) was higher than the samples with lesser score (n = 17/group). However, expression of these miRs from plasma exosome or from whole plasma of post-KT patients with different severity of IF/TA as determined by percentage of IF/TA including; grade I (5-25%) (n = 15), grade II (26-50%) (n = 15), grade III (≥ 50%) (n = 6) versus stable graft function (no IF/TA) (n = 15) was not different. However, high expression of miR-21 in exosome, but not from whole plasma, was demonstrated in IF/TA grade II and III compared with IF/TA grade I. In contrast, serum creatinine (Scr) and proteinuria, the current standard biomarkers, could not differentiate IF/TA grade I out of grade II/III. There was no correlation between exosome miR-21 versus the current standard renal injury biomarkers, including Scr, blood urea nitrogen and proteinuria, in IF/TA grade II or grade III.

**Conclusion:** High miR-21 in plasma exosome, but not in whole plasma, indicated high grade IF/TA in post-KT patients. This non-invasive monitoring biomarker allows the more frequent evaluation on IF/TA than renal biopsy (a standard but more invasive procedure) resulting in the earlier management. More studies on patients are warrant.

**Key words:** Interstitial Fibrosis, Tubular Atrophy, Kidney transplantation, MicroRNA, Exosome

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## Introduction

Interstitial fibrosis and tubular atrophy (IF/TA), a major cause of chronic allograft dysfunction in post kidney transplantation (post-KT), demonstrates in 40% and 65% of patients after 3-6 and 24 months post-KT, respectively.[1] Although kidney biopsy is the gold standard diagnosis of IF/TA,[2] the procedure is too invasive for the frequent test. Hence, the non-invasive biomarkers for IF/TA will be beneficial. As such, microRNAs (miRs) is the small non-coding RNA molecules containing 22-25 nucleotides that regulated

gene expression by complementary binding 3'UTR of mRNA leading to the degradation or inhibition of gene expression. Interestingly, miRs are more stable, easily detectable and frequently enriched in the exosome,[3-7] the 40-200 nm extracellular vesicles protecting several cell compartments including miRs.[8-14] Indeed, the communication between cells by cellular production of miRs through excreted-exosome is demonstrated.[15,16] Hence, exosomal miRs is one of the interesting biomarkers. Due to the uncertainty of the normalization of urinary biomarker including be urine creatinine, total number of exosome, urine protein or time normalization (eg. 24 h urine collection),[17,18] miRs in plasma exosome might be the proper biomarkers and were explored here.

Indeed, several miRs are correlated with the pathogenesis of fibrosis including; miR-21, miR-142-3p and miR-221. The increased expression of miR-21 in post-KT IF/TA from urine exosome[19] and in plasma[20] possibly due to the activation by TGF-β resulting in the increased activated Akt and mesangial expansion is mentioned.[21] In addition, the expression of miR-142-3p from urine, plasma and renal tissue from post-KT IF/TA is increased[22-24] and miR-221 involves in TGF-β dependent fibrosis.[25] Then, we explored the expression of these miRs from renal tissue and in plasma for the discovery of IF/TA biomarker.

## Materials and Methods

### *Patient samples*

The samples of patients with post-KT from the King Chulalongkorn Memorial Hospital, Bangkok, Thailand were collected following the approved protocol by the Ethical Institutional Review Board, Faculty of Medicine, Chulalongkorn University, according to the Declaration of Helsinki, with written informed consent from each individual patient (IRB No.230/62). The samples were snap frozen in the liquid nitrogen and were kept in -80°C before use. Renal histology from the protocol biopsy of patient post-KT (**Table 1 and Figure 1**) with the high score of fibrosis as reported by positive score in chronic glomerular (CG), chronic tubular (CT) and chronic vascular (CV) versus lower score were collected for determination of kidney miRs. Renal tissue was put in the miRNeasy Mini Kit (QIAGEN, Hilden, USA) before the further miRs analysis (see below). In addition, the peripheral bloods of another group of patients (**Table 2**) with the IF/TA score identified by kidney biopsy in concordance with the Banff classification[26] were collected. Plasma samples were categorized into 4 groups including IF/TA grade I (5-25%), grade II (26-50%), grade III (≥ 50%) and stable graft function based on renal histopathology. Patients with serology positive for BK virus, cytomegalovirus (CMV) and re-transplanted patients were excluded.

### *Plasma exosome isolation, Nanoparticle tracking and Western blot analysis*

All samples from patients (**Table 2**) were kept in -80°C until analyzed. As such, plasma samples were incubated with of thrombin to remove fibrin clot, centrifuged for supernatants collection, precipitated with 24% polyethylene glycol (PEG) (Manufacturer number DB0433; Thermo Scientific, Waltham, MA, USA) and incubated at 4°C overnight. Although several percentage of PEG has been successfully used for the plasma exosome extraction, PEG at 16-24% is repeatedly used in the previous publications.[27,28] After that, the samples were centrifuged at 1,500 g for 30 minutes for retrieval of exosome pellets, eluted by phosphate buffer solution (PBS) and kept in RNA later for miRs identification. The presentation of exosome in suspended-pellets was supported by nanoparticle tracking and Western blot analysis. For nanoparticle tracking, the suspended exosome pellets were homogeneously diluted in 1:200 particle-free PBS (0.02 μm filtered) and the size and concentration of exosome were measured by NanoSight NS300 (Malvern Panalytical Instruments Company, Malvern, United Kingdom).

**Table 1. The demographic data of patients with less or more fibrosis (kidney tissue)**

| Parameters | Less fibrosis (17) | More fibrosis (17) |
|---|---|---|
| Recipient age (years) | 62 ± 8 | 72 ± 11 |
| Recipient gender (F/M) | 11/6 | 10/7 |
| Dialysis vintage (months) | 62 ± 46 | 71 ± 54 |
| Post-transplantation time, months | 2 ± 1** | 112 ± 21 |
| Type of donor (living/deceased) | 11/6 | 7/10 |
| Total ischemic time (min) | 628 ± 346 | 763 ± 411 |
| HLA mismatch | 1.5 ± 0.8* | 2.8 ± 0.9 |
| Highly sensitized PRA (PRA > 30), n (%) | 2 (12) | 7 (41) |
| Native kidney disease, n (%) | | |
| ADPKD | 3 (18) | 2 (12) |
| Chronic glomerulonephritis | 5 (29) | 4 (22) |
| Diabetic nephropathy | 8 (47) | 10 (59) |
| Obstructive nephropathy | 1 (6) | 2 (12) |
| Unknown | | |
| Immunosuppression, n (%) | | |
| Cyclosporine A-based | 0 (0) | 5 (29) |
| Tacrolimus-based | 14 (82) | 9 (53) |
| Rapamycin-based | 2 (12) | 3 (17) |
| Everolimus-based | 0 (0) | 0 (0) |
| Azathioprine | 0 (0) | 5 (29) |
| Mycophenolate mofetil | 14 (82) | 10 (59) |
| Prednisolone | 15 (88) | 16 (94) |
| Advagraft | 2 (12) | 3 (18) |

*, $p < 0.01$; **, $p < 0.001$

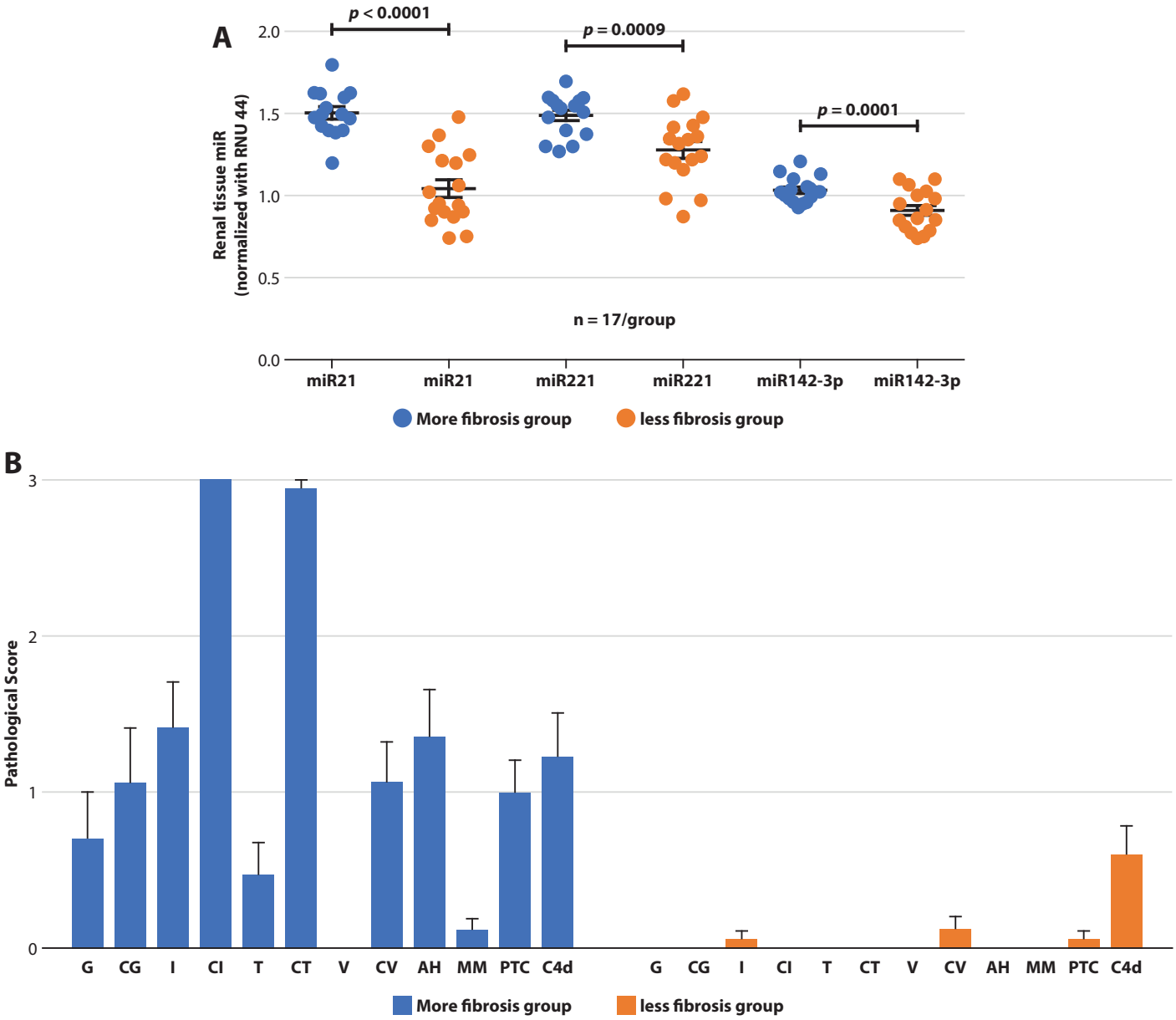


**Figure 1. The expression of microRNA in renal tissue with more or less fibrosis (A) and the pathological score following Banff criteria of "more fibrosis" versus "less fibrosis" following the record from pathologist reports (B) were demonstrated.**
G, glomeruli; CG, chronic glomeruli; T, tubular; CT, chronic tubular; V, vascular; CV, chronic vascular; AH, arteriolar hyaline thickening; MM, mesangial matrix expansion; PTC, cells in peritubular capillary area; C4d, complement 4d

**Table 2. The demographic data of patients (plasma samples)**

| Parameters | Stable (n = 14) | IF/TA I (n = 15) | IF/TA II (n = 17) | IF/TA III (n = 6) |
|---|---|---|---|---|
| Recipient age (years) | 47 ± 4 | 46 ± 4 | 53 ± 3 | 54 ± 5 |
| Recipient gender (F/M) | 10/4 | 5/11 | 7/11 | 2/4 |
| Dialysis vintage (months) | 64 ± 34 | 58 ± 36 | 77 ± 45 | 65 ± 38 |
| Post-transplantation time, months | 19 ± 6** | 89 ± 19 | 102 ± 20 | 79 ± 27 |
| Type of donor (living/deceased) | 10/4 | 10/16 | 4/14 | 3/3 |
| Total ischemic time (min) | 580 ± 632 | 517 ± 221 | 617 ± 317 | 677 ± 322 |
| HLA mismatch | 2.6 ± 0.5 | 1.7 ± 0.3 | 2.6 ± 0.4 | 2.7 ± 0.2 |
| Highly sensitized PRA (PRA > 30), n (%) | 1 (7.1) | 1 (6.7) | 5 (29.4) | 1 (16.7) |

**Table 2. (Continued)**

| Parameters | Stable (n = 14) | IF/TA I (n = 15) | IF/TA II (n = 17) | IF/TA III (n = 6) |
|---|---|---|---|---|
| Native kidney disease, n (%) | | | | |
| ADPKD | 0 (0) | 2 (13) | 0 (0) | 1 (17) |
| Chronic glomerulonephritis | 3 (22) | 3 (20) | 6 (35) | 2 (33) |
| Diabetic nephropathy | 7 (50) | 9 (60) | 8 (47) | 3 (50) |
| Obstructive nephropathy | 2 (14) | 0 (0) | 2 (13) | 0 (0) |
| Unknown | 2 (14) | 1 (7) | 1 (5) | 0 (0) |
| Immunosuppression, n (%) | | | | |
| Cyclosporine A-based | 3 (21.4) | 4 (25) | 6 (33.3) | 1 (16.7) |
| Tacrolimus-based | 10 (71.4) | 10 (62.5) | 8 (44.4) | 5 (83.3) |
| Rapamycin-based | 1 (7.1) | 3 (18.8) | 2 (11.1) | 0 (0) |
| Everolimus-based | 2 (14.3) | 0 (0) | 0 (0) | 0 (0) |
| Azathioprine | 0 (0) | 1 (6.3) | 2 (11.1) | 1 (16.7) |
| Mycophenolate mofetil | 14 (100.0) | 11 (68.8) | 14 (77.8) | 0 (0) |
| Prednisolone | 14 (100.0) | 15 (93.8) | 15 (83.3) | 5 (83.3) |
| Advagraft | 2 (14.3) | 2 (12.5) | 3 (16.7) | 0 (0) |

**, $p < 0.001$ vs. other groups

In addition, the standard procedure of Western blot analysis was performed. In short, the starting plasma volume for the exosome extraction in all samples was 250 µL following a previous publication.[27,28] Then, the suspended pellet was mixed with 100 mM of dithiothreitol (DTT) (Thermo Scientific) at 95°C 5 min, loaded in 10% of SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane (PVDF) (GE Healthcare, UK) and blocked with 5% of bovine serum albumin (BSA). Then primary antibody against exosome proteins[29] (Abcam, Cambridge, MA, USA) including anti-CD9 (ab92726), anti-CD63 (ab193349) and TSG101 (ab83) was incubated overnight at 4°C, washed, incubated with secondary antibody linked horseradish at room temperature for 1 hour and detected the signal by Super Signal West Femto substrate (Thermo Scientific) and analyzed with Image Quant TL (GE Healthcare, UPPSALA, Sweden). The concentrations of primary and secondary antibody were 1:1,000 in 5% BSA and 1:5,000 in 2.5% BSA, respectively.

### *Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real-time PCR*

Total RNA was isolated from renal tissue or plasma exosome pellets by the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, USA). Then, quantification of miR was based on the total RNA quantification as measured by NanoDrop 1000 (Thermo Scientific). The ratio of absorbance at optical density (OD) 260 divided by OD 280 more than 1.8 indicated the adequate purity for the further test. Total RNA (10 ng/µl) was reverse-transcribed into cDNA using reagent from TaqMan® MicroRNA Reverse Transcription Kit and specific stem loop primer from TaqMan microRNA assay (Thermo Scientific) including; miR-21 (ID 000397), miR-142-3p (ID 000464), miR-221(ID 000524), RNU-44 (house-keeping miR for renal tissue, ID 001094) and cel-39 (exogenous control for plasma sample, ID 000200). Real-time qPCR is performed on the Applied Biosystems™ 7500 Real-Time PCR Systems.

### *Statistical analysis*

All data was analyzed by SPSS software (version 22, IBM Corporation, New York, USA) and Graph Pad Prism version 7.0 software (LaJolla, CA, USA). The results were presented as mean ± standard error (SE). The differences between groups were examined for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey's analysis or Student's t-test for comparisons of multiple or 2 groups, respectively, and $p < 0.05$ was statistically significant.

## Results

Expression of the interesting miRs in renal tissue of patients with post-renal transplantation was explored and the miRs from the whole plasma (plasma) or the exosome-fraction of plasma (exosome) was examined to see if these miRs could be used as the invasive biomarker for the identification of IF/TA.

The expression of miRs in renal histology from 34 patients, including 17 samples from the first time of protocol biopsy (2 ± 1 months post-KT) (less severe fibrosis) versus 17 samples of the more severe IF/TA (112 ± 21 months post-KT) (**Table 1**) were evaluated. Indeed, the post-transplantation period and the HLA mismatch score were higher in the patients with more fibrosis in comparison with the patients with less fibrosis (**Table 1**) supporting the previous publications.[30] On the other hand, the expression of all selected miRs in renal tissue with

the higher fibrosis score was more than the sample with less severe fibrosis (**Figure 1**). However, the difference in the expression of miR-21 was higher than miR-221 and miR-142-3p (**Figure 1A**). The fold change between the average value of miR in patients with more fibrosis compared with less fibrosis in miR-21 and miR-221 were 1.50 and 1.25, respectively, and in miR-142-3p was 1.15. Due to the increased expression of the miRs in IF/TA kidney samples, these miRs in plasma and in exosome fraction were tested in the samples from the other groups of patients with the IF/TA classification following Banff criteria[26] (**Table 2**). The demographic data of these patients was similar except for the lesser post-translation period in patients with stable graft function supporting the previous publications.[31] A simple plasma exosome extraction by PEG with the centrifugation (as in method section) is selected because of procedure simplicity and low cost which is suitable for the clinical use. As such, the existence of exosome in the extracted fraction from prepared plasma by this method was demonstrated by i) the visualization of exosome (40-200 nm diameter) by nanoparticle tracking analysis identified most plasma exosome in 131 ± 85 nm in diameter similar to other articles[32,33] (**Figure 2A**) and ii) the abundance of several exosome markers including CD9, CD63 and TSG101 following the previous publications[34] (**Figure 2B**). This data supported the clinical use by this simple exosome extraction. Of note, protein abundance of CD63 from the exosome fraction of IF/TA grade I was highest among other groups (**Figure 2D**) suggesting a possible role of inflammatory process in early IF/TA.[35]

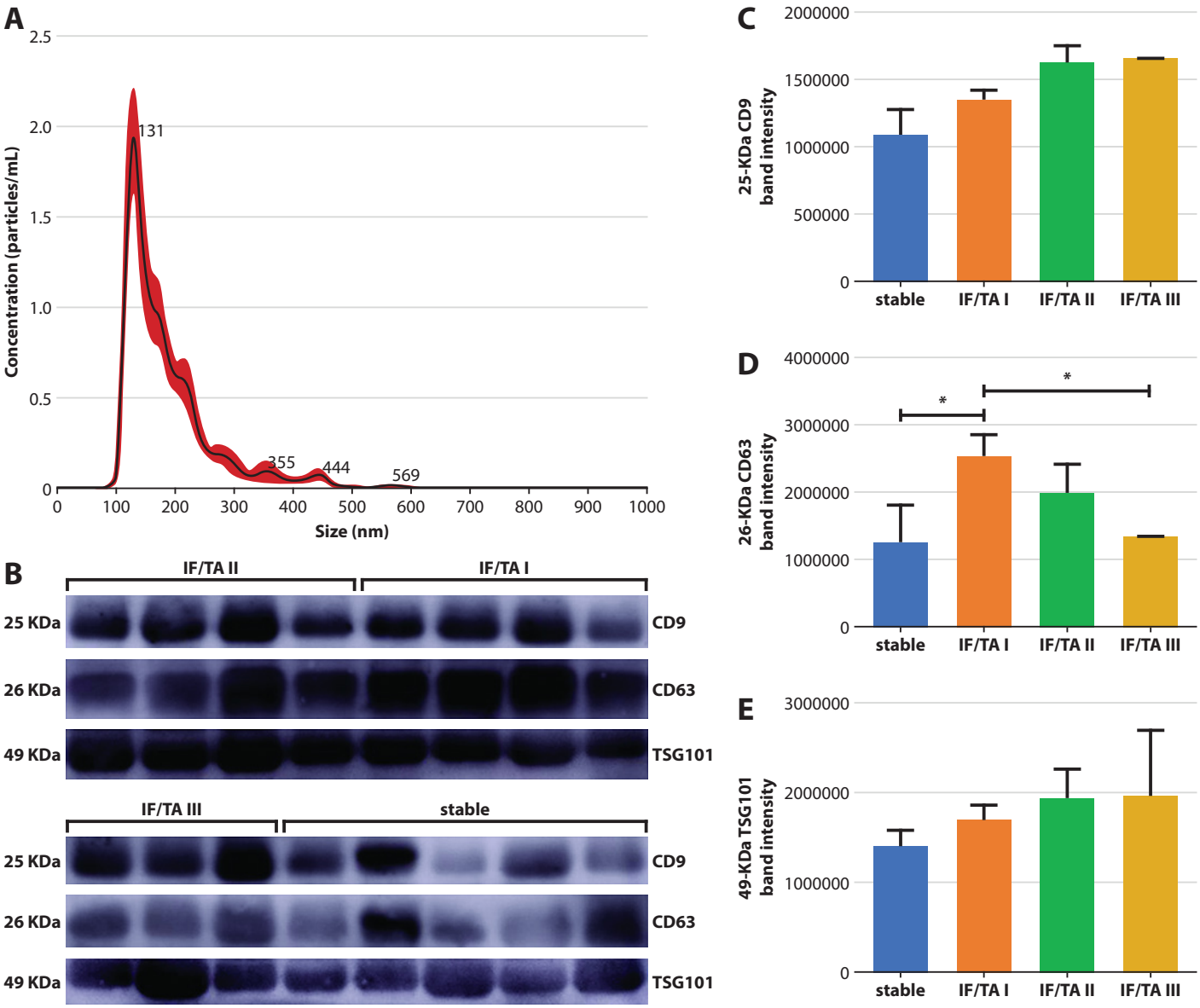


**Figure 2. The representative figure of microvesicles in plasma as evaluated by nanoparticle tracking analysis demonstrated the vesicle range of exosome (30-150 nM) (A), the Western blot analysis to determine exosome by the protein abundance of exosome biomarkers (B) and the band intensity score (C-E) (n = 3-6/group for C-E) were demonstrated. *, $p < 0.05$**

From plasma sample, the allograft dysfunction in patients with IF/TA could be identified by the conventional biomarkers; blood urea nitrogen (BUN) and serum creatinine (Scr), but not proteinuria (UPCI), in comparison with samples from patients with stable allograft function (**Figure 3A-C**). However, both BUN and Scr could not differentiate the different severity of IF/TA (**Figure 3A-B**). In parallel, the expression selected-miRs in neither whole plasma nor plasma exosome of patients with IF/TA was not different from the control (**Figure 3D-I**), despite the higher expression in kidney samples (**Figure 1**).

As such, miR-21 in whole plasma (plasma miR-21) of patients with IF/TA II was lower than the group with stable graft function (**Figure 3D**) and miR-21 in plasma exosome fraction (exosome miR-21) could differentiate between IF/TA I and IF/TA III (**Figure 3G**), different from the previous studies.[19,20] The differentiation between high grade IF/TA (grade II and III) out of IF/TA with the lower severity (grade I) with exosome miR-21 (**Figure 3G**) might be beneficial in the clinical practice. In addition, the expression of exosome miR-21 was not correlated with any current biomarkers of renal injury, including Scr, BUN and proteinuria (**Figure 4**), but showed a tendency to be positively correlated with BUN and Scr in patients with IF/TA grade II and grade III, respectively (**Figure 4B, C**).

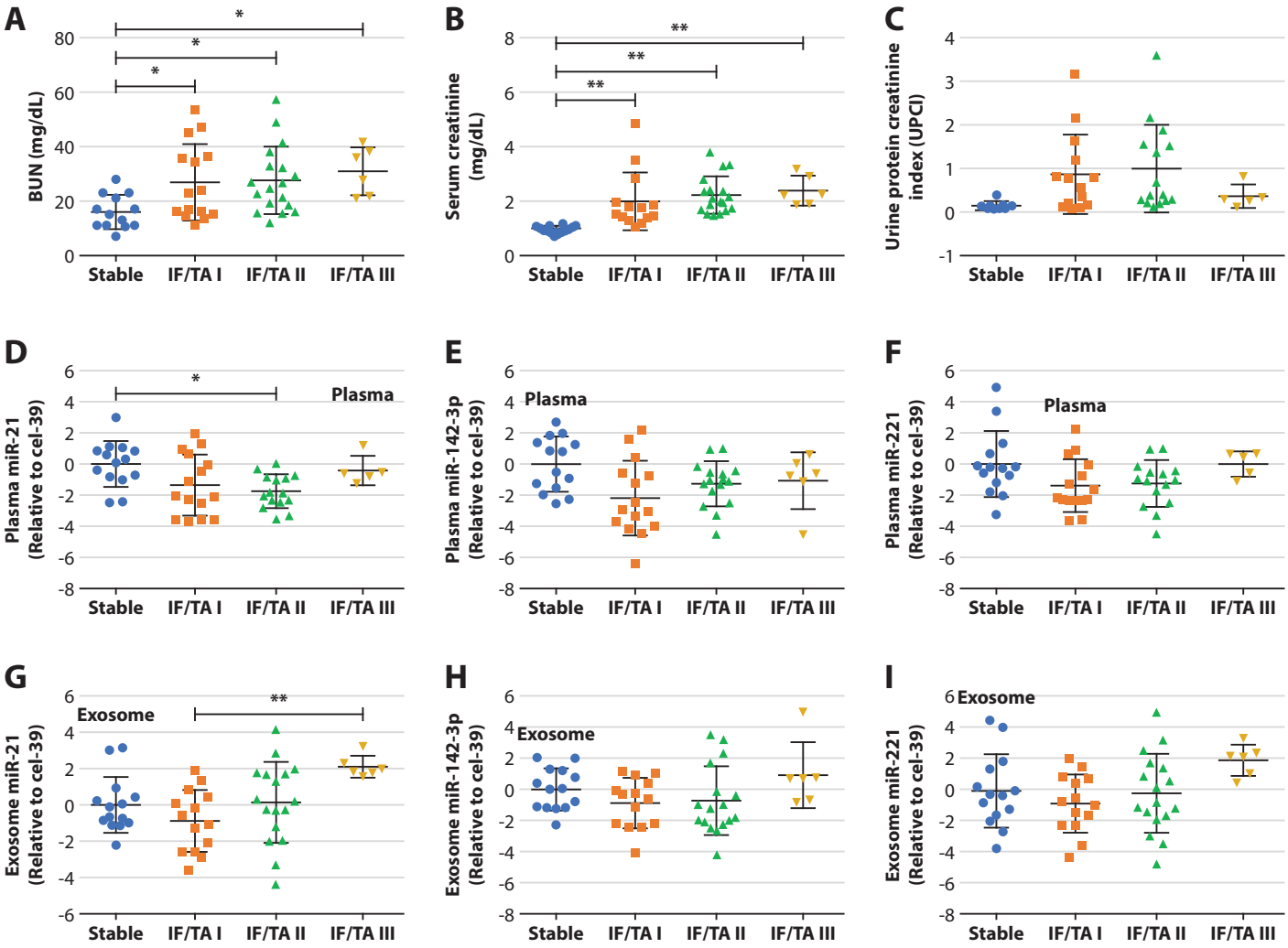


**Figure 3. The evaluation of IF/TA as determined by blood urea nitrogen (BUN) (A), serum creatinine (B), urine protein creatinine index (UPCI) (C), microRNAs from whole plasma (D-F) and microRNA from plasma exosome (G-I) from patients with stable allograft function (Stable: n = 14), interstitial fibrosis/ tubular atrophy (IF/TA) grade I (n = 15), IF/TA grade II (n = 17) and IF/TA grade III (n = 6) were demonstrated. * $p < 0.05$, ** $p < 0.01$**

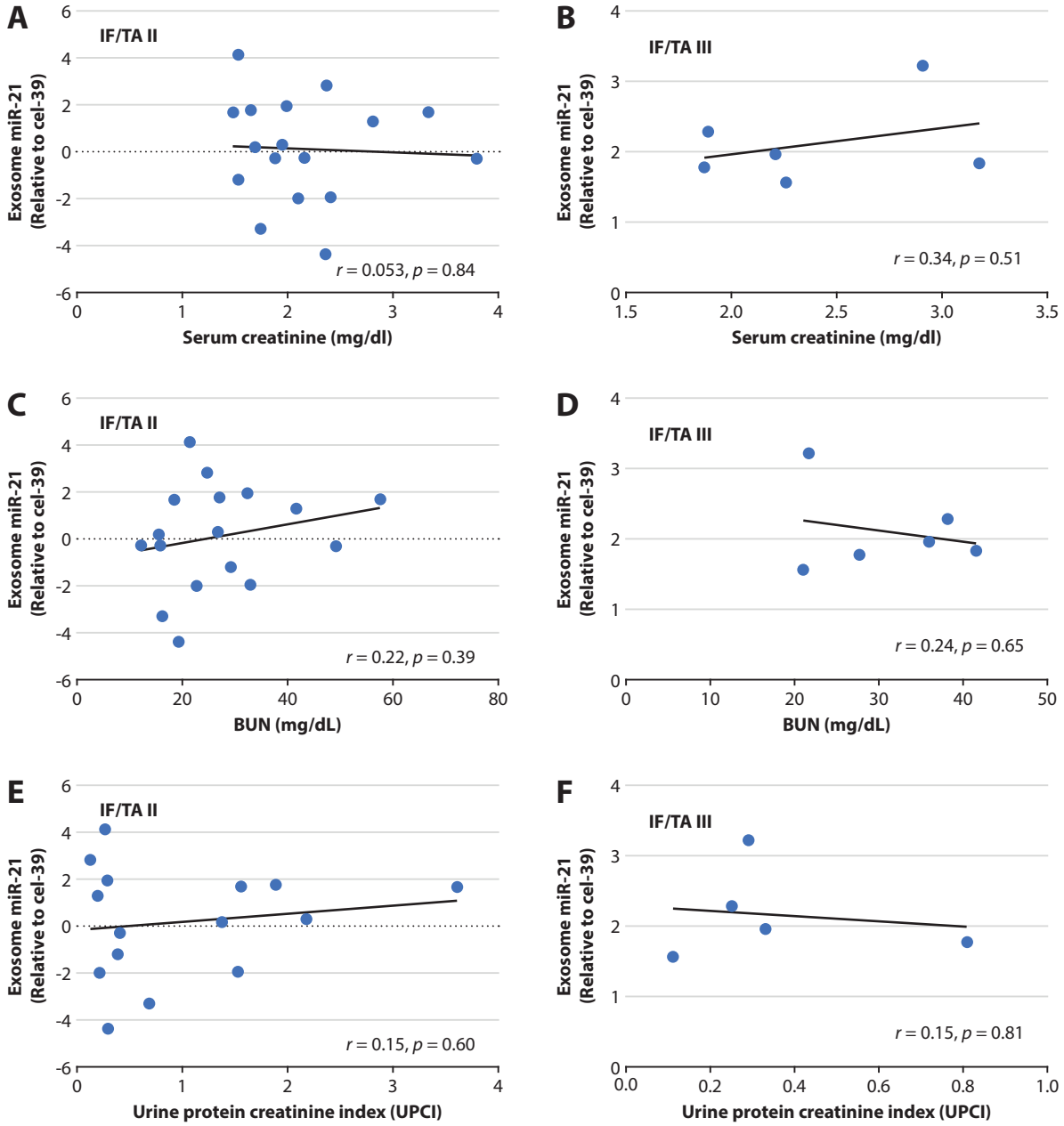


**Figure 4. The Pearson's correlation of plasma exosome microRNA-21 versus serum creatinine, blood urea nitrogen (BUN) and urine protein creatinine index (UPCI) in patients with interstitial fibrosis/ tubular atrophy (IF/TA) grade II (A, C, E) and III (B, D, F) were demonstrated.**

## Discussion

The frequent monitoring of renal fibrosis after renal transplantation, as refer to IF/TA, with the non-invasive monitoring procedures will be clinically beneficial. The expression of miR-21 in plasma exosome, but not from whole plasma, could be used for the differentiation between high versus low grade IF/TA which possibly allow prompt therapeutic managements.

### *Plasma exosome, an interesting non-invasive biomarker of kidney transplantation*

Most nephrologists avoid frequent renal biopsy which is an invasive procedure causing pain and some complications. However, the detection of post-KT IF/TA currently depends on the kidney biopsy. The frequent follow-up of IF/TA progression is tremendous beneficial to the patients because the earlier manipulation of IF/TA provides the better renal allograft function.[1] Exosome is an interesting source of biomarker because it contains interesting molecules on plasma membrane and protects the degradable contents (eg. nucleic acid and transcription factors) that make it easier for the detection as refer to "cell biopsy" or "liquid biopsy".[36] Because the normalizations of urine biomarkers are necessary due to the possibility of the alteration in urine concentration from the accumulation of urine in bladder before urine collection,[37] plasma exosome might be an easier and proper biomarker for the real clinical use. Although miRs are quite stable, several reports prefer the determination of exosome miRs.[38,39] Interestingly, the burdens of exosome proteins by CD63 was lower in stable graft function and IF/TA grade III in comparison

with IF/TA grade I, while the burdens of CD9 and TSG101 were not different between groups suggesting the limitation of using exosome proteins for the determination of exosome numbers. Also, it is possibly that microvesicle is one of the inflammatory process in the early phase of fibrosis as CD63 (membrane protein that associated with intracellular vesicles) is an essential cofactor to leukocyte recruitment.[35] Moreover, the evaluation of exosome number by Nanosight also showed the high variability in the measurement on the same sample (data not shown). Hence, the development on quantitatively measurement of plasma exosome is interesting.

***Exosome miR-21 in plasma and renal fibrosis***

The selected miRs associate with the pathogenesis of fibrosis by different pathways[22-25] which is supported by the detection of these miRs in renal tissue with IF/TA (**Figure 1**). Indeed, exosomal miR-21 has been previously mentioned as a fibrosis biomarker as identified by the micro-array data from the renal histology of the post-KT patients.[22,23,40,41] As such, miR-21 associates with TGF-β, a well-known cytokine of fibrosis pathogenesis,[21] and miR-21 could be detectable in urine exosome[19] and in plasma[20] of post-KT patients with IF/TA. Here, miR-21, from plasma exosome fraction but not from whole plasma, could differentiate IF/TA grade I out of grade II and III, despite level of the gene expression was not different between stable allograft control group and IFTA grade I. Nevertheless, our data support the fibrosis production through miR-2 and the micro-vesicles transportation of miR-21 into blood circulation.[21] As such, the delivered miR-21 is demonstrated to decrease the Phosphatase and tensin homolog (PTEN) leading to phosphorylation of Protein kinase B (AKT) signaling which decreases the expression E-cadherin and increases the expression of α-SMA and fibronectin in renal tubules.[42] Although we expected to see increased plasma miR-21 due to the impact of miR-21 in both inflammatory process[42] and fibrosis generation[43] as previously reported,[20] plasma miR-21 in IF/TA was not higher than the stable allograft. In contrast, plasma miR-21 in IF/TA II was lower than the control group (**Figure 3D**), while exosome miR-21 in IF/TA II show a tendency to be higher than the control but did not reach the significant level (**Figure 3G**). The inconsistency in the benefit of miR-21 in whole plasma as the IF/TA post-KT biomarker that is previously demonstrated[20] and the less benefit in our data might due to the limited number of patients in our cohort. In addition, the advantage of exosome miR-21 over plasma miR-21 implies that miR-21 in the exosome fraction with, protective exosome-membrane, might be more stable than miR-21 in the fraction of whole plasma. More studies in the larger number of patients are interesting.

***Proposed of the clinical use of exosome miR-21 in plasma for high grade IF/TA detection***

Although exosome miR-21 could not be used for the diagnosis of IF/TA post-KT from our data, the detection of high grade IF/TA by this non-invasive biomarker will be clinically beneficial. The increased exosome miR-21 from the baseline indicates the prompt management to explore several causes of IF/TA such as calcineurin inhibitor toxicity, high urine protein excretion and several renal underlying diseases. Currently, the frequency for IF/TA evaluation is limited by the invasiveness of renal biopsy. The frequent monitoring by plasma exosome will be tremendously helpful. In addition, miR-21 was not correlated with any of the current biomarkers in the clinical practice. Then, the value of miR-21 could not be postulated from the more severe injury by these parameters and miR-21 should be analyzed along with the current biomarkers, not only in the patients with the more severe renal injury. Despite several ongoing-researches for the discovery of post-KT IF/TA biomarker[44] the solid conclusion is still lacking. Therefore, we propose to add the regular monitoring of plasma exosome miR-21 for the early detection of IF/TA grade II/III into the current post-KT clinical follow-up as an indicator for the additional urgent renal biopsy to confirm IF/TA diagnosis. Further clinical study on miR-21, at least in Thai patients, for IF/TA monitoring is warrant.

In conclusion, plasma exosome miR-21 is an interesting non-invasive biomarker of IF/TA grade II/III which is better than the current biomarkers (serum creatinine and proteinuria). The increased exosome miR-21 indicates for urgent renal biopsy to confirm high grade IF/TA and the prompt IF/TA managements.

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## References

1. Boor P, Floege J. Renal allograft fibrosis: biology and therapeutic targets. Am J Transplant. 2015;15(4):863-86.
2. Korbet SM. Nephrology and the Percutaneous Renal Biopsy: A Procedure in Jeopardy of Being Lost Along the Way. Clin J Am Soc Nephro. 2012; 7(10):1545-7.
3. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA -mediated gene silencing. Cell. 2008;132(1):9-14.
4. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 2010;79:351-79.
5. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post -transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9(2):102-14.
6. Kasinath BS, Feliers D, Sataranatarajan K, Ghosh Choudhury G, Lee MJ, Mariappan MM. Regulation of mRNA translation in renal physiology and disease. Am J Physiol Renal Physiol. 2009;297(5):F1153-65.
7. Vasudevan S, Tong YC, Steitz JA. Switching from repression to activation: MicroRNAs can up-regulate translation. Science. 2007;318(5858):1931-4.
8. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol. 2007;179(3):1969-78.
9. Gallo A, Tandon M, Alevizos I, Illei GG. The Majority of MicroRNAs Detectable in Serum and Saliva Is Concentrated in Exosomes. Plos One. 2012;7(3):e30679.
10. Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, et al. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int. 2007;72(9):1095-102.
11. Lawson C, Vicencio JM, Yellon DM, Davidson SM. Microvesicles and exosomes: new players in metabolic and cardiovascular disease. J Endocrinol. 2016;228(2):R57-R71.
12. Li QL, Bu N, Yu YC, Hua W, Xin XY. Exvivo experiments of human ovarian cancer ascites-derived exosomes presented by dendritic cells derived from umbilical cord blood for immunotherapy treatment. Clin Med Oncol. 2008;2:461-7.

13. Qiu SQ, Duan XB, Geng XR, Xie JX, Gao H. Antigen-specific activities of CD8+T cells in the nasal mucosa of patients with nasal allergy. Asian Pac J Allergy. 2012;30(2):107-13.
14. Street JM, Barran PE, Mackay CL, Weidt S, Balmforth C, Walsh TS, et al. Identification and proteomic profiling of exosomes in human cerebrospinal fluid. J Transl Med. 2012;10:1-7.
15. Alexander M, Hu RZ, Runtsch MC, Kagele DA, Mosbruger TL, Tolmachova T, et al. Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. Nat Commun. 2015;6:1-16.
16. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6): 654-9.
17. Koritzinsky EH, Street JM, Chari RR, Glispie DM, Bellomo TR, Aponte AM, et al. Circadian variation in the release of small extracellular vesicles can be normalized by vesicle number or TSG101. Am J Physiol Renal Physiol. 2019;317(5):F1098-110.
18. Street JM, Koritzinsky EH, Glispie DM, Star RA, Yuen PS. Urine Exosomes: An Emerging Trove of Biomarkers. Adv Clin Chem. 2017;78:103-22.
19. Zununi Vahed S, Omidi Y, Ardalan M, Samadi N. Dysregulation of urinary miR-21 and miR-200b associated with interstitial fibrosis and tubular atrophy (IFTA) in renal transplant recipients. Clin Biochem. 2017;50 (1-2):32-9.
20. Glowacki F, Savary G, Gnemmi V, Buob D, Van der Hauwaert C, Lo-Guidice JM, et al. Increased Circulating miR-21 Levels Are Associated with Kidney Fibrosis. Plos One. 2013;8(2):e58014.
21. Dey N, Ghosh-Choudhury N, Kasinath BS, Choudhury GG. TGFbeta -stimulated microRNA-21 utilizes PTEN to orchestrate AKT/mTORC1 signaling for mesangial cell hypertrophy and matrix expansion. Plos One. 2012;7(8):e42316.
22. Ben-Dov IZ, Muthukumar T, Morozov P, Mueller FB, Tuschl T, Suthanthiran M. MicroRNA sequence profiles of human kidney allografts with or without tubulointerstitial fibrosis. Transplantation. 2012;94(11): 1086-94.
23. Scian MJ, Maluf DG, David KG, Archer KJ, Suh JL, Wolen AR, et al. MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. Am J Transplant. 2011;11(10): 2110-22.
24. Zununi Vahed S, Poursadegh Zonouzi A, Ghanbarian H, Ghojazadeh M, Samadi N, Omidi Y, et al. Differential expression of circulating miR-21, miR-142-3p and miR-155 in renal transplant recipients with impaired graft function. Int Urol Nephrol. 2017;49(9):1681-9.
25. Morinaga J, Kadomatsu T, Miyata K, Endo M, Terada K, Tian Z, et al. Angiopoietin-like protein 2 increases renal fibrosis by accelerating transforming growth factor-beta signaling in chronic kidney disease. Kidney Int. 2016;89(2):327-41.
26. Roufosse C, Simmonds N, Clahsen-van Groningen M, Haas M, Henriksen KJ, Horsfield C, et al. A 2018 Reference Guide to the Banff Classification of Renal Allograft Pathology. Transplantation. 2018;102(11):1795-814.
27. Lv CY, Ding WJ, Wang YL, Zhao ZY, Li JH, Chen Y, et al. A PEG-based method for the isolation of urinary exosomes and its application in renal fibrosis diagnostics using cargo miR-29c and miR-21 analysis. Int Urol Nephrol. 2018;50(5):973-82.
28. Rider MA, Hurwitz SN, Meckes DG, Jr. ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles. Sci Rep. 2016;6:1-14.
29. Lu J, Li J, Liu S, Wang T, Ianni A, Bober E, et al. Exosomal tetraspanins mediate cancer metastasis by altering host microenvironment. Oncotarget. 2017;8(37):62803-15.
30. Zachary AA, Leffell MS. HLA Mismatching Strategies for Solid Organ Transplantation - A Balancing Act. Front Immunol. 2016;7:1-14.
31. Rose C, Gill J, Gill JS. Association of Kidney Transplantation with Survival in Patients with Long Dialysis Exposure. Clin J Am Soc Nephro. 2017;12(12):2024-31.
32. de la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M. "Exosomics"-A Review of Biophysics, Biology and Biochemistry of Exosomes With a Focus on Human Breast Milk. Front Genet. 2018;9:1-11.
33. Yu LL, Zhu J, Liu JX, Jiang F, Ni WK, Qu LS, et al. A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples. Biomed Res Int. 2018;2018:1-9.
34. Tang YT, Huang YY, Zheng L, Qin SH, Xu XP, An TX, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. Int J Mol Med. 2017;40(3):834-44.
35. Doyle EL, Ridger V, Ferraro F, Turmaine M, Saftig P, Cutler DF. CD63 is an essential cofactor to leukocyte recruitment by endothelial P-selectin. Blood. 2011;118(15):4265-73.
36. Kahlert C. Liquid Biopsy: Is There an Advantage to Analyzing Circulating Exosomal DNA Compared to cfDNA or Are They the Same? Cancer Res. 2019;79(10):2462-5.
37. Ralib AM, Pickering JW, Shaw GM, Devarajan P, Edelstein CL, Bonventre JV, et al. Test characteristics of urinary biomarkers depend on quantitation method in acute kidney injury. J Am Soc Nephrol. 2012;23(2):322-33.
38. Endzelins E, Berger A, Melne V, Bajo-Santos C, Sobolevska K, Abols A, et al. Detection of circulating miRNAs: comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. Bmc Cancer. 2017;17:1-13.
39. Moldovan L, Batte K, Wang Y, Wisler J, Piper M. Analyzing the circulating microRNAs in exosomes/extracellular vesicles from serum or plasma by qRT-PCR. Methods Mol Biol. 2013;1024:129-45.
40. Glowacki F, Savary G, Gnemmi V, Buob D, Van der Hauwaert C, Lo-Guidice JM, et al. Increased circulating miR-21 levels are associated with kidney fibrosis. PLoS One. 2013;8(2):e58014.
41. van den Akker EK, Dor FJ, JN IJ, de Bruin RW. MicroRNAs in Kidney Transplantation: Living up to Their Expectations? J Transplant. 2015;2015: 1-10.
42. Zhou Y, Xiong MX, Fang L, Jiang L, Wen P, Dai CS, et al. miR-21 -Containing Microvesicles from Injured Tubular Epithelial Cells Promote Tubular Phenotype Transition by Targeting PTEN Protein. Am J Pathol. 2013;183(4):1183-96.
43. Huang Y, He Y, Li J. MicroRNA-21: A Central Regulator of Fibrotic Diseases Via Various Targets. Curr Pharm Design. 2015;21(17):2236-42.
44. Salvadori M, Tsalouchos A. Biomarkers in renal transplantation: An updated review. World J Transplant. 2017;7(3):161-78.