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Blood MicroRNA-346 and 92a as Potential Non-invasive Biomarkers for Diabetic Nephropathy

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Abstract
Diabetic nephropathy (DN) is a progressive kidney disease, and it is estimated to develop in one third of both main types of diabetes.New biomarkers are needed to improve the identification of individuals at increased risk of developing diabetes mellitus complications particularly DN. The currentstudy aimed to evaluate the diagnostic potential of two circulating microRNAs (miR-346 and miR-92a) as a non-invasive biomarker for DNintype 2 diabetesmellitus Egyptian patients.Atotalof90plasmasampleswerecollected (15 control,25normoalbuminuriapatients and50patients with DN that divided equally into microalbuminuria group and macroalbuminuria group, n= 25).The expressionlevelsof thetwo miRNAswerequantitatively detected usingreal-timePCR.Results revealed significant decrease inmiR-346expressionlevel,while miR-92a expression level was significantly upregulated in the DN groupscomparedtocontrolgroup.Correlations between the studied miRNAs levels and the tested biochemical parameterswasconducted, in addition toreceiver operatingcurveanalysistodetectthepotentialapplication of miR-346 and miR-92a for discriminating diabetic nephropathy patients fromnon-diabetic nephropathypatients.Higher sensitivity and specificity of plasma miR-92a than miR-346 was observed.Inconclusion,miR-92aandmiR-346mightbe apotentialnon-invasive biomarkerfor DN with miR-92a showing privilege over miR-346 that should inspire additional research.

Keywords: *Diabetes, Diabetic nephropathy,microRNA, miR-92a, miR-346*

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I. Introduction

Diabetes is increasingly becoming a major chronic disease burden all over the world. It represents a fast-growing health problem in Egypt with a significant impact on morbidity, mortality and health care resources[1].

If left untreated, diabetes can cause many complications due to damage in small blood vessels including damage to the eyes, kidneys, and nerves. Damage to the kidneys, known as diabetic nephropathy(DN), can lead to tissue scarring, urine proteins loss, and eventually chronic kidney disease, sometimes requiring dialysis or kidney transplant[2, 3].

Diabetic kidney disease and DN are the dominant cause of end stage renal disease (ESRD) in the United States and most developed countries.DN raises the overall 10-year mortality and morbidity among diabetic patients at least 6 folds compared to healthy age matched non-diabetic people[4, 5].

About 20%–40% of type 2 diabetics with microalbuminuria progress to overt nephropathy; and about 20% will develop ESRD after the development of overt nephropathy[6].

Most guidelines suggest screening DN with a spot urinary albumin/creatinine ratio (normal < 30 mg alb/g creatinine), from either first morning (preferred) or random urine samples[7].

There are many limitations in using albuminuria as a biomarker of DN as many patients experience glomerular filtration rate loss without progression in albuminuria and even normoalbuminuria. Therefore, there is interest in finding novel biomarkers for diagnosis and identify progression risk[8, 9].

Miearly DN

croRNAs (miRNAs) are a short single-stranded non-coding RNAs with length about 19-24 nucleotides, implicated in post-transcriptional gene regulation, biological and pathological systems of every cell type, including kidney cells[10].

The role of miRNAs in DN progression is through the development of renal fibrosis. Previous studies hypothesized that some miRNAs cause renal fibrosis[11, 12].

Recently, circulatingmiRNAs have been considered as very promising biomarkers both to detect human pathologies earlier and to enhance the outcome of patients with specific drug therapies[13].Therefore, the quantitative measurement of miRNA in urine and plasma samples can be used as a diagnostic biomarker for controlling the progression of diabetic complications[14].

SMAD3/4 plays a significant role in TGF-β-mediated kidney fibrosis. miR-346 was identified as a SMAD family-related miRNA that has not been well characterized in this regard[12, 15].

Previous studies stated that atherosclerosis-related CKD cause oxidative stress, in turn leading to miR-92a expression, endothelial dysfunction, and renal impairment[16-18].

The aim of the current study is to explore the potential usefulness of some kidney diseases-associated plasma miRNAs namely miR-346 and miR-92a as a potential non-invasive markers for diagnosis of DN in type 2 diabetes mellitus Egyptian patients.

II. Subjects & Methods

2.1.Experimental subjects

This study was carried out the guidelines of World Medical Associationand has been approved by the Theodor Bilharz institute, Cairo, Egypt ethical committee. Informed consent was obtained from patients before blood sampling.

Complete history record, full clinical examination, laboratory investigations and radiological examinations was carried out for all patients.

The presence of type 2 diabetes mellitus (T2DM) in the patients was established according to World Health Organization criteria: Fasting glucose (FG) levels ≥7.0 mmol/l, glucose levels ≥11.1 mmol/l (200 mg/dl) as determined by a 2 h oral glucose tolerance test (OGTT) when glycated hemoglobin (HbA1c) levels >6.5%, or when the subjects had a clinical diagnosis of the disease. Individuals with FG levels of 6.1-6.9 mmol/l (110-125 mg/dl) or 2 h OGTT glucose levels of 7.8-11.0 mmol/l (140-199 mg/dl) were designated as exhibiting pre-diabetes. The healthy controls were defined as subjects with FG of 4.8-5.2 mmol/l (110 mg/dl), and 2-h OGTT glucose levels of < 7.8 mmol/l (140 mg/dl)[19].

Diabetic nephropathy diagnosis was proved according to the kidney disease improving global outcomes guideline for diabetes management in chronic kidney disease. Patients with ACR less than 30 mg/g were classified as normoalbuminuria, those with values more than 30 mg/g and less than 300 mg/g were defined as microalbuminuria, while the group with an ACR of more than 300 mg/g was classified as macroalbuminuria[20]

2.2. Patient selection

- Inclusion criteria

- Patients with a history of type 2 diabetes mellitus for greater than or equal to 5 years and albuminuria >30 mg/24 hours.

- Exclusion criteria

- Patients presenting with other causes of increased urinary albuminuria (urinary tract infection, hypertension, pregnancy, menstruation, and genital infections, any systemic or local infection).
- Patients with thyroid, cerebrovascular or cardiovascular disease; patients with history of intake of steroids, statins medications, estimated Glomerular filtration rate (eGFR) <60 mL/min/1.73 m2, and patients with present or past hemodialysis/peritoneal dialysis or history of renal transplant.
- Patients with type1 diabetes mellitus, renal stones, or history of nephrotoxic drug usage in the last 3 months.
- Patients with underlying malignancies, kidney disease history and other systemic diseases.

2.3. Study population

- A total of ninety Egyptian subjects were enrolled in this study and were divided into four groups:

- *Group 1* (control): healthy volunteers that were not suffering from any disease or take any medications that might affect their kidney function (n=15: 7♂ 8♀; mean age 53.2 ± 1.56 years).
- *Group 2* (Normo): Normoalbuminuria group that include diabetic patients without any kidney impairment (n=25: 6♂ 19♀; mean age 55.36 ± 2.07 years).
- *Group 3* (Micro): Microalbuminuria group that include diabetic patients with moderate kidney impairment (n=25: 11♂ 14♀; mean age 55.48 ± 1.77 years).
- *Group 4* (Macro): Macroalbuminuria group that include diabetic patients with severe kidney impairment (n=25 8♂ 17♀; mean age 59.8 ± 1.36 years).

2.4. Specimen collection

- Five milliliters of blood were collected from all the previous groups in two tubes one of them contain anticoagulants (EDTA) the other one contain clot activator and centrifuged at maximum speed for 20 min.

- The plasma and serum was removed, transferred to a polypropylene, capped tube in 1 ml aliquots, and frozen at -80 °C for pending assay

2.5. Laboratory investigations

- Glucose, HbA1c, creatinine, urea, uric acid levels was determined using specific diagnostic kits obtained from Spectrum diagnostics, Egypt (Cat. No. 250 001; 602 001 – I; 237 001; 320 004 and 323 001 respectively). UACR was determined using specific assay kit purchased from LifeSpan BioSciences, USA (Cat. No. LS-K562-100). Also, plasma insulin level was measured using ELISA kit acquired from Eagle Biosciences, USA (Cat. No. LS-K562-100).
- Insulin resistance index (HOMA IR) was calculated according to Salgado et al. [21]
  - HOMA IR = (Fasting blood glucose in mmol/L × insulin in m IU /ml) ÷ 22.5

2.6. Quantification of plasma miRNAs level

1- MicroRNA extractionwas isolated according to manufacturer's instructions using miRNeasy Serum/Plasma Kit (cat no. 217184) purchased from Qiagen, Germany.

2- Complementary DNA (cDNA) synthesis was carried out using miScirpt 11 RT kit (cat No.218161), obtained from Qiagen, Germanyas directed by the manufacturer.

3- MicroRNAs quantitation using real-time PCR was performed using miScript SYBR Green PCR Kit (cat.no.218075) and miScript primer (**Table 1**) Assays obtained from Qiagen, Germany based on manufacturer's guidelines.

Table 1: Oligonucleotide forward miRNA primers for real-time PCR

Primer's name	**Sequence**
Hsa-miR-346	UGUCUGCCCGCAUGCCUGCCUCU
Hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU
U6 (Housekeeping miRNA)	CAAAGTCAGTGCAGGTAGGCTTA

4- Target miRNA expression level was calculated in relative to U6 using Livak's equation[20].

- Fold change= $2^{-\Delta\Delta C}{}_{T}$

2.7. Statistical analysis

- Analysis of data was done by IBM computer using SPSS version 22. Data was presented as the mean ± standard error (SE). Multiple comparisons between more than two groups have been conducted by one way analysis of variance (ANOVA) with a post hoc Tukey HSD test. The Pearson correlation test was used to examine correlation relationships between serum miRNAs and clinical parameters. Receiver operator characteristic (ROC) curve was done to determine the diagnostic value of serum miRNAs in diabetic patients with DN and to find out the best cut off value of the studied diagnostic biomarkers. A P-value <0.05 was considered to be statistically significant.

III. Results

3.1. Blood glucose and related parameters

Table (2) results indicated significant increase in blood glucose, fasting blood glucose, 2-hours post prandial blood glucose, glycosylated Hb and HOMA IR levels in comparison with control group.

Table 2: Blood glucose and related parameters in control, diabetic nephropathy groups

Groups / **Parameters**	**Control**	**Normo**	**Micro**	**Macro**
Fasting blood glucose (mg/dl)	79.51±3.51	151.56 ± 5.67^{ad}	175.42±7.84^{ad}	225.30 ± 12.70^{abc}
2-hours post prandial blood glucose (mg/dl)	106.60± 2.50	265.6± 10.50^{ad}	282.43±14.55^{ad}	336.00±15.30^{abc}
Glycosylated Hb (%)	4.71± 0.16	8.36± 0.31^{acd}	9.60±0.24^{abd}	10.60±0.31^{abc}
HOMA IR	1.14±0.039	3.30±0.073^{acd}	3.98± 0.131^{ab}	3.98±0.147^{ab}

- Values are expressed as means ± standard error of the mean in each group
- Data with different superscript are significantly different at p≤0.05. ^{a} significance vs control group, ^{b} significance vs Normo-group, ^{c} significance vs Micro- group, ^{d} significance vs Macro-group

3.2. Kidney function parameters

Results in table (3) revealed that creatinine level was significantly increased in microalbuminuria and macroalbuminuria compared to healthy control. Additionally, significant elevation in urea and uric acid were significantly elevated in all kidney impairment groups than normal group. Significant increase in UACR in macroalbuminuria group was shown compared to control subjects.

Significant decrease in eGFR level was observed in all groups in comparison with normal group.

Table 3: Kidney parameters levels in different groups

Parameters \ Groups	Control	Normo	Micro	Macro
Creatinine (mg/dl)	0.97 ±0.07	1.27 ± 0.05[d]	1.48 ± 0.05[ad]	1.95 ± 0.16[abc]
eGFR (mL/min/1.73 m^2)	91.7 ±4.15	67 ±3.38[ad]	58.8 ±3.24[ad]	41.2±3.26[abc]
Urea (mg/dl)	32.3± 1.6	48.9 ± 1.53[acd]	60.50±1.68[abd]	91.60 ± 4.50[abc]
Uric acid (mg/dl)	4.19 ±0.179	5.00 ± 0.214[acd]	6.22 ± 0.195[abd]	6.50 ± 0.191[abc]
UACR (mg Alb/ gm creatinine)	18.57 ± 1.50	13.06 ± 1.23[d]	139.20 ± 14.50[d]	702.81 ± 84.38[abc]

- Values are expressed as means ± standard error of the mean in each group
- Data with different superscript are significantly different at p≤0.05. [a] significance vs control group, [b] significance vs Normo-group, [c] significance vs Micro- group, [d] significance vs Macro-group

3.3. The expression levels of miR-346 and miR-92a in healthy and diabetic nephropathy groups

The expression level of plasma miR-346 and miR-92a in control and diabetic nephropathy (DN) groups are indicated in figure(1). Significant downregulation in plasma miR-346 in normoalbuminuria, micro-albuminuria and macro-albuminuria compared with the normal control group was observed. On the other hand, miR-92a expression level was significantly upregulated in microalbuminuria and macroalbuminuria in comparison with healthy subjects.

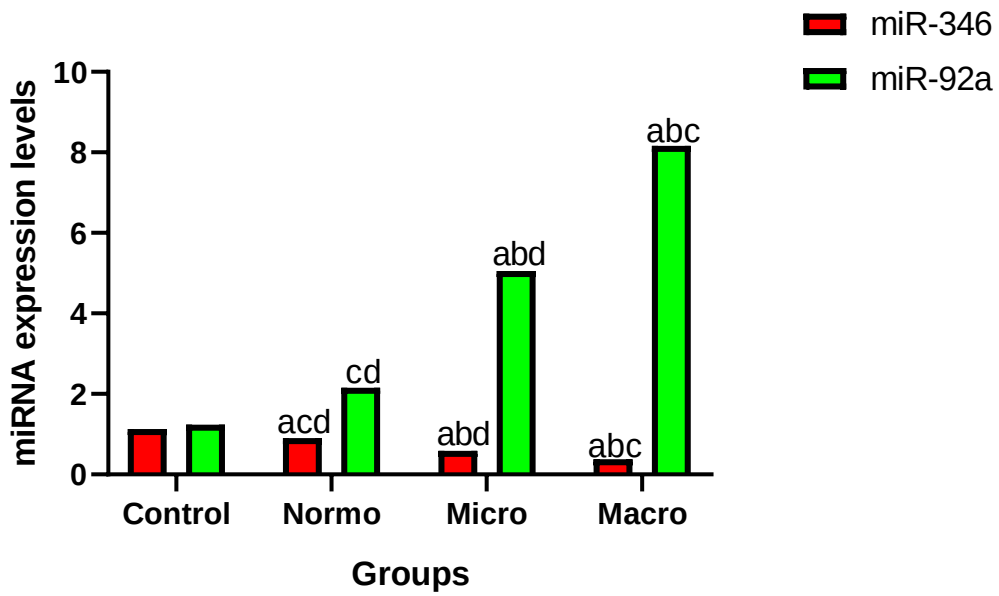


Figure1: Means of miR-346 and miR-92a expression levels in different groups. [a] significance vs control group, [b] significance vs Normo-group, [c] significance vs Micro- group, [d] significance vs Macro-group

3.4. Correlations between miR-346 and miR-92a with the other studied parameters in diabetic nephropathy groups

Table (4) reveals correlation results between miR-346 and other parameters in normoalbuminuria, microalbuminuria, macroalbuminuria.

In normoalbuminuria groups, miR-346 showed non-significant correlations between all studied parameters, while miR-92a was positively correlated with UACR.
In DN patients with microalbuminuria and macroalbuminuria both miR-346 and miR-92a showed significant correlations with kidney function parameters (creatinine, urea and uric acid).
Additionally, miR-92a showed significant positive correlations with FBG, HbA1c, HOMA-IR and UACR in microalbuminuria patients, while in macroalbuminuria group there was a positivesignificant correlations between miR-92a and kidney function parameters (creatinine, urea, uric acid and UACR) and a negative significant correlations with eGFR while miR-346 have a negative significant correlations with kidney function parameters (creatinine, urea and UACR).

Table 4: Correlation study between miR-346/miR-92a and the other tested parameters in diabetic nephropathy groups

Groups / Parameters	Normo		Micro		Macro	
	miR-346	miR-92a	miR-346	miR-92a	miR-346	miR-92a
	r	r	r	r	r	r
Fasting blood glucose	-	-	-	0.496*	-	-
HbA1c	-	-	-	0.561**	-	-
HOMA IR	-	-	-	0.518**	-	-
Creatinine	-	-	-0.415*	0.499*	-0.420*	0.803**
eGFR	-	-	-	-	-	-0.663***
Urea	-	-	-0.475*	-	-0.463*	0.680***
Uric acid (mg/dL)	-	-	-	0.402*	-	0.515***
UACR	-	0.723***	-	0.763***	-0.469*	0.9***

- Statistically significant at: * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001

3.5. The diagnostic accuracy of miR-346 and miR-92a expression levelsbetween groups
To analyze the diagnostic accuracy of miR-346 and miR-92a expression in DN, receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated to evaluate the sensitivity and specificity for predicting DN patients based on the expression of each individual miRNA.
The ROC curve analysis of miR-92a showed best sensitivity and specificity than miR346 relative gene expression in differentiating patients with DN from healthy control (table 5 and figures 2).
The ROC curve analysis of miR-346 showed best sensitivity and accuracy than miR-92a relative gene expression in differentiating patients with DN from normoalbuminuria patients (table 5 and figures 3).

Table 5: Diagnostic values of miR-346 and miR-92a

Groups	DN vs Control		DN vs Normoalbuminuria	
	miR-346	miR-92a	miR-346	miR-92a
AUC	0.915	0.928	0.954	0.89
P-value	0.000	0.000	0.000	0.000
Cut-Off	0.84	1.66	0.67	3.85
Sensitivity (%)	77.3	82.7	88	72
Specificity (%)	100	100	92	96
PPV (%)	63.3	68.9	61.3	49.3
NPV (%)	36.7	31.1	38.7	50.7
Accuracy(%)	80	85.5	89.3	80

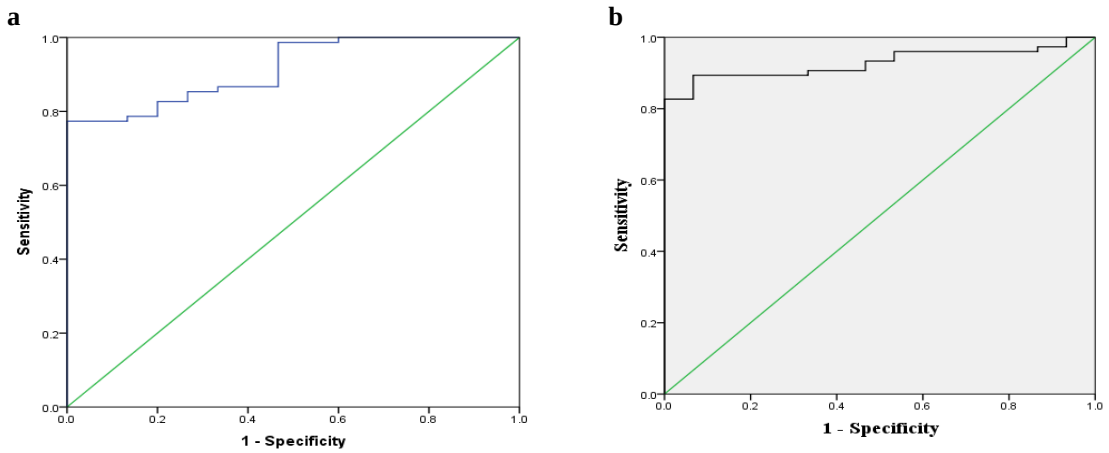
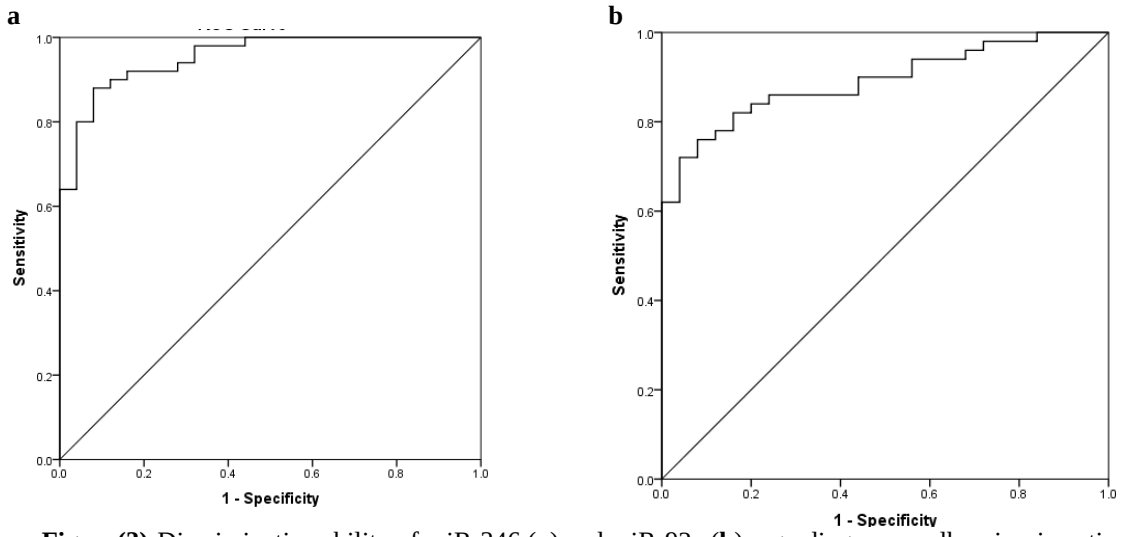


Figure (2): Discriminative ability of miR-346 (**a**) and miR-92a (**b**) regarding normal subjects



Figure(3): Discriminative ability of miR-346 (**a**) and miR-92a (**b**) regarding normoalbuminuria patients

IV. Discussion

Type 2 diabetes mellitus (T2DM) is a metabolic disorder with a prevalence expecting to rise from 285 million individuals in 2010 to 439 million by the year 2030[23]. The disease is associated with multiple complications, with chronic kidney disease being the most prevalent which accounts for 50% of all cases of end stage kidney diseaserequiring kidney transplantation[24, 25].

Diabetic nephropathy (DN) accounts for a significantly increased morbidity and mortality in T2DM patients and account for about 40% of T2DM patients. Although microalbuminuria is regarded as the gold standard for diagnosis of an early and reversible DN, the sensitivity to precisely detect disease progression remains unsatisfied[26].

Previous studies defined the role of miRNAs in normal renal function and development and various renal diseases suggesting specific renal miRNAs might be good targets to monitor DN pathogenesis[27-29].

Present study results demonstrated that the level of miR-346 was significantly decreased in subjects with overt proteinuria compared with the patients diagnosed with either normal albuminuria or microalbuminuria.

Also, correlation analysis revealed that the level ofmiR-346 was negatively correlated with serum creatinine and UACR and positively correlated with eGFR these results are in accordance with previous studies that showed the relation between miR-346 and kidney disease.

In experimental mice kidney study, miR-346 was reported to be expressed in mesangial fibrosis which is a major process during the pathogenesis of DN. Additionally, miR-346 was identified as a SMAD family-related miRNA has 6-11 complementary binding sites in the 3'-untranslated region (3'-UTR) of SMAD3/4[15, 30].

SMAD signaling pathway has been reported to has a key role in renal fibrosis through TGF-β activation which is considered an important mediator in the development of renal nephropathy in diabetes. Administration of miR-346 attenuated SMAD3/4 expression in the renal tissue and ameliorated the renal function and glomerular histology in the DN mice model suggesting the therapeutic efficacy of miR-346 in DN [12, 30-32].

The current study results also showed significant up-regulation in miR-92a expression level in all diabetic groups, which was more pronounced with increased albuminuria.

Significant negative correlation between miR-92a and eGFR, while positive correlations between miR-92a and blood glucose indices and the other kidney function was also observed.

These observations agree with the information that elevated glucose causes significant alterations in proximal tubular epithelial cells gene expression and is implicated as an important casual cause stimulus in DN[33, 34].

Supporting the above findings data publishedby Shang et al.[16] showed increased circulating levels of miR-92a in chronic kidney disease (CKD) andend stage renal disease with negative correlation with eGFR.

Shang et al.[16] also stated that endothelial dysfunction is a common complication of renal failurecaused due to increased oxidative stress in the vessel wall of CKD patients andthat miR-92a was induced in endothelial cells by oxidative stressindicating an endothelial origin of the circulating miR-92a in CKD patients.

Loyer et al.[35] showed that higher expression level of miR-92a and pro-inflammatory biomarkers were identified under the exposure condition of endothelial cells to oxidized LDL and low share stress. Moreover, they stated that suppression of miR-92a prevents endothelial dysfunction and atherosclerosis in mice.

Fujioka [18] reported that atherosclerosis-related CKD causes oxidative stress, in turn leading to miR-92a expression, endothelial dysfunction, and renal impairment. In addition, especially in CKD with late stage, oxidative stress derived from uremic toxin mainly contributes to microRNA-92a gene expression profile, endothelial dysfunction, and renal failure.

According to Wiese et al.[17], renal damage extremely increased endothelial miR-92a-3p and dual inhibition of miR-92a-3p and miR-489-3p mainly decreased atherosclerotic lesion compared to control.

In addition, Tsai et al.[36]noted significantly higher level of urinary exosomal miR-92a-1-5p in diabetics than in non-diabetics.

V. Conclusion

From the aforementioned results both miR-346 and miR-92a reflected the severity of DN with miR-92a showing good sensitivity and specificity to diagnose established DN progression. Therefore, it can be concluded that both miR-346 and mir-92a might be potential biomarkers for DN that shall encourage additional studies.

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Authors contributions

All the authors have contributed in designing, preforming, analyzing the data and writing of the manuscript.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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