MicroRNA-499a (rs3746444A/G) gene variant and susceptibility to type 2 diabetes-associated end-stage renal disease

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Abstract. Diabetic nephropathy (DN) is a major risk factor for end-stage renal disease (ESRD). MicroRNAs (miRNAs/miRs) and their variants may be implicated in health and disease, including DN. The present study aimed to investigate the association of the miRNA-499a gene (MIR499A) A/G seed region variant (rs3746444) with DN-associated ESRD susceptibility in patients with diabetes mellitus, and to determine whether there was an association between the different genotypes and the patients' laboratory and clinical data. A case-control pilot study was conducted on 180 adult patients with type 2 diabetes mellitus. A total of 90 patients with ESRD on regular hemodialysis were considered as the cases, and 90 age-, sex- and ethnicity-matched diabetic patients with normo-albuminuria were considered as the controls. MIR499A genotyping was performed using a TaqMan Real-Time allele discrimination assay. Results demonstrated that the MIR499A rs3746444*G variant conferred susceptibility to the development of ESRD under co-dominant [(odds ratio (95% confidence interval): 2.49 (1.41-3.89) and 2.41 (1.61-6.68) for heterozygous and homozygous comparison, respectively], dominant [2.30 (1.18-3.90)] and allelic [1.82 (1.17-2.83)] models. Different genotypes of the specified variant did not exhibit significant associations with the clinic-laboratory data of the studied patients or the circulating miR-499a plasma levels. In conclusion, results of the present study suggested that MIR499A rs3746444 may be a susceptibility variant for DN-associated ESRD in the study population. However, larger sample size studies with different ethnicities are warranted to verify these findings.

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Abbreviations: DN, diabetic nephropathy; ESRD, end-stage renal disease; miRNAs/miRs, microRNAs; SNP, single-nucleotide polymorphism; T2DM, type 2 diabetes mellitus

Key words: DN, ESRD, miR-499a, SNP

Introduction

Diabetic nephropathy (DN)-associated end-stage renal disease (ESRD) is a growing public health concern worldwide (1). Over the past three decades, DN-associated ESRD has been identified as a major cause for dialysis in several countries, including Saudi Arabia, in which type 2 diabetes mellitus (T2DM) accounts for >33% of all cases of ESRD (1,2). Several promising studies have identified genes associated with the occurrence and progression of DN, including genes associated with microRNAs (miRNAs/miRs) (3,4). This family of non-coding RNAs is highly conserved, tissue-specific and short in length (20-24 nucleotides). miRNAs play key roles in numerous physiological and pathological conditions in human organs, including the kidneys. For example, upregulation of miR-192, miR-194, miR-204, miR-215 and miR-216, and downregulation of miR-133a, miR-133b, miR-1d, miR-296, miR-1a, miR-122 and miR-124a have been observed in human kidneys (5). Also, miR-192 has been reported to be 20-fold higher expressed in cortical kidney tissue compared with medullary kidney tissue, where it is implicated in sodium transport regulation; in addition, miR-155 could suppress the expression of the angiotensin II receptor type 1, which could influence the blood pressure (5,6). By binding to mRNA targets, miRNAs have the ability to cause translational repression, mRNA destabilization and/or degradation (7). The

thermodynamics of miRNA-mRNA target interactions may be affected by single-nucleotide polymorphisms (SNPs) occurring in the precursor-miRNA (pre-miRNAs), resulting in target gene dysregulation and, subsequently, phenotype variations or disease susceptibility (8). The miRNA-499a gene (MIR499A) is located in the human myosin heavy chain 7B cardiac muscle β gene on chromosome 20q11.22 34990376-34990497 (+) and consists of a single exon that encodes for a single transcript (MI0003183). It is 122 base pairs in length and was reported to be a marker of cardiomyocyte injury (9). Although the number of studies focusing on the precise role of miR-499a in the pathogenesis and development of ESRD due to DN is limited, miR-499a has been proposed to affect several biological processes, such as cellular senescence, inflammation, apoptosis and immune response (10), that may play roles in ESRD biogenesis (Table SI) (11-14). Moreover, it was previously reported that miR-499a upregulation may regulate insulin resistance, glycogenesis and modulate insulin signaling by inhibition of phosphatase and tensin homolog (15).

A common variant (rs3746444), which is located within the miR-499a-3p seed region AC(A/G)UCAC at the position 20:34,990,448 (GRCh38), has been reported to be associated with several diseases, particularly in the Middle Eastern population, such as cardiovascular disease (9), autoimmune diseases (16) and cancer (17). In our previous study, significant dysregulation of circulating miR-499a levels was identified in the current DM-associated ESRD cohort (18). Therefore, the present study aimed to explore the association of the MIR499A (A/G) seed region variant (rs3746444) with DN-associated ESRD susceptibility in patients with T2DM, and to determine whether there is an association between the different genotypes and the patients' clinic-laboratory data. To the best of our knowledge, this is the first study to investigate the potential association of the specified variant with ESRD susceptibility in a Middle Eastern patient sample.

Materials and methods

Study population. A total of 90 consecutive patients with T2DM-associated ESRD on regular hemodialysis (three times/week) were recruited as the present study's patient group. The patients were attending the Nephrology Center of Mohammed bin Saud Al-Kabeer for renal dialysis at Arar Central Hospital (Arar, Saudi Arabia). The inclusion/exclusion criteria for the ESRD patient group selection have been described in detail in our previous work on the same ESRD patient cohort (18). Patients presenting with any chronic disease, autoimmune disorders, cancers and renal disease other than DM-associated nephropathy (diagnosed by renal biopsy according to the local standard protocols adopted from the international standards for diagnosis of non-diabetic renal disease) (19) were excluded. As it is highly likely that, eventually, a significant proportion of the patients diagnosed with diabetes early will develop ESRD, patients with T2DM with a matching duration of diabetes and normoalbuminuria (urinary albumin/creatinine ratio, <30 μg/mg) (20,21) were considered as the control group. All biomedical research involving human participants conformed to the guidelines of the Helsinki Declaration. The protocol of the present study was approved by the Research Ethics Committees of Northern Border University (approval no. 6/340/H), and written informed consent was obtained from all participants.

Laboratory analysis. Fasting venous samples were collected on plain and ethylenediaminetetraacetic acid-vacuum tubes before the specified second dialysis session for patients with ESRD. Serum was separated by centrifugation for 12 min at 1,300 x g at room temperature, immediately from the former tube for biochemical analysis using commercially available kits for glucose (cat. no. 44044831190), kidney function tests [creatinine (cat. no. 4810716190) and urea (cat. no. 4460715190)] and lipid profiling [total cholesterol (cat. no. 3039773190), triacylglycerol (cat. no. 20767107322), HDL-c (cat. no. 7528566190) and LDL-c (cat. no. 7005717190)] on a Cobas Integra Biochemical analyzer (all Roche Diagnostics GmbH).

Genotyping for MIR499A (rs3746444). Genomic DNA was extracted from whole blood using a Wizard Genomic DNA Purification kit (cat. no. A1120; Promega Corporation) according to the manufacturer's protocol, followed by DNA concentration and purity assessment using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and agarose gel (2%) electrophoresis to assess the DNA integrity. Samples were subsequently stored at -20˚C until the time of genotyping. A MIR499A polymorphism assay (rs3746444, assay ID: C_2142612_30) was run using TaqMan allele discrimination real-time PCR with the quality measures as described previously (16). In brief, PCR was run in a 25-μl reaction volume containing gDNA (20 ng) diluted to 11.25 μl with nucleases-free water, 12.5 μl TaqMan Universal PCR Master Mix (2X) and 1.25 μl 20 x TaqMan SNP Assay provided from the same supplier. A no-template and a no-polymerase enzyme samples were run each time with the study samples as negative controls. Genotyping was performed in a manner blinded to the case/control status. PCR amplification was performed on a StepOne Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), using the following conditions: Two-phases initial hold (2 min at 50˚C, and 10 min at 95˚C), followed by a 40-cycle two-step PCR (denaturation for 15 sec at 95˚C and annealing/extension for 1 min at 60˚C). Allelic discrimination was determined by analysing the fluorescence data files from each run using automated allele-calling software (SDS version 1.3.1; Applied Biosystems; Thermo Fisher Scientific, Inc.; Fig. S1). The overall genotype call rate was 100%.

Statistical analysis. Sample size and power calculations using G power-3 software (http://www.gpower.hhu.de/) demonstrated that, with the specified study design, allowable error rates, α error=0.05, a medium effect size=0.5 and a sample size of 90/group, may yield 91% power of the study. Data distribution and variance homogeneity were assessed by the Shapiro-Wilk test and Levene's test, respectively. Continuous data are expressed as the mean ± standard deviation, and categorical variables are presented as frequency counts. Data were compared using the χ^2 test, while unpaired Student's t-test, one-way ANOVA or Kruskal-Wallis tests were applied to compare continuous variables. The deviation of the observed genotype distribution from the Hardy-Weinberg equilibrium was analyzed using a χ^2 goodness-of-fit using the Online Encyclopedia for Genetic Epidemiology (http://www.oege.org/). Genotype-specific

Table I. Clinical and biochemical characteristics of patients with ESRD and the corresponding controls.

	Men			Women		
Clinical characteristic	Controls (n=48)	Patients with ESRD (n=48)	P-value	Controls (n=42)	ESRD (n=42)	P-value
Age, years	41.7±15.4	46.6±12.3	0.112	50.5±14.3	47.0±14.7	0.247
Hemodialysis duration, years	NA	3.52±1.68	NA	NA	3.95±2.06	NA
eGFR, ml/min/1.73 m^2	86.4±18.8	6.33±2.19	<0.001[a]	76.5±21.4	5.29±1.23	0.001[a]
Hypertension, n (%)	13 (32.5)	24 (57.1)	0.029[a]	24 (51.1)	12 (25.0)	0.011[a]
Biochemical findings						
FBS, mmol/l	4.53±0.43	5.57±2.45	0.009[a]	4.62±0.35	5.49±1.38	<0.001[a]
BUN, mmol/l	3.94±1.08	19.6±8.2	<0.001[a]	3.38±0.89	18.3±6.58	<0.001[a]
Creatinine, μmol/l	93.7±16.9	848.1±366.4	<0.001[a]	80.0±18.2	781.8±149.1	<0.001[a]
UA, μmol/l	267.5±78.8	376.3±73.0	<0.001[a]	238.0±78.7	383.7±91.8	<0.001[a]
Total cholesterol, mmol/l	4.10±0.76	3.53±0.77	0.001[a]	4.92±1.82	3.84±0.86	<0.001[a]
Total triglyceride, mmol/l	1.26±0.83	1.17±0.40	0.554	1.02±0.66	1.59±0.95	0.001[a]
HDL-c, mmol/l	1.01±0.39	0.97±0.34	0.654	1.33±0.44	0.89±0.26	<0.001[a]
LDL-c, mmol/l	2.68±0.61	2.89±1.23	0.321	3.12±1.45	2.75±0.84	0.139
TC/HDL ratio	4.57±1.77	4.10±1.94	0.246	3.98±1.56	4.61±1.48	0.046[a]

Data are displayed as the mean ± standard deviation, unless otherwise indicated. Two-sided χ^2 and Student's t-tests were used for comparisons. [a]P<0.05. NA, not available; ESRD, end-stage renal disease; eGFR, estimated glomerular filtration rate; FBS, fasting blood sugar; BUN, blood urea nitrogen; UA, uric acid; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; TC, total cholesterol.

adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the different genetic models. The study variables, including age, sex, number of hemodialysis sessions, presence of hypertension and duration of the disease, were adjusted. P<0.05 was considered to indicate a statistically significant difference. SPSS version 23 (IBM Corp.) was used for the statistical analyses.

MIR499A (rs3746444) variant in silico analysis. Gene structural and functional analysis were performed using Ensembl Genomic database (ensembl.org) and miRBase.org; target prediction was performed using DIANA-microT-CDS v5.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), miRBase (http://www.mirbase.org/) and DIANA-TarBase v7.0 algorithm (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index) databases; and annotation clustering and pathway enrichment analysis were performed using miRPath v3.0 (http://www.microrna.gr/miRPathv3) and all were extensively detailed in our previous studies (8,12). Predicted miR-499a targets were compared with the presence of both A and G alleles using the miR2Go program (http://compbio.uthsc.edu/miR2GO) at a medium hierarchical filtering level (P<0.05).

Results

Characteristics of the study population. The baseline characteristics of the study subjects are summarized in Table I. The mean age of the patients with ESRD was 46.6±12.3 years for men and 47.0±14.7 years for women. The mean duration of dialysis was comparable for both sexes in the study participants. There were no sex-specific differences in the clinical and biochemical characteristics of the participants, except those associated with lipid profile parameters, which were worse in female patients compared with those in men (Table I).

MIR499A variant (rs3746444) in the study population. In the study population, the genotype frequencies of MIR499A (rs3746444) SNP followed the Hardy-Weinberg equilibrium (P=0.149 in controls and P=0.398 in patients). The minor allele frequencies for rs3746444*G were 0.28 and 0.42 in controls and patients with ESRD, respectively. A significant difference in MIR499A genotypes was observed between the study groups. The frequency of the GG genotype was 11.1% in controls, compared with 20% in patients with ESRD (P=0.030). Carrying the rs3746444*G allele conferred a nearly 2-fold increase in the susceptibility to the development of ESRD, with an OR (95% CI) of 1.82 (1.17-2.83) under the allelic genetic association model. Consistently, homozygote/heterozygote individuals (rs3746444*GG/AG) were more likely to develop ESRD under the homozygous/heterozygous comparison and dominant models [GG vs. AA: OR=2.41, 95% CI: 1.61-6.68; AG vs. AA: OR=2.49, 95% CI: 1.41-3.89; (AG + GG) vs. AA: OR=2.30, 95% CI: 1.18-3.90; Table II].

MIR499A variant (rs3746444) and clinic-laboratory characteristics of patients with ESRD. When investigating the association between different genotypes of the specified variant and the clinical and biochemical characteristics of the patient group, no significant associations were demonstrated, as demonstrated in Table III.

Table II. Genotype and allele frequencies of MIR499A (rs3746444) polymorphism in patients with ESRD and the corresponding controls.

Genetic model	Genotype	Controls, n=9 0 (%)	Patients with ESRD, n=90 (%)	P-value	OR (95% CI)
Co-dominant model	AA	49 (54.4)	32 (35.6)	0.030[a]	1.0
	AG	31 (34.5)	40 (44.4)		2.49 (1.41-3.89) [a]
	GG	10 (11.1)	18 (20.0)		2.41 (1.61-6.68) [a]
HWE P-value		0.149	0.398		
Dominant model	AA	49 (54.4)	32 (35.6)	0.011[a]	1.0
	AG + GG	41 (45.6)	58 (64.4)		2.30 (1.18-3.90) [a]
Recessive model	AA + AG	80 (88.9)	72 (80.0)	0.099	1.0
	GG	10 (11.1)	18 (20.0)		1.9 (0.82-4.62)
Allelic model	A	129 (71.7)	104 (57.8)	0.006[a]	1.0
	G	51 (28.3)	76 (42.2)		1.82 (1.17-2.83) [a]

χ^2 analysis was used for comparisons. OR and CI adjusted for confounding factors, such as age, sex, hypertension, duration of the disease and the number of hemodialysis sessions. The co-dominant model represents both heterozygous and homozygous comparison models. [a]P<0.05. ESRD, end-stage renal disease; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

Effect of MIR499A (rs3746444) on gene structure and function. Although miR-499a forms a secondary hairpin loop with complementary sequences in its structure (Fig. S2), different genes were predicted to be targeted by both mature forms synthesized from either arm. A total of 1,890 genes were predicted to be affected by miR-499a (919 genes by 3p, 810 by 5p and 161 genes by both), as detailed previously (8). The most significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with miR-499a targets that may be involved in DN progression into ESRD are summarized in Table IV.

The presence of the rs3746444 SNP within the seed region of the passenger miR-499a strand may lead to disruption of targets and the creation of a different set of genes (8,16). Also, it may shorten the stem-loop structure; thus, affecting the processing of the entire miRNA.

Our previous study reported dysregulation of the circulatory levels of miR-499a in the same patient cohort (18); therefore, the present study focused on determining whether these circulatory levels exhibited significant variations among different genotypes of the same gene. The findings, however, did not reveal significant associations between the different genotypes of MIR499A (rs3746444) and the circulating miR-499a plasma levels detected previously using the Livak method (22) (P=0.173; Fig. 1).

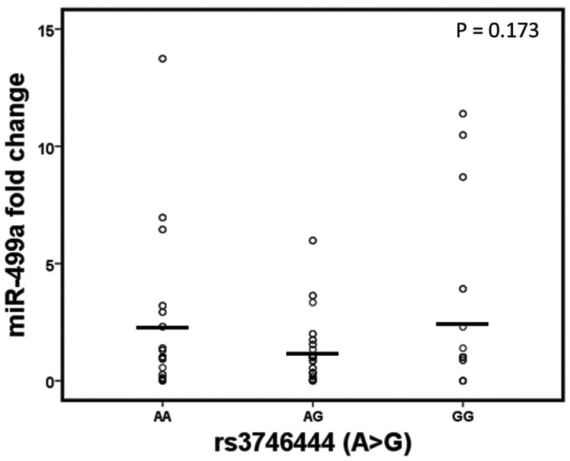

Figure 1. Association between the MIR499A (rs3746444) variant and miR-499a plasma expression levels in patients with ESRD. Solid lines represent medians. The relative expression levels of plasma miR-499a were normalized to those of the internal control RNU6B in our previous study (14). The calculation of the relative gene expression compared with the corresponding control was performed using the following equation: Relative quantity=$2^{-\Delta\Delta Cq}$, where $\Delta\Delta C_q$=(C_q MIR499A-C_q RNU6B) ESRD-(C_q MIR499A-C_q RNU6B) control cohort (22). The Kruskal-Wallis test was used for comparison. miR/MIR, microRNA; ESRD, end-stage renal disease.

Discussion

SNPs have been reported to be the most common type of genetic variation associated with population diversity, disease susceptibility and individual therapeutic response (23). SNPs may affect miRNA expression and/or maturation at the level of transcription of the primary transcript, post-transcriptional processing of primary transcripts or pre-miRNAs, or affecting miRNA-mRNA interactions (24).

The present study demonstrated that carrying the rs3746444*G allele conferred susceptibility to ESRD in patients with DM compared with non-carriers under the allelic, co-dominant and dominant genetic association models. Results of our previous study demonstrated that the rs3746444 polymorphism results in an A>G mismatch in the miR-499a precursor stem region (located opposite the mature sequence), affecting the mature miR-499a-3p seed region implicated in correct miRNA-mRNA binding, with subsequent disruption and creation of several targets (8). Carriers of the homozygote genotypes (AA or GG) yield two mature forms, miR-499a-5p and miR-499a-3p*A or *G, respectively, with each having its specific panel of target genes. In comparison, the heterozygous (AG) carriers produce three mature forms, miR-499a-5p, -3p*A and -3p*G, with three subsets of target genes. The *in silico*

Table III. Association of MIR499A variant with clinical and biochemical characteristics of patients with ESRD.

	Rs3746444 genotypes				
Clinical characteristic	AA (n=32)	AG (n=40)	GG (n=18)	P-value	OR (95% CI) AG/GG vs. AA
Age, years (%)				0.385	
<30	3 (9.4)	4 (10.0)	0 (0.0)		1.0
≥30	29 (90.6)	36 (90.0)	18 (100)		1.39 (0.29-6.66)
Sex, n (%)				0.514	
Female	15 (46.9)	24 (60.0)	9 (50.0)		1.0
Male	17 (53.1)	16 (40.0)	9 (50.0)		0.66 (0.28-1.59)
Hemodialysis duration, years	3.8±1.8	3.8±1.9	3.3±1.9	0.577	
eGFR, ml/min/1.73 m^2	5.9±1.9	5.8±1.8	5.4±1.4	0.656	
Hypertension, n (%)				0.069	
Negative	23 (71.9)	25 (62.5)	7 (38.9)		1.0
Positive	9 (28.1)	15 (37.5)	11 (61.1)		0.48 (0.19-1.21)
Biochemical findings					
FBS, mmol/l	6.0±2.6	5.3±1.4	5.2±2.1	0.219	
BUN, mmol/l	19.2±7.4	17.9±7.7	20.9±6.2	0.349	
Creatinine, μmol/l	829±226	763±302	894±271	0.220	
UA, μmol/l	371±85.5	380±83.9	395±80.0	0.628	
Total cholesterol, mmol/l	3.82±0.8	3.6±0.71	3.5±1.1	0.520	
Total triglyceride, mmol/l	1.4±0.6	1.3±0.6	1.5±1.1	0.500	
HDL-c, mmol/l	0.9±0.3	0.9±0.3	0.8±.2	0.821	
LDL-c, mmol/l	2.7±0.9	2.7±0.9	3.0±1.4	0.676	
TC/HDL ratio	4.6±2.0	41±1.3	4.2±1.8	0.527	

Data are presented as the mean ± standard deviation, unless otherwise indicated (n=90). χ^2 and one way ANOVA test was used for comparison. OR, odds ratio; CI, confidence interval; ESRD, end-stage renal disease; eGFR, estimated glomerular filtration rate; FBS, fasting blood sugar; BUN, blood urea nitrogen; UA, uric acid; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; TC, total cholesterol.

analyses of the present study revealed that the presence of these different alleles may disrupt and create 667 and 744 gene targets, respectively. Among validated gene targets that have been investigated are nucleolar protein 4, nuclear receptor interacting protein-1, Bcl-2-like protein 14, chemokine (C-C motif) ligand 8 and sex-determining region Y-box 4 (20). To the best of our knowledge, this is the first study to investigate the MIR499A rs3746444 variant for ESRD susceptibility in patients with DM in a sample of the Middle Eastern population. Consistent with the findings of the present study, Misra *et al* (25) reported that the heterozygous and the homozygous genotypes of MIR-499A rs3746444 conferred risk for ESRD development, which was induced by other disorders apart from DN, and may be associated with an almost 3-fold increased risk for acute allograft rejection in a North Indian population (25).

Given that SNPs in pre-miRNAs may alter miRNA processing and/or expression, on analyzing the association of MIR499A polymorphism with miR-499a circulating plasma levels and different clinic-laboratory characteristics of patients, no significant associations were observed, as previously described. It is hypothesized that the presence of a miR-499a variant may affect the thermodynamics of the RNA-RNA interactions and the affinity of this miRNA by interfering with optimal binding to target mRNAs, thereby resulting in the dysregulation of target genes that mediate disease susceptibility to ESRD without affecting the relative expression and/or disease phenotype (22). The *in silico* analyses of the present study revealed some of the top significant KEGG pathways involving miR-499a targets that may be involved in DN progression into ESRD (26-32). For example, the (TGF-β1) pathway was implicated in DN etiopathology (26,28). The TGF-β1 pathway activates key signaling pathways, including the PI3K/AKT pathway that mediates the phosphorylation and inactivation of the transcriptional FOXO, leading to oxidant stress, mesangial cell expansion and accumulation of extracellular matrix proteins, key components of DN-associated chronic kidney disease (26). Upregulation of epidermal growth factor receptor (ErbB) tyrosine kinase activity is important in mediating several complications of diabetes, including renal pathologies, cardiac fibrosis and vascular dysfunction (29). Moreover, the role of the TNF pathway in the progression of DN in diabetes mice and rat models was evident. For example, using a soluble TNF receptor-2 fusion protein has been reported to improve the early stage of DN in DM model of the KK-Ay mouse (30). Also, TNFR-Fc TNF inhibitor had been found to attenuate the renal hypertrophy without affecting the metabolic profile in rats with induced diabetes (31). mTOR has

Table IV. Key Kyoto Encyclopedia of Genes and Genomes pathways involving miR-499a targets.

Gene targets of miR-499a-3p, n	Gene targets of miR-499a-5p, n	Biological pathway or disease
0	35	Adherence junction
0	91	Focal adhesion
0	41	Phosphatidylinositol signaling system
47	40	GABAergic synapse
74	73	FoxO signaling pathway
0	11	Biosynthesis of unsaturated fatty acids
0	2	Biotin metabolism
41	33	TGF-β signaling pathway
18	17	Mucin type-O-glycan biosynthesis
62	54	Thyroid hormone signaling pathway
15	0	Prion disease
61	52	Sphingolipid signaling pathway
48	50	ErbB signaling pathway
59	0	TNF signaling pathway
74	0	Ubiquitin-mediated proteolysis
5	0	Phenylalanine, tyrosine and tryptophan biosynthesis
35	0	mTOR signaling pathway
13	0	Glycosphingolipid biosynthesis
39	0	ECM-receptor interaction

Each number represents the number of gene targets in each pathway or disease affected by miR-499a-3p or miR-499a-5p. Result intersection and filtration after false discovery rate correction was applied. Fisher's exact test (hypergeometric distribution) was used for statistical analysis. The two mature forms of the MIR499 gene are involved in FoxO, TGF-β and ErbB signaling pathways that are implicated in DN-associated chronic renal disease (21-24). Additionally, miR-499a-3p is involved in the TNF and mTOR signaling pathways (25-27). miR, microRNA; GABA, γ-aminobutyric acid; ErbB, epidermal growth factor receptor; ECM, extracellular matrix.

been implicated in maintaining the glomerular podocyte function; increased activity contributed to glomerular hypertrophy and hyperfiltration associated with progressive DN (32).

Notably, the aforementioned pathways were targeted in numerous experimental investigations to validate their therapeutic implication in DN (29,30,33,34). Ziyadeh *et al* (33) reported an improvement in glomerular filtration rates induced by the monoclonal anti-TGF-β antibody in db/db diabetic mice (33). Furthermore, long-term treatment of streptozotocin-induced diabetic rats with AG825, a specific inhibitor of receptor tyrosine-protein kinase ErbB-2, was reported to significantly correct the hyperreactivity of the perfused mesenteric vascular bed to norepinephrine and the attenuated responsiveness to carbachol (29). Inhibition of TNF-α with the soluble TNF receptor (TNFR)2 fusion protein, etanercept, improved the progression of the early stage of DN in a diabetic model established in the KK-A(y) mouse, mainly through inhibition of the anti-inflammatory action of the renal TNF-α-TNFR2 pathway (30). Moreover, rapamycin, the first reported compound to inhibit mTOR, inhibits glucose-induced phosphorylation of p70S6 kinase and its substrate, S6 ribosomal protein, in mesangial cells, attenuating the morphological and functional disorders of diabetic kidneys in a T2DM mouse model (34).

Consistent with the findings of the present study, Ciccacci *et al* (35) revealed the involvement of miR-499a in DN susceptibility in an Italian T2DM cohort (35). Results of the aforementioned study revealed that this association was due to the impact of miR-499a on two apoptotic players, calcineurin and dynamin-related protein, by which miR-499a dysregulation caused mitochondrial dysfunction and cell apoptosis, which may have contributed to disease susceptibility (33). Notably, the latter processes have been implicated in DN (36,37), which may in part support the association of the miR-499a variant with ESRD susceptibility in the present cohort. However, an increasing number of studies involving larger independent cohorts with different ethnicities are required to confirm the findings of the present study.

In conclusion, to the best of our knowledge, the present study was the first to propose the impact of the MIR499A A/G seed region variant (rs3746444) on DN-associated ESRD susceptibility. It may be included in the list of molecular susceptibility genes that may support patient-risk stratification and early preventive measurement implementations. However, it is worth noting that the implication of other potential confounders could not be conclusively ruled out, such as exposure to different environmental factors (such as type of treatment or nutrition) and the additional genetic factors. Further larger-sample size and long-term follow-up replication studies, particularly in populations with different ethnicities, are warranted. Functional studies are also required to validate the role of miR-499a in DN-associated ESRD etiopathology.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MSF, BTAA, EAA, SAAQ, SWK, WA and EAT conceived and designed the experiments. BTAA and MSF recruited the study samples and recorded clinical patient data. MSF and BTAA confirm the authenticity of all the raw data. MSF, BTAA and EAT contributed to the experiments. EAT contributed to the statistical analysis. All authors contributed to obtaining the reagents and materials of the present study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Research Ethics Committees of Northern Border University approved the present study. Patients who participated in this research exhibited complete clinical data, and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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