Urinary MicroRNAs, Possible Biomarkers for Early Detection of Patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD)

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Introduction. Autosomal dominant polycystic kidney disease (ADPKD) is a prevalent renal disorder that causes abnormal growth of renal epithelial cells. The excessive expansion of renal epithelial cells can lead to cyst formation that is associated with serious renal complications. The early diagnosis of ADPKD makes the control of the disease somehow attainable. Regarding the diagnostic potential of microRNAs (miRs) as robust clinical biomarkers, the present study aimed to examine the potential of urinary miRs in early diagnosis of ADPKD in asymptomatic patients.
Methods. Urine samples were obtained from 20 asymptomatic ADPKD patients and 20 healthy control individuals and the miR content of the samples was extracted and converted to cDNA for the qRT-PCR experiment. The relative expressions of miR-17, miR-21, miR-143, and miR-223 were evaluated in ADPKD cases and healthy individuals. Serum levels of kidney function markers were also evaluated in the study participants.
Results. The urine samples of patients with ADPKD demonstrated higher levels of miR-17, miR-21, and miR-143 along with a lower miR-223 level compared to the healthy control group.
Conclusion. This study revealed the differential expression of the studied miRs in ADPKD patients. Detection of miRs in urinary samples might provide a useful platform for early diagnosis of ADPKD in asymptomatic patients.

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INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the fourth most prevalent inherited kidney disease affecting approximately 1/400 to 1/1000 of the population worldwide. Mutations in *PKD1* and *PKD2* genes are responsible for 85 and 15 percent of ADPKD cases, respectively. Moreover, mutations in *GANAB* account for approximately 0.3% of total ADPKD cases.[1,2] The common complications of ADPKD encompass pain, high blood pressure, and even kidney failure. The ADPKD-associated pain is usually due to cyst growth, cyst infection, bleeding, and kidney stones. Almost all ADPKD cases develop high blood pressure in the future and subsequent follow-up is necessary. Besides, hepatic, and pancreatic cysts in addition to vascular complications, and brain aneurism are among the co-morbidities of ADPKD. There are also implications regarding ADPKD-associated reproductive problems in men and

women. It has been shown that early diagnosis of ADPKD helps in better management of the disease and permits the therapeutic approaches to prevent the development of organ-failure complications. Hence, the early detection of ADPKD is critical to prevent the establishment of renal failure.

ADPKD is clinically diagnosed primarily through physical examinations and a review of the family history.[3] Due to a series of overlapping symptoms with other clinical conditions, diagnostic imaging or genetic tests are mandatory to confirm ADPKD. The molecular approaches including the amplification-based assays, probe-based tests, and sequencing platforms could be employed to detect the mutations in *PKD1* and *PKD2* genes.[4]

MicroRNAs (miRs) are regulatory and non-coding RNAs that modulate cellular functions by exerting regulatory effects commonly on the expressions of distinct genes. Although miRs are intracellular molecules, they could be leaked out into extracellular fluids during a myriad of cellular events. Accordingly, their existence in body fluids and clinical samples such as blood, serum, and urine has been widely verified. Body fluid miRs are protected from extracellular nucleases within a miR ribonucleoprotein complex.[5] Since the body fluid miRs originate from the tissue cells, they could reflect the status of the cells they have originated from to some degree. So that, the diagnostic and prognostic values of some miRs have been demonstrated in a series of clinical conditions.

Urine is a feasibly accessible clinical sample that contains various miRs especially those that originated from the urinary tract cells such as various renal epithelial and endothelial cells.[66] There are examples of urinary miRs that are associated with the progression of ADPKD. For example, the elevated levels of miR-17-92 cluster and miR-21 are reported to be associated with renal cyst formation in a murine model of ADPKD.[7,8] Besides, dysregulated urinary miRs including miR-1, miR-133, miR-223, and miR-199 have been suggested to be associated with ADPKD.[9] Other miRs such as miR-143 have been shown to regulate the proliferation of renal epithelial cells.[10]

Regarding the notable potential of miRs in the early diagnosis of various clinical conditions, the present study aimed to evaluate the possible efficacy of miR-17, miR-21, miR-143, and miR-223 in detection of the asymptomatic ADPKD patients.

MATERIALS AND METHODS

This study was conducted under ethical approval from the local ethics committee at Tabriz University of Medical Sciences (registration code: IR.TBZMED.REC.1396.1278). Written informed consent was obtained from all the study participants. The recently diagnosed ADPKD cases were confirmed by an experienced nephrologist according to familial history and the confirmed kidney cysts in the ultrasonographic examination. A total of 20 ADPKD cases and 20 healthy control individuals were included in the study.

Inclusion criteria for ADPKD patients were the diagnosis of the disease within the recent three months, normal kidney function, and normal urine analysis. Exclusion criteria for the AKPKD group were BMI above 30, kidney stones, acute kidney injury, proteinuria, bacteriuria, hematuria, history of malignancies, diabetes mellitus, autoimmune diseases, and consumption of anti-inflammatory medicines during the last three months.

The control group was age-matched to the ADPKD group with the inclusion of normal kidney function and normal urine analysis results. Exclusion criteria for the control group were a BMI above 30, a history of inflammatory and autoimmune diseases, any history of malignancies, other kidney diseases, diabetes mellitus, and use of non-steroidal anti-inflammatory drugs (NSAIDs), or steroid medications.

A total of 5 mL of the urine sample and 5 mL of peripheral blood sample were collected from each study participant. The collected urine samples were centrifuged at 400×*g* for 5 minutes to remove any debris before miR extraction. The miR content of the urine supernatants was extracted by the miRCURY biofluids miR extraction kit (Exiqon, Denmark). The quantity of the extracted miRs was measured by a Nanodrop instrument (Thermo Fisher, USA). Specific stem-loop reverse transcription primers were exploited for specific cDNA synthesis of the miRs using a ReverAid kit (Thermo Fisher, USA). The cDNA samples were amplified using specific primers for miR-17, miR-21, miR-143, and miR-223 by a RotorGene qPCR thermal cycler (Qiagen) and SYBR green master mix (Takara, Japan). The relative expressions of miRs were normalized to the expression of U6 small nuclear RNA using the Livak ($2^{-\Delta\Delta CT}$) method. The sequences of the exploited primers are represented in Table 1.

Table 1. Sequences of the Primers Exploited in the qRT-PCR Experiment

Primer		Sequence (5' > 3')
miR-17	F:	GCCGGCGTCAGAATAATGTCAAAGTGC
	R:	CACCATAATGCTACAAGTGCCTTCACTGC
miR-21	F:	AAAGGATCCGCCATAGAAACCCAGTTTC
	R:	GTGCAGGGTCCGAGGT
miR-143	F:	ACAGACCGGTACAAGTGCAGA
	R:	GGTCGGCATACAGCTAATACA
miR-223	F:	ATGGTTCGTGGGTGTCAGTTTGTCAAAT
	R:	GCAGGGTCCGAGGTATTC
U6 snRNA	F:	GCTTCGGCAGCACATATACTAAAAT
	R:	CGCTTCACGAATTTGCGTGTCAT

Abbreviations: F, forward; R, reverse.

To evaluate kidney function-related markers in the blood, the clotted peripheral blood samples were centrifuged at 300 × *g* for 10 minutes for serum separation. The serum levels of creatinine, BUN, and uric acid were measured using a BT3000 analyzer (Biotecnica).

Statistical Analysis

The normality of the obtained data was first analyzed by the Kolmogorov-Smirnov test, then the data was analyzed by Student's *t*-test. Data are represented as mean ± SD. Statistical analysis was carried out by Graph Pad Prism software version 6. *P* values below .05 were considered statistically significant.

RESULTS

The mean age of ADPKD patients was 37.9 ± 9.49 and for controls was 36.8 ± 10.57. There was no significant difference regarding age, gender, and BMI between the studied groups ($P > .05$; Table 2).

Table 2. Demographic Characteristics of the Study Participants Data are represented as mean ± SD.

Characteristics	ADPKD Group	Control Group	*P*
Age, y	37.9 ± 9.49	36.8 ± 10.57	.89
Sex (Male / Female)	1.6 ± 0.50	1.6 ± 0.50	.99
BMI, Kg/m^2	26.26 ± 3.24	27.47 ± 3.39	.67
Creatinine (mg/dL)	1.051 ± 0.09	1.041 ± 0.08	.89
Blood Urea, mg/dL	32 ± 3.58	31.75 ± 3.30	.93
Uric Acid, mg/dL	5.2 ± 0.51	4.995 ± 0.40	.62

Kidney Ultrasonography

An abdominal and pelvic ultrasonographic examination revealed enlarged kidneys with multiple fluid-filled cysts in the ADPKD group. In some cases, the renal parenchymal tissue was partially replaced by cysts. The ultrasonographic images of ADPKD and healthy kidneys are illustrated in Figure 1.

Blood Profiling

The mean level of urea was 32 ± 3.58 mg/dL in ADPKD patients and 31.75 ± 3.30 mg/dL in the control individuals. The serum level of creatinine was 1.051 ± 0.09 mg/dL in ADPKD patients and 1.041 ± 0.08 mg/dL in control participants. The serum levels of creatinine, urea, and uric acid in ADPKD and control groups did not differ significantly (Table 2).

Expression of Mirs in ADPKD Urinary Samples

The urinary samples of ADPKD patients

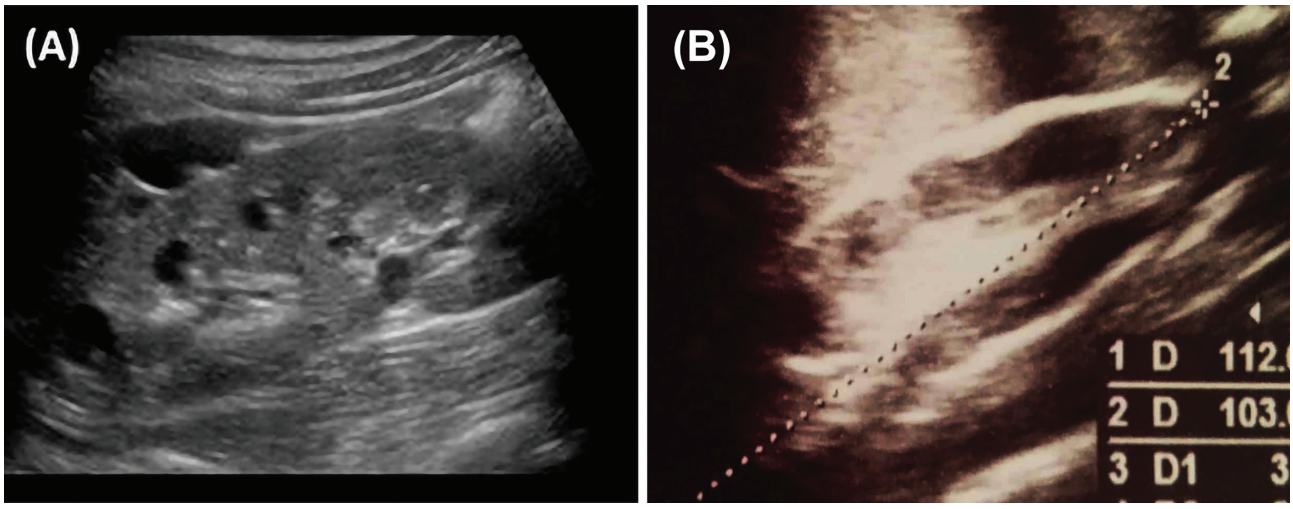

Figure 1. Ultrasonographic Images of the ADPKD (A) and Healthy Kidneys (B) [Abnormal enlargement of the kidneys and multiple cysts are obvious in the kidneys of ADPKD patients.]

exhibited elevated levels of miR-17 (P = .033), miR-21 (P = .010), and miR-143 (P < .001) accompanied by a decreased expression of miR-223 (P = .015) compared to the urine samples of the healthy individuals. Table 3 represents the fold change values obtained for each miR in ADPKD patients compared to its expression in control group. Figure 2 illustrates the relative expressions of the studied miRs among healthy control individuals and ADPKD patients.

DISCUSSION

Early diagnosis of ADPKD is critical for the proper management of renal cysts to prevent severe forms of the disease that are associated with kidney failure. A reliable screening test might improve the early detection of the disease in asymptomatic patients, especially in familial clusters with a history of renal cysts. The present study demonstrated that healthy and asymptomatic ADPKD patients exhibit differential expressions of miR-17, miR-21, miR-143, and miR-223 in their urine samples.

ADPKD is a common genetic disorder in which the dysregulated epithelial cells growth contributes to the formation of cellular aggregates. This involves the growth of benign cysts within the kidney which grow in size over time and have a significant impact on kidney function. Although these cysts are benign, they can get enlarged by filling with liquid and reducing the portion of renal parenchymal tissue. The inheritance pattern of polycystic kidney disease could be either dominant or recessive. The dominant autosomal type is the most common hereditary renal disease. Mutations in *PKD1* and *PKD2* cause differential onsets of renal cysts. So that, the average age for end-stage renal disease in *PKD1* mutations is approximately 55 while it is 75 in the case of *PKD2*.[11]

MiRs are non-coding RNAs with an approximate length of 18 to 22 nucleotides that control distinct cellular events through the regulation of gene expression at post-transcription and protein translation levels.[12] It has been shown that the expression signatures of various miRs might mirror the various clinical conditions. Accordingly, the diagnostic potential of miRs has been evaluated by numerous researchers all around the world for multiple clinical conditions such as cancer, autoimmunity, and infections. MiRs are also known as one of the key game-players in the pathogenesis of renal cysts through the modulation

Table 3. The Fold Change Values Obtained for the Studied miRs in ADPKD Patients

Relative Fold Change* in ADPKD Group (Mean ± SD)	miR-17	miR-21	miR-143	miR-223
	1.22 ± 0.43	1.48 ± 0.75	3.24 ± 1.12	0.71 ± 0.47

*The fold change values are compared to control group and are obtained by Livak formula for calculation of the relative gene expression.

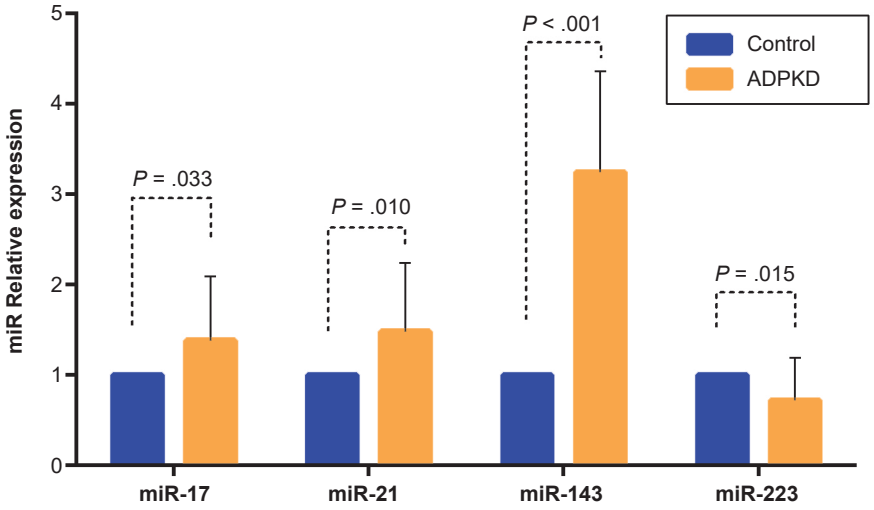

Figure 2. Relative Expressions of the Studied miRs in Control and ADPKD Groups [The ADPKD patients demonstrated significant increases in urinary expressions of miR-17, miR-21, and miR-143. On the other hand, the urine samples of ADPKD patients contained lower levels of miR-223.]

of renal epithelial cell proliferation and apoptosis. For instance, miR-17, miR-21, miR-143, and miR-223 have regulatory effects on tumor suppressor genes and oncogenes and are involved in renal cyst formation.[13]

In murine models of polycystic kidney disease, high expression of miR-17 promotes the formation of cysts. This miR also provokes the proliferation of HEK293T cells.[14] It has been shown that miR-17 causes cyst inflammation and growth through the activation of mammalian target of rapamycin (mTOR) signaling.[15] MiR-21 is an oncogenic miR preventing apoptosis in cystic epithelial cells. Accordingly, it has been shown that the down-regulation of miR-21 inhibits the progression of renal cysts.[7] Other miRs such as miR-143 and miR-223 also participate in the regulation of cell proliferation and apoptosis.[16-22] Ben-Dov *et al.* demonstrated that urine epithelial cells from ADPKD patients express higher levels of miR-143 than that of healthy individuals.[9] MiR-223 is a regulator of the expressions of inflammatory genes and is an indicator of kidney injury in sepsis.[23]

In the present study, we tried to exploit various miRs involved in the pathology of kidney diseases and cyst formation in a way toward discriminating the asymptomatic ADPKD patients over healthy individuals. We demonstrated here that while the serum levels of creatinine, BUN, and uric acid were normal in the asymptomatic ADPKD cases, their urine levels of the studied miRs were substantially different than the normal individuals. This differential expression of miRs in urine samples might provide a platform for early diagnosis of ADPKD to improve the management of the disease in its early stages.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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