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MiR-423-5p as Optimal Endogenous Control for Quantification of Circulating MicroRNAs in Patients With CKD

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Received 12 June 2023; revised 20 July 2023; accepted 24 July 2023; published online 11 August 2023

Kidney Int Rep (2023) **8,** 2150–2152; https://doi.org/10.1016/j.ekir.2023.07.018
KEYWORDS: CKD; microRNA; normalization; qRT-PCR; reference genes

INTRODUCTION

Liquid biopsy has gained interest in recent years as a valuable, noninvasive source of biomarkers for both diagnostic and prognostic purposes for a wide range of diseases. MicroRNAs (miRNAs) are small, nonprotein coding RNAs that regulate gene expression by targeted binding to the 3′-untranslated regions of messenger RNA, thereby inducing degradation and translational repression.[1] They are abundant and highly stable in biological fluids and are promising candidates for disease biomarkers. Quantitative real-time polymerase chain reaction (PCR), the most commonly used technique to assess circulating miRNA levels, requires a normalization step involving uniformly expressed endogenous miRNAs. Small, nucleolar RNAs (SNORD44 [RNU44] and SNORD48 [RNU48]) have been used as reference miRNAs for quantification in cell and tissue samples[2]; however, these are not suitable for normalizing circulating miRNAs due to their variability of expression in both plasma and serum. The introduction of an exogenous miRNA as spike-in control (e.g., cel-mir-39) helps control technical biases related to sample preparation, including reverse transcription and/or PCR conditions, without adjusting for variation in the quantity of biological material and other technical variables. At this point, there is no consensus on standard reference serum miRNAs for quantitative real-time PCR normalization in patients with chronic kidney disease (CKD). Therefore, our study's aim was to identify a panel of reliable miRNAs suitable for this clinical context.

RESULTS

First, we retrieved a publicly available miRNA data set investigating serum miRNA profiles in patients with autosomal dominant polycystic kidney disease from Gene Expression Omnibus Repository (GSE101811) for analysis.[3] This study included 10 healthy controls, 10 nondialysis-dependent and 10 dialysis-dependent patients with autosomal dominant polycystic kidney disease. A total of 752 miRNAs plus control assays were quantified using the miRCURY LNA ExiqonRT-PCR human miRNA arrays I and II (Exiqon, Copenhagen, Denmark). There were 54 miRNAs detected in ≥90% of every group and in ≥90% of all samples. These were ranked according to their expression stability across healthy controls and CKD patients (Supplementary Methods). The top 8 miRNAs were miR-23b-3p, miR-425-5p, miR27b-3p, miR-23a-3p, let-7b-5p, miR-151a-3p, miR-223-3p, and miR-92a-3p (Figure 1a); the first 3 of which are well known for their oncogenic and tumor suppressive roles in cancer progression.[4–6] The 7 most stable and highly expressed miRNAs were selected (miR-151a-3p was excluded due to a higher mean Ct value) for validation using quantitative real-time PCR. Both miR-26a-5p and miR-423-5p were also included because both are recommended by the manufacturer as one of the miRNA normalizers for plasma and serum samples. The abundance stability of these 9 miRNAs was assessed in the sera of healthy controls and other CKD patients.

Serum samples were collected from 46 individuals and were divided into 3 groups based on the estimated glomerular filtration rate as follows: ≥60 ml/min per 1.73 m^2, 15 to 59 ml/min per 1.73 m^2 and dialysis-dependent

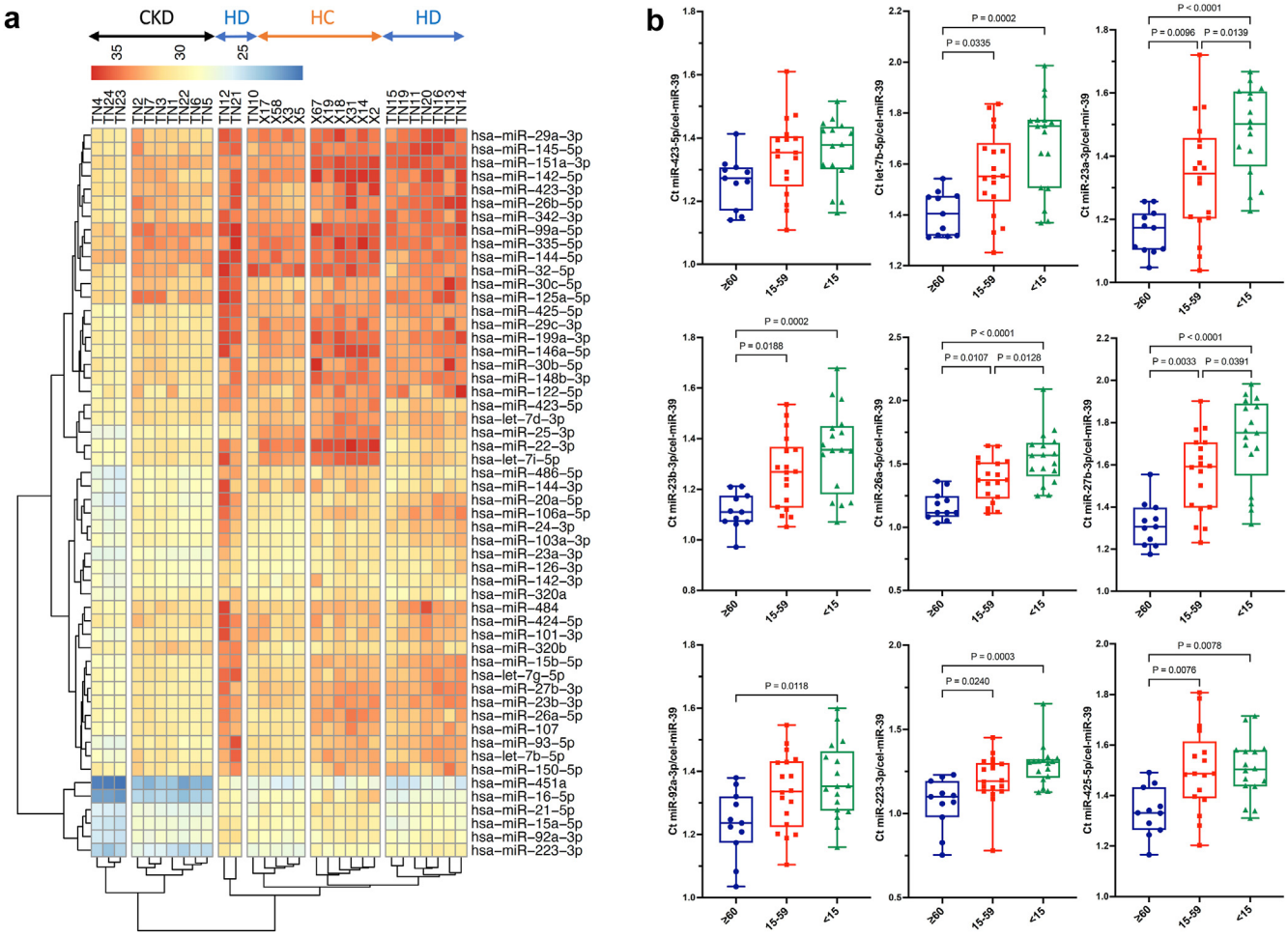

Figure 1. (a) Heatmap of Ct values for 54 serum miRNAs across healthy controls, nondialysis-dependent and dialysis-dependent patients with autosomal dominant polycystic kidney disease (Gene Expression Omnibus Repository, GSE101811); (b) Differential expression (normalized Ct values) of candidate reference miRNAs in sera from healthy controls and patients with chronic kidney disease.

(Table 1). The primary etiologies of CKD include diabetes, hypertension, cardiorenal syndrome, obstructive uropathy, reflux nephropathy, cystic kidney diseases, glomerulonephritis (including IgA nephropathy, antineutrophil cytoplasmic antibody-associated vasculitis, and focal and segmental glomerulosclerosis), and unknown. All samples showed good reverse transcription efficiency as measured by exogenous cel-miR-39 amplification. The differential expression of the candidate reference miRNAs was evaluated (Figure 1b) and, interestingly, all but miR-423-5p showed significant differences in expression among the 3 groups with consistently higher normalized Ct values in the nondialysis and dialysis-dependent groups. In contrast, no significant between-group differences in expression were detected in miR-423-5p, suggesting that it is stably expressed in healthy controls, patients with CKD, and dialysis-dependent patients. The results were similar when we repeated the analysis based on 4 estimated glomerular filtration rate groups: ≥60 ml/min per 1.73 m^2, 30 to 59 ml/min per 1.73 m^2, 15 to 29 ml/min per 1.73 m^2 and dialysis-dependent (Supplementary Figure S1).

Table 1. Clinical characteristics of the 3 analyzed groups

Parameter	eGFR ≥60 ml/min per 1.73 m^2 (n = 11)	eGFR 15–59 ml/min per 1.73 m^2 (n = 18)	eGFR <15 ml/min per 1.73 m^2 (n = 17)
Age (mean, SD)	64.3 ± 8.7	72.1 ± 11.5	64.2 ± 14.2
Sex (male, %)	5 (45.5)	13 (72.2)	11 (64.7)
Mean eGFR (ml/min per 1.73 m^2, SD)	71.8 ± 8.3	31.8 ± 12.6	7.4 ± 3.1
BMI (kg/m^2, SD)	29.0 ± 9.5	27.1 ± 3.6	25.1 ± 5.1
ACEi/ARB use (yes, %)	8 (72.7)	14 (77.8)	6 (35.3)
Duration of dialysis (yrs, SD)	NA	NA	2.7 ± 3.2

ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; eGFR, estimated glomerular filtration rate; NA, not applicable.

DISCUSSION

In the present work, we focused on both healthy controls and patients with CKD with a broad range of kidney disorders representing those most commonly encountered in clinical practice. We investigated the suitability

of a panel of miRNAs for serum miRNA expression normalization. Despite the abundance of studies on circulating miRNAs, publicly available datasets in CKD patients are scarce. We found a sizable sample set that suited our exploration analysis, which enabled us to define a panel of miRNAs that are highly stable and abundant in both healthy controls and patients with autosomal dominant polycystic kidney disease. We also investigated miR-26a-5p and miR-423-5p, which are considered stable in plasma or serum both in other studies and by the manufacturer.[7,8] They were ranked 11th and 26th, respectively among the most stable miRNAs from our initial analysis.

Notably, we found that all miRNA candidates except miR-423-5p were significantly different in our samples with lower expression levels across the board in non-dialysis and dialysis-dependent CKD patients. The possible explanations for the observed findings include a reduced concentration of circulating miRNAs in CKD on the whole compared to other disease states,[9] removal of circulating miRNAs by dialysis treatment, and/ or the presence of PCR inhibitors in the CKD serum. The latter was considered less likely given the absence of technical issues with the spike in control and we therefore effectively excluded PCR inhibition for endogenous miRNAs. Previous studies reported contradictory results regarding elimination of circulating miRNA during hemodialysis treatment.[S1,S2] Overall, our data suggest that miR-423-5p does appear to be a suitable reference miRNA for expression normalization across serum samples from both healthy controls and patients with CKD. In turn, and in its role as a reference miRNA, it may help improve the accuracy and validity of results in any given set of experiments.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENT

Funding

LW is supported by the 2021 and 2022 Royal Australasian College of Physicians Jacquot Research Establishment Fellowship and the APH Philanthropic Foundation Research and Innovation Grant.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary Reference.

Figure S1. Differential expression (normalized Ct values) of candidate reference miRNAs in sera from healthy controls and CKD patients.

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