Mastering non-invasive predictive biomarkers to follow up renal transplant patients

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ABSTRACT

Renal transplantation is the treatment of choice for end-stage renal disease (ESRD) patients. Several cellular processes are regulated via non-coding RNAs by silencing target gene expression. Previous investigations have established a linkage between a number of human microRNAs and kidney failure. This study aims to identify the expression of urinary miR-199a-3p and miR-155-5p as non-invasive biomarkers during post and pre-transplantation over a six-month follow-up period. In addition to the classic chronic renal disease markers (estimated glomerular filtration rate eGFR, Serum creatinine, serum electrolytes, and Antinuclear antibodies ANA test). Urinary miR-199a-3p and miR-155-5p expression levels in 72 adults with diabetic nephropathy and, 42 adults with lupus nephropathy renal transplant recipients. Both were compared with 32 healthy controls prior and post-transplantation. miRNAs were evaluated by quantitative reverse transcription-polymerase chain reaction. Urinary miR-199a-3p significantly (p<0.0001) downregulated in diabetic and lupus nephropathy prior to transplantation and significantly upregulated post-transplantation compared to the control. While urinary miR-155-5p quantities were significantly higher in prior renal transplant patients in comparison with the same patients’ post-renal transplantation (p<0.0001). In conclusion, Urinary miR-199a-3p and miR-155-5p can be used as a non-invasive biomarker with high specificity and sensitivity to follow up the renal transplant patients before and post-transplantation instead of biopsy which is complicated by a non-negligible factor.

Introduction

The main surgical indication of end-stage renal disease (ESRD) (defined as reducing the estimated glomerular filtration rate (eGFR) to 20–25% over six months or increasing serum creatinine) is kidney transplantation to prolong and improve the patient’s life (1). Several conditions damage the kidney and contribute to the progression of disease that may lead to renal failure. Diabetes is the leading cause of renal disease, followed by vascular disease (specifically hypertension) (2). Glomerulonephritis, polycystic kidney disease, pyelonephritis, and systemic lupus erythematosus are additional conditions that cause chronic kidney disease (CKD). The classical method to assess the etiology and severity of kidney graft damage is a renal biopsy examination, which has an approximately 3% complication risk (3). Otherwise, for biopsies in individuals with acute renal failure and higher creatinine, the risk of bleeding is predicted to rise (4). Moreover, the use of biomarkers for early disease detection and follow-ups complicates the ability to provide diagnoses on time. Invasive biopsies, which are used to check rejection, are convenient, albeit hazardous, for renal transplant recipients. To detect the development of disease earlier, assessing the unique and widely obtainable biomarkers of a renal transplant patient’s follow-up might make it possible to discover new, potential target treatments. Recently, gene expression profiling has played a critical role in medicinal selection for many diseases. MicroRNAs (miRNAs) are short non-coding RNA (20–24 nucleotides that are synthesized from eukaryotic genomes, which control the expression gene by blocking protein formation from mRNAs translation (5) via partial sequence binding similarity to the 3´ untranslated region (3´UTR) of mammalian target mRNAs (6). The impact of miRNAs in maintaining the homeostasis of the human body is demonstrated in the fact that any difference in their expression causes pathological consequences. miRNAs, unlike mRNA, are stable in several fluids in the body. After being released into circulation, they are bound to proteins or surrounded by microvesicles, thereby protecting them with ribonuclease from degradation (7).

It is thought that miRNAs are a part of the regulation of a variety of essential pathways, such as apoptosis and cell proliferation (8), regulation of fat metabolism (miR-14) (9), acute kidney injury (AKI) (10), and others. In addition, they have also been linked to disorders including autoimmune and neurodegenerative diseases, as well as cancer (11), and have been seen to have a role in the maturation, function and growth of the cells of the adaptive immune system in lupus nephritis (LN) (12). miRNA acts as a key factor in kidney development, maintenance of kidney function and development of renal failure (13). Several studies have mentioned that miR-886, miR-194, miR-192, miR-146a, miR-204, miR-215, and miR-216 all

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have a role related to the kidneys. In fact, the dysregulation of some miRNAs (e.g., miR-30 and miR-200) leads to faults in the cell proliferation rate in kidney terminal differentiation, as well as deviations in pattern and retarded terminal differentiation of kidney tubules. A bioinformatics study of miRNAs’ target genes demonstrated that they were enhanced in the pathway to cell proliferation and apoptosis. Furthermore, studies found that the Mammalian target of rapamycin mTOR was the miR-199a-3p target gene; the mTOR 3'-UTR position 129-135 demonstrated complementary pairing with miR-199a-3p (14), and miR-199a-3p mimics were found to suppress mTOR expression. The mammalian target of rapamycin, mTOR—a serine/threonine-protein kinase—is a pathway that regulates several mechanisms, like proliferation, survival, cell growth and metabolism (15). mTOR maintains homeostasis of the renal tubular, as has been reported by several studies (16-18).

Nowadays, studies have focused on some of the conserved miRNA families, such as those that involve miR-199. Non-coding miR-199 (199a and 199b), especially 199a, performs a crucial role in assisting the maintenance of proper homeostasis and disease pathogenesis regulation. Presently, a considerable number of studies have suggested that normal cell activation plays a role in the diverse physiological or pathological processes by regulation of two mature types of miR-199. For example, miR-199a-3p expression is raised in gastric malignancies by promoting cell proliferation (19), while it is relatively downregulated in breast cancer (20) (21) and is extremely low in bladder cancer (22). Additionally, miR-199a-5p lowers the amount of apoptosis that is detectable compared to miR-199a-3p in cancer cells, such as PC3, A549, KB and MCF7 (23). miRNA 199a plays a pivotal role in autophagy, which involves the accumulation of damaged organelles or protein aggregates (24). In addition, multiple studies have demonstrated that the miR-199a dysregulation is seen in a variety of malignancies, particularly ovarian cancer (25), hepatocellular carcinoma (26), renal cell carcinoma (27), osteosarcoma (28) and others (29) (30). Different target genes are manipulated with miR-199a-3p, such as CD44 (31), caveolin-2 (32) and e-Met (23). miR-199a-3p exogenous expression reduced the proliferation and survival of cancer cells (33).

Among other miRNAs that play a vital role in the initiation and advancement of malignancies is miR-155-5p (34). Urine and serum miR-155-5p are used as a predictor of significance for diabetic nephropathy (DN) (35). miR-155 expression in DN patients increased due to the contribution of inflammation-mediated glomerular injury (36). Zhang et al in 2020 study found that miR-155-5p improves the survival of DN mice through SIRT1 as a targeted regulator. Moreover, miR-155-5p stimulates renal fibrosis by activation of STAT3 via targeting SOCS116 (37).

However, there are relatively few studies on the clinical significance of urinary miR-199a-3p and miR-155-5p in kidney transplant patients before and after transplantation. The object of this study is to quantify urinary miR-199a-3p and miR-155-5p expression as possible non-invasive biomarkers for follow-up renal transplant recipients instead of biopsies. Furthermore, the participants’ urine samples were acquired.

**Materials and Methods**

Two patient groups were created in this study, compared with 32 healthy controls. as in Fig. 1.

In Group I, we estimated urine miR-199a-3p and miR-155-5p in 72 DN patients’ prior renal transplantation over a 6-month follow-up period when compared with the 32 healthy control participants. In Group II, we analysed the expression of selected miRNAs in 42 LN patients prior and post-transplantation then compared them with the same control participants. All participants were patient volunteers recruited at the Renal Transplant Centre Hospital, Sulaymaniyah, Iraq, from October 2020 until January 2022. Each transplant center assessed its guidelines for deciding if the candidate required a kidney transplant. Depending on our previous work with the centre, in our study, each patient had an overall 6 visits, with 15 days after 1, 2, 3, 4, and 6 months after kidney transplantation.

At each visit, safety parameters were assessed. Patient samples were received from healthy volunteers aged 18–56 years. The Medical Ethical Committee of Sulaimani University approved the study. A detailed medical history with a description of the age of onset of renal injury from diabetes and lupus was collected for each individual’s evaluation. The clinical trials and laboratory data for all participants that were gathered: eGFR with ESRD follow-up, age, sex, serum creatinine, blood sugar, diastolic blood pressure, cytosolic blood pressure, urine pH, urine specific gravity, and Na+ and K+ concentrations. Calcium), ANA and PCR tests were taken into consideration in the assessment of the LN group.

**Urine samples**

Samples of urine were collected in a 15 ml universal RNase-free tube from DN and LN patients at ESRD prior to renal transplant and at each one of the six post-transplantation visits. They were transported in an ice bath to the molecular lab, then centrifuged at 2000 g for 10 minutes at 4 °C to remove debris. For RNA extraction, the supernatant was split into 400 microlitre aliquots. Until the molecular lab, then centrifuged at 2000 g for 10 minutes at 4 °C to remove debris. For RNA extraction, the supernatant was split into 400 microlitre aliquots. Until the molecular lab, then centrifuged at 2000 g for 10 minutes at 4 °C to remove debris. For RNA extraction, the supernatant was split into 400 microlitre aliquots.

**Experimental methods**

**RNA extraction**

miR-199a-3p and miR-155-5p from urine were ex-
tracted using the miRNeasy Kit (Qiagen, cat. no. 217184, GmbH, Germany). The thawed frozen samples were centrifuged at 2000 g for 2 min at 4 °C. 1 ml lysis solution (TRizol) was added to the 200 microlitre urine specimens together with 3.5 microl (1.6 × 108 copies/microlitre) of a spike in lyophilized C. elegans miR-39 miRNA mimic as a normalizing internal control. The materials were combined in a tube, then 200 l of chloroform was added after vigorous mixing, which was then centrifuged at 12000 g for 15 min at 4 °C. The manufacturer’s technique was followed at that point, with each sample’s whole aqueous phase placed into a single affinity column. The cleaned RNA was then eluted from the membrane with nuclease-free water into a collection tube and refrigerated at −80 °C at 260 nm and 280 nm (A260/A280). The concentration and amount of total RNA were determined with a NanoDrop (Scientific Fischer).

Reverse transcriptase-polymerase chain reaction (qRT-PCR)

Urinary miR-199a-3p and miR-155-5p expression were determined by qRT-PCR using the MystiqMT microRNA cDNA synthesis Mix Kit (SIGMA-ALDRICH, Cat no. MIRRT). 3 µl of sample extracted RNA was taken, then cDNA reverse transcription was completed following the kit’s instructions using 2 µl poly-A tailing buffer (5X), 1 µl poly-A polymerase, 4 µl of nuclease-free water. With a poly-A polymerase reaction, the urinary miRNAs were first polyadenylated with Ready Script reverse transcriptase and other required materials for the synthesis of cDNA by using an oligo-dT adapter primer which was later added to change the poly-A tailed miRNAs into the first-strand cDNA. The following were the reaction variables: 20 min at 42 °C, 5 min at 85 °C. The cDNA was stored long-term at −20 °C. Afterward, urinary miR-199a-3p and miR-155-5p were amplified by qRT-PCR (Qiagen/Corbett Rotor-Gene6000, Germany), using the SYBR Green (SIGMA-ALDRICH, Cat. No. MIRRMO3), which is used frequently for this reason. has-miR-199a-3p and has-miR-155-5p primers were purchased from SIGMA-ALDRICH, cat. no. MIRAP00244 and MIRAP00202, respectively, compiled with the instructions of the manufacturer. qRT-PCR was used with a maximum of 40 cycles and the number of cycles at which the amplification curve passed the threshold (Ct) was calculated. miRNA expressions were normalised with the endogenous internal control C. elegans miR-39 miRNA mimic. The data were analysed and the differences in expression amounts for every target among samples were determined using the ∆∆Ct method (38).

Experimental Data Processing

In the present study, the Prism6 software was used as the statistical data analysis program. The parameters were recorded as mean ± standard deviation. The p-value was used to evaluate the variations between the two groups; p values <0.05 were considered significant. One-way ANOVA was used to compare quantitative parametric variables among more than two groups. Origin Pro 2020 was used to draw the experimental receiver operating characteristics (ROC), to calculate the area under the curve (AUC) for urinary miR-199a-3p and miR-155-5p, and to find the best cut-off values.

Results and Discussion

This study suggests that urinary miRNA expression levels could produce valuable diagnostic biomarkers for follow-up in patients who were subjected to renal transplants. With high specificity and sensitivity, we discovered that the urine expression of miR-199a-3p and miR-155-5p was a statistically significant biomarker in this study before and after renal transplantation throughout a six-month follow-up period. Reliable, accurate, economical, yet non-invasive biomarkers that can estimate the effectiveness of a renal transplant will be necessary for the development of tailored treatment plans.

As a preliminary stage in our investigation, in the DN and LN cohorts, there was a significant variation in serum creatinine levels, eGFR, systolic blood pressure, diastolic blood pressure, urine pH, specific gravity and Na+ prior to and post-transplantation with P<0.0001. Unlike the LN Group, K+ electrolytes showed no significant difference in DN. CRP elevated pre-transplantation and then declined post-transplantation in LN patients. The clinical characteristics and some biochemical parameters of all patients and control groups are illustrated in Fig. 2 for both groups. Table 1-S and Table 2-S.

From this investigation, we established that there was a significant expression of urinary miR-199a-3p and miR-155-5p before and after renal transplant. The qRT-PCR technique was used to measure the urinary miRNA expression levels. Urinary miR-199a-3p expression levels in DN and LN patients were significantly lower in pre-transplant than in the control group, whereas miR-155-5p presented the opposite trend. Levels of urinary miR-199a-3p expression, represented as the fold change (FC) in prior transplant DN (2.16±0.76), were significantly lower than those in the first 3 months of post-transplantation (3.45±0.43) and reached an approximately normal range in the second 3 months (4.12±0.55) when compared with the control healthy group’s CTR (4.08±0.50).

Likewise, in LN patients, miR-199a-3p expression downregulated in prior transplantation (2.15±0.70) when compared to the same patients after the first 3 months and
second 3 months–3.62±0.46 and 5.32±0.46, respectively—with a p-value <0.0001, as seen in Fig. 3.

The urinary expression levels of miR-155-5p in DN and LN renal transplant patients. miR-155-5p expression was significantly higher in DN and LN pre-transplant, 6.02±1.19 and 7.19±1.29, respectively when compared with the healthy control, 4.08±0.50, p value<0.0001 Fig (4). The urine levels of miR-155-5p returned over the 6 months follow-up period (3.16±0.44 and 4.06±0.50) in the first and second 3 months post-transplantation (Table 3-S).

Urinary miR-199a-3p and miR-155-5p separately showed a moderate power (sensitivity and specificity) by ROC curve analysis for distinguishing the value for early progress of the patients after renal transplantation. For miR-199a-3p DN patients, various areas under the curve AUC were observed (AUC=0.90, 0.83 and 0.53) during pre- and post-transplantation, while in LN patients the AUC=0.95, 0.72 and 0.96. Urinary miR-155-5p showed a lower value of AUC (AUC=0.89, 0.92 and 0.95) in DN patients when compared with LN patients (0.96, 0.92 and 0.92), as seen in Fig 5 and Fig 6. Table 4-S.

The above data imply that urinary miR-199a-3p and miR-155-5p profiles may serve as practical noninvasive biomarkers for the follow-up of renal transplant recipient patients. We believe this project is the first to sequentially track the urinary miR-199a-3p and miR-155-5p expression of a profile of miRNAs in renal transplant recipients before transplantation and for more than six months after transplantation. Recent researches in the field of urine microRNAs (39-42) have demonstrated their potential as a biomarker for renal impairment. Urinary miRNAs varied as glomerular sclerosis progressed in renal illness, depending on the kind of renal disease (43). With increasing age, renal growth is expected to slow as it approaches the fifth decade of life, a state called kidney senescence. To date, there are a number of miRNAs that are identified as renal miRNAs, such as miR-146a, miR-886, miR-192, miR-194, miR-204, miR-215, miR-216, miR-let-7ag, miR-196a/b, miR-10a/b, miR-130, miR-143, miR-21, miR-200a, miR-30a-e and miR-872 (44) (45) (46). MiRNAs with differential expression after renal transplantation have been found in many investigations, demonstrating their potential as diagnostic biomarkers (47) (48) (49) (50). Among them, urinary miR-155 has been linked to renal function and hypoxia/ischemia disorders. Jonathan et al found that miR199a-3p in combination with the other eight miRNAs has a prognostic value for delayed graft function after kidney transplantation (51).
miR-199a-3p targets the MET proto-oncogene and ERK-2, thereby inhibiting proliferation and apoptosis that play important role in controlling renal function (52). Additionally, miR-199a targets the AKT/mTOR signalling pathway (53). mTOR signalling promotes podocyte and tubular cell homeostasis in physiologic situations. Furthermore, many investigations have shown that mTOR is a miR-199a-3p target gene and that miR-199a-3p mimics can suppress mTOR expression, resulting in lower stimulation of its downstream proteins 4EBP1 and p70s6k. AKT is a member of the AGC family of threonine-serine kinases, which are involved in cell proliferation, development and protein translation (54). Furthermore, according to several studies, urine miR-155 expression levels in nephrolithiasis are much greater than in healthy people, and there is a negative association between urinary miRNA levels and IL-1, IL-6 and TNF-expression (55, 56).

On the other hand, Olga et al showed that regularly monitoring urine miR-155p and CXCL10 in the early post-transplantation period can aid in predicting the probability of AR and graft dysfunction (57). The current results combined with the obvious data on urine analysis of miRNAs suggest that the kidneys are involved in the physiological of urinary miRNAs expression. Our study investigated the urinary levels of miR-155-5p and miR-199a-3p in DN patients with renal failure disease prior to and post renal transplant. First, we noticed that miR-199a-3p expression was significantly lower in post-transplantation patients than in pre-transplantation patients, while miR-155-5p levels showed high expression before transplant when compared with post-transplantation.

Evaluation of the importance of urine samples from kidney transplant recipients as a non-invasive technique to assess the outcome of the transplantation process by using an mRNA profile instead of a biopsy is a significant component of this study. In addition, and to the higher of our knowledge, here we discuss two significant cases (DN and LN) which lead to end-stage renal disease, prior to transplantation and then follow them post-transplantation. Finally, we can expect use of the urinary miR-199a-3p and miR-155-5p miRNAs as therapy for renal transplant patients in the future.

There is still much to learn and understand about miRNAs themselves and their interactions with their target genes. It is required to create more reliable techniques, enhance their repeatability and specify the potential miRNAs in the context of prospective, randomized, multicenter clinical studies that enable the classification of transplant patients according to a particular clinical occurrence, such as DN and LN. However, to prevent differences across patients some methodologically important issues in the detection of miRNAs need to be resolved. Our analysis is merely a single-center study, so it has to be verified by additional centers. Furthermore, there are several restrictions on our study. The extract sizes and limited patients are modest; in fact, this is a drawback shared by all published investigations of this kind of biomarker up to now. Our results should be confirmed by additional validation studies. In light of these limitations, this large prospective research suggests that consecutive pre- and post-transplantation monitoring of the urine expression of miR-199a-3p and miR-155-5p may very well give important prognostic biomarkers for renal transplant patient follow-up.

To summarise, urinary miR-199a-3p and miR-155-5p were expressed in different ways during a renal transplant when compared with the healthy control when monitoring the renal graft. It can be regarded as a non-invasive biomarker for clinical diagnosis without any morbidity to the patients. On the other hand, they can be used to distinguish between DN and LN in renal transplant patients.

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Authors’ Contribution
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