Regulatory Role of Long Non-Coding RNA “Maternally-Expressed Gene-3” in Diabetic Nephropathy through Targeting microRNA-21 and Transforming Growth Factor-Beta

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Abstract

Introduction: Diabetic nephropathy (DN) is a leading cause of chronic kidney disease and end-stage renal disease worldwide. The maternally expressed gene-3 (MEG3), a lncRNA, is implicated in the development of diabetic microvascular complications like retinopathy and nephropathy. MicroRNA-21 (miRNA-21) and Transforming Growth Factor-Beta (TGF- β) are established playmakers in fibrogenesis in DN. Research stated that MEG3 can sponge miRNA-21 inhibiting it. Additionally, MEG3 modulates the activity of TGF- β genes by binding to promotor-distal regulatory elements.

Aim: Investigate MEG3 regulatory role in DN pathogenesis, and its possible inhibitory effect on miRNA-21 and TGF- β signaling pathway.

Methods: The study was performed on 75 subjects divided into 3 groups: 25 controls, 25 diabetics, and 25 DN patients. Participants were subjected to routine laboratory investigations, estimation of gene expression of MEG3 and miRNA-21 by RT-qPCR and measurement of serum TGF- β levels by ELISA.

Results: DN group shows a highly significant decrease in MEG-3 relative gene expression, and a significant increase in both miRNA-21 relative gene expression and TGF- β sera levels, when compared to diabetic and control groups. In addition, MEG3 is significantly negatively correlated to miRNA-21, TGF- β 1, and HbA1c confirming the sponging effect of MEG3 on miRNA-21 and predicting its potential protective role in DN. On the other hand, there is a statistically significant positive correlation between miRNA-21 gene expression and TGF- β 1 levels indicating the up-regulatory role of miRNA-21 on TGF- β 1 protein expression levels.

Conclusion: Our data suggests that MEG3 is implicated in DN pathogenesis through a possible loop encompassing miRNA-21 and TGF- β 1.

Highlights

- MicroRNA-21 and TGF- β are established playmakers in the pathogenesis of fibrosis in DN.
- MEG3, a lncRNA, can sponge miRNA-21 inhibiting it.
- MEG3 modulates the activity of TGF- β genes by binding to promotor-distal regulatory elements.
- Downregulation of MEG3 expression occurs upon TGF- β 1 treatment.
- MEG3 is greatly implicated in DN pathogenesis through a possible loop encompassing miRNA-21 and TGF- β 1.
- MEG-3 could impede the pathogenesis and the progression of DN, potentially through obturating miRNA-21 and TGF- β , with a potential negative feedback loop between elevated TGF- β and decreased MEG3 expression in DN.

Introduction

Diabetic nephropathy (DN) - which is one of the most serious complications of Diabetes Mellitus (DM) - has become the second cause of end-stage renal disease [1]. The pathophysiological abnormalities in the kidneys of DN patients are characterized by thickening of the basement membranes, accumulation of the extracellular matrix (ECM) proteins, widening of the podocytes slit membranes, and activation of myofibroblasts [2,3]. Consequently, renal dysfunction occurs in patients with DN in the form of impaired renal perfusion, glomerulopathy, proteinuria and hypertension [4]. Therefore, in depth comprehension of the molecular mechanisms of DN progression is of great importance for the improvement of therapeutic protocols for such disease.

MicroRNAs (miRNAs) are small non-coding RNAs of about 22 nucleotides. Their role in the incidence and progression of various diseases has become a topic of great importance in the medical field. They participate in gene expression regulation by translation suppression and mRNA stability modulation. Consequently, it has been demonstrated lately that various miRNAs share in DN pathogenesis and progression [5]. Usually, miRNAs act by binding to certain sites within the 3' untranslated region (UTR) of its target mRNAs, without complementary base pairing, to inhibit protein synthesis [6].

MicroRNA-21 (miRNA-21) is a multipotent miRNA involved in angiogenesis, cell proliferation promotion, immune destruction, and inflammation [7]. McClelland and colleagues confirmed that miRNA-21 is a key modulator in DN pathogenesis, as its expression has been found to be increased in biopsies from diabetic patients' kidneys that show advanced fibrotic stages of DN. Researchers also stated the important role of miRNA-21 in renal fibrosis through its effect on TGF- β and Phosphatase and tensin homolog (PTEN) [8]. Thus, miRNA-21 may represent a new therapeutic approach for treating DN through its antifibrotic effects [9].

A previous study demonstrated that miRNA-21 may be responsible for endothelial-to-mesenchymal transition and endothelial dysfunction, linking the increased miRNA-21 expression levels to the appearance of microalbuminuria, the occurrence of nephropathy and the deterioration of chronic kidney disease (CKD) leading to renal fibrosis. This could be explained by the modulation of transforming growth factor-beta (TGF- β) and its signaling pathway inhibitor smad7. Increased miRNA-21 level downregulates its well-established target smad7, resulting in the deposition of collagen types IV and I, the thickening of the glomerular basement membrane, and the hyperplasia of the mesangial matrix which are key pathological characteristics of DN [10].

Long non-coding RNA (lncRNA) is a type of RNA transcripts of more than 200 untranslatable nucleotides [11]. Lately, lncRNAs are gaining much interest in the medical research field. They play vital roles in different physiological processes. In addition, lncRNAs may be involved in the pathogenesis of many diseases through their sponging effect on miRNAs, according to the competing endogenous RNA (ceRNA) hypothesis [12]. Although the ceRNAs are the most recently discovered entrants in the different mechanisms of miRNA mediated gene regulation, we need more studies to fully understand their mechanisms of action [13].

An identified lncRNA called Maternally Expressed Gene-3 (Meg3) has been involved in diabetic retinopathy [14], and DN development [12]. It has been found that MEG3 serum levels are significantly lower in diabetic retinopathy patients and diabetic patients without retinopathy than in normal control, suggesting that MEG3 down-regulation may be involved in diabetic retinopathy development and other microvascular complications of diabetes [14].

Recently, Using Bioinformatic analysis, Lin et al., illustrated binding sites between MEG3 and miRNA-21. This was further validated by dual-luciferase reporter gene assay in cell lines of nasopharyngeal carcinoma (HK-1) [15]. According to these results, we performed the current study to investigate the underlying regulatory role of the lncRNA MEG3 in the pathogenesis of diabetic nephropathy, through targeting the miRNA-21 and TGF- β signaling pathway.

Subjects And Methods

4.1 Ethical approval

This study is authorized by Kasr Al-Ainy hospital's local ethical committee in compliance with the Declaration of Helsinki principals. Written informed consent from all participants was obtained before sample collection.

4.2 Design and Participants

This cross-sectional study was performed on 75 subjects of matched age and sex. They were divided into 3 groups: 25 control subjects, 25 diabetic patients, and 25 DN patients. All patients were recruited from the diabetes and internal medicine clinics, faculty of medicine, Cairo university, during the period between June 2020 to June 2021.

All patients in this study underwent comprehensive history taking including duration and type of diabetes mellitus, grade of DN, and the type of treatment of the disease, in addition to, thorough clinical examination. Patients were excluded from the study if they were suffering from other chronic systemic inflammatory diseases, autoimmune diseases, malignancy, or concurrent use of corticosteroids. All participants were subjected to routine laboratory investigations including kidney function tests, fasting blood glucose (FBG) and 2 hours post prandial blood glucose (2-hPPG) tests, glycated hemoglobin (HbA1c), and urinary albumin/creatinine ratio (ACR). Five ml of peripheral venous blood samples were withdrawn from each subject and were kept at room temperature for 1 h. Centrifugation was then done to collect sera at 2,500 x g for 10 min. Sera were stored at -80°C until further analysis.

4.3 Estimation of gene expression of lncRNA-MEG3 and miRNA-21 by QRT-PCR

Total RNA extraction from sera was done using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Quality check of RNA was done using NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Afterwards complementary DNA (cDNA) synthesis using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher

Scientific) was done according to the instructions. Real time-qPCR was then performed to amplify MEG3 and miR-21 using SYBR Premix Ex Taq™ II (Perfect Real Time, TaKaRa, Japan). The following primers were used: MEG3 forward primer: 5'-GCATTAAGCCCTGACCTTTG-3' and reverse primer: 5'-TCCAGTTTGCTAGCAGGTGA-3'; miRNA-21 forward primer: 5'-TAGCTTATCAGACTGATGTTGA-3' and reverse primer, 5'-AGTGCGTGTCGTGG-3'. PCR reaction conditions were as follows: 95°C for 5 min, then 40 cycles on 95°C for 15 seconds then on 60°C for 60 seconds. The $2^{-\Delta\Delta Ct}$ method was applied to quantitatively analyze results. Normalization to U6 snRNA was done.

4.4 Measurement of serum TGF-β levels by ELISA (MyBioSource, San Diego, California, USA): This was done according to the manufacturer’s recommendations.

4.5 Statistical Analysis

Statistical analyses were executed using IBM SPSS Statistics software version 23. A (P value <0.05) was considered the cut-off value for statistical significance. GraphPad Prism Software version 7.0.0 was used to plot the graphs. We used One-way ANOVA (parametric test, P> 0.05). The chi-square (χ²) test was used for categorical variables. Continuous variables are expressed as mean ± SD. Analysis of correlation between variables was assessed using pearson correlation [16].

Results

5.1 Comparisons of the demographic features in all studied groups:

As shown in Table 1, regarding age, gender, and disease duration, there is no statistically significant difference between the studied groups (p value > 0.05), indicating that the studied groups are properly matched.

Table (1): Demographic data among the studied groups:

	Control (n=25)	Diabetic patients (n=25)	Diabetic nephropathy patients (DN) (n=25)	P-value
Age (years)	50 ± 7	56 ± 11	54± 8.1	0.1
Gender:				
Male	6 (32%)	5 (20%)	8 (24%)	0.61
Female	19 (68%)	20 (80%)	17(76%)	
Disease duration	————	3M-5.1Y 1.5	5M-3.5Y 2.9	0.055

Data are expressed as mean ± SD for age but expressed as minimum and maximum values with the median for the disease duration. P-value < 0.05 is considered statistically significant.

5.2 Comparisons of the routine laboratory investigations in all studied groups:

As shown in table (2), significantly higher values of serum FBG, 2-hPPG and HbA1c are seen in diabetic and DN patients when compared to control. Also, FBG and HbA1c levels in DN are significantly higher than in diabetic patients.

As regards kidney function tests, serum urea and creatinine show significantly higher levels in diabetic and DN patients when compared to the control group. Moreover, serum urea levels and the urinary ACR show significantly higher levels in DN when compared to diabetic patients. On the other hand, there is no significant difference regarding the serum uric acid levels among the studied groups.

Table (2): Routine laboratory investigations among the studied groups:

Routine laboratory investigations	Control (n=25)	Diabetic patients (n=25)	Diabetic nephropathy patients (n=25)	P-value
FBG (mg/dl)	85 ± 8.8	148 ± 33*	178 ± 57*#	0.03
2-hPPG (mg/dl)	114± 14	266± 54*	280± 66*	<0.0001
HbA1c (%)	5.5 ± 0.78	8.4 ± 1.6 *	9.7 ± 1.5*#	<0.0001
Urea (mg/dl)	33 ± 6.7	50 ± 21*	66 ± 20*#	< 0.0001
Creatinine (mg/dl)	0.82 ± 0.15	1.3± 0.26*	1.4± 0.85*	<0.002
ACR (mg/g)	9.4 ± 4	13 ± 4.7	207 ± 97*#	<0.0001
Uric acid (mg/dl)	5.8 ± 1.1	5.1± 1.5	5.9± 1.5	0.12

Data expressed as mean ± SD or n (%). P-value < 0.05 is considered statistically significant. FBG: Fasting blood glucose, 2-hPPG: 2 hours postprandial glucose, HbA1c: HemoglobinA1c, ACR: Albumin creatinine ratio.

* Significant difference versus the control group.

Significant difference versus the diabetic group.

5.3 Relative gene expression of MEG3 and miRNA-21 in all studied groups:

As regards MEG3 relative gene expression levels, Figure (1) shows a significant decrease in diabetic and DN patients as compared to the control group. In addition, DN patients show significantly decreased levels as compared to diabetic patients ($p < 0.001$).

The results of miRNA-21 relative expression demonstrate a significant increase in diabetic and DN groups as compared to the control group. Moreover, a significant increase of miRNA-21 relative expression in DN is seen when compared to diabetic patients ($p < 0.001$).

5.4 Serum TGF- β 1 levels among the studied groups

Figure (2) shows a significant increase in TGF- β 1 levels in diabetic and DN patients in comparison with the control group. Furthermore, a significant increase is detected in the DN group as compared to the diabetic group.

5.5 Correlations between MEG3, miRNA-21, TGF- β 1, HbA1c, and ACR in all studied groups

As shown in Figure (3), there is a strong significant negative correlation between MEG3 on one hand, and miRNA-21 and TGF- β 1 gene expressions on the other hand, with p-values (< 0.0001) and r values (-0.75 and -0.63) respectively. This indicates the inhibitory effect of MEG3 on miRNA-21 and TGF- β 1 in DN patients.

There is also a statistically strong significant positive correlation between miRNA-21 gene expression and TGF- β 1 protein expression levels ($r = 0.91$ & $p\text{-value} < 0.0001$), indicating the up-regulatory role of miRNA-21 on TGF- β 1 protein levels, causing progression of DN.

Moreover, there is a positive correlation between miRNA-21 with HbA1c and ACR. This correlation is statistically strong with HbA1c and non-significant with ACR ($p\text{-values} < 0.0001$, 0.09 – $r = 0.76$ and 0.26 respectively) in DN patients, reflecting the effect of increased miRNA-21 gene expression on the severity of the DN. In addition to that, MEG3 shows a strong negative correlation with HbA1c with a $p\text{-value} < 0.0001$ and an $r = -0.55$, showing the potential protective effect of MEG3 in Diabetes (figure 4).

Discussion

Not only that Diabetic Nephropathy (DN) is one of the most frequent complications of Diabetes Mellitus, but it also increases morbidity and mortality [17]. Approximately 40% of DN patients yearly develop End-Stage Renal Disease (ESRD) [18]. Established DN hallmarks are persistent progressive albuminuria and declining renal functions, with coexisting microvascular complications and absence of other kidney diseases [19]. Current therapies are not able to competently prevent disease progression to ESRD in most patients. This concludes the importance of investigating molecular mechanisms – including long non-coding RNAs (lncRNAs), and mediators underlying DN aiming for the development of better therapeutic protocols [20, 21].

Various microRNAs have been studied extensively over the past few decades in DN pathogenesis [22]. Some microRNAs are upregulated in DN such as miRNA-21, miR-34a-5p, miR-141, and miR-370 [23]. Among these, miRNA-21 is found to provoke renal injury and fibrosis by impairing the cell cycle and inducing mesangial hypertrophy. The molecular mechanism thought to be involved in this is the

downregulation of SMAD7 and PTEN which activates TGF- β 1 and Akt signaling pathways respectively [22].

The current study aims to better understand MEG3 lncRNA's regulatory involvement in the pathogenesis of DN, via targeting miRNA-21 and TGF- β signaling pathways.

MEG3 is a maternally imprinted gene in the Dlk1-Gtl2 locus on chromosome 14q32.3 in human. MEG3 is known as a tumor suppressor gene that plays an important role in the development and the progression of various diseases [24]. Our results regarding MEG-3 clearly demonstrate significant reduction of MEG3 expression in diabetic and DN patients as compared to the control group. In addition, MEG3 expression is significantly decreased in DN patients compared to diabetic patients which suggests the involvement of MEG3 in the pathogenesis of DM complications.

Our results coincide with a previously reported study where MEG3 expression was significantly reduced in renal tissues of DN rats compared to normal renal tissues. Moreover, MEG3 expression was significantly decreased in a time and dose dependent manner while investigating the effect of high glucose treatment. High glucose stimulation was found to activate β -catenin and upregulate the expression of its target genes (α -SMA and snail1) in podocytes. These results indicate a correlation between MEG3 and podocyte injury due to the activation of Wnt/ β -catenin pathway following high glucose treatment [25].

In line with the same context, a study on ischemic brain injury showed that reduced MEG3 expression was responsible for accelerated angiogenesis [26], a key step in the progression of DN [14, 27]. Similarly, **Qiu et al., (2016)** stated significant down-regulation of MEG3 expression in STZ-induced diabetic mice retina and endothelial cells upon high glucose exposure and oxidative stress [28]. On the contrary, **Zha et al., (2019)** found upregulated MEG3 in DN rats and in vitro. The team suggested that the progression of cell fibrosis and inflammatory response in mesangial cells were mediated by modulating toll-like receptor 4 axis through MEG3 sponging effect on miR-181a [29]. An explanation of this controversy could be the discrete human-specific gene expression patterns that formulate gene networks complex enough to tune different pathways.

Our results regarding miRNA-21 relative gene expression obviously demonstrate significant upregulation of miRNA-21 gene in diabetic patients, with further increase in DN group. In addition, our results show a negative but non-significant correlation between MEG3 and miRNA-21 gene expression levels. A possible explanation would be the limited sample size, as statistical significance depends on sample size rather than on degree of correlation. Consequently, for small sample sizes, very high correlations can be detected without statistical significance.

Coinciding with us, **Zhong et al., (2013)** confirmed that miRNA-21 level in fibrosed renal tissue is upregulated. In renal tubules, miRNA-21 could regulate mesangial expansion, interstitial fibrosis, macrophage infiltration, podocyte loss, albuminuria, and induced fibrotic and inflammatory genes' expressions related to diabetic nephropathy [30]. In addition, upregulation of miRNA-21 expression

induces endothelial dysfunction and low-grade inflammation seen in diabetic retinopathy, an eminent microvascular complication of DM frequently associated with DN [31].

In agreement with our results, **Dan et al., (2018)** and **Jia et al., (2019)** proved the presence of a negative regulatory tie between MEG3 and miRNA-21 suggesting a direct binding site on miRNA-21 for MEG3. Interestingly, Dan and colleagues performed their studies on gastric cancer tissue and cell lines. They applied the luciferase report assay to elaborate miRNA-21 as a MEG3 target. their qRT-PCR study elucidated negative regulation of miRNA-21 expression by MEG3. Moreover, MEG3 overexpression in GC cells counteracted miR-21 effects illustrating the inhibitory effect of MEG3 on miR-21 expression [32,33].

In this study, the expression of TGF- β 1, a key mediator of DN and a pleiotropic cytokine [34], is significantly increased in diabetic and DN patients in comparison with the control group with accentuated results in DN patients. Furthermore, A statistically significant positive correlation is established between miRNA-21 gene expression and the TGF- β 1 protein levels, confirming the up-regulatory role of miRNA-21 on the TGF- β 1.

Concurring with our results, **Zhou et al., (2018)** investigated serum TGF- β 1 levels in type-2 diabetes mellitus (T2DM) patients. Macroalbuminuric T2DM patients had increased TGF- β 1 levels when compared with microalbuminuric T2DM patients. The latter group also showed increased TGF- β 1 levels when compared with normoalbuminuric T2DM patients [35]. DN is a disease that shows progressive podocyte injury. It was stated that TGF- β 1 is strongly expressed in podocytes of DN kidneys indicating a role of TGF- β in podocyte injury [36]. The modulatory action of miRNA-21 on TGF- β 1 may result in the pro-sclerotic effect that ultimately results in renal fibrosis [8].

Additionally, a previous report demonstrated that miRNA-21 overexpression - by hindering its target gene smad7 - may actuate TGF- β 1-induced Epithelial-to-mesenchymal transition, exacerbating renal damage [7]. Furthermore, a positive feedback loop where TGF- β can activate Smad3, a direct inducer of miRNA-21 transcription, is suggested. The elicited miRNA-21 then inhibits the translation of TGF- β inhibitor 'smad7'. This eventually increases the availability of TGF- β and promotes fibrosis in affected tissue. It is worth mentioning that miRNA-21 can aggravate the progression and direct the phenotypes of DN. A positive correlation was documented in previous research between upregulated miRNA-21 expression and biomarkers of inflammatory and fibrotic pathways like ACR, collagen IV, fibronectin, tissue inhibitor of metalloproteinase 1 [37] and HbA1c [10].

Interestingly, **Mondal and colleagues** Using a modified chromatin oligo affinity precipitation (ChOP) method, mapped genome-wide chromatin-binding sites for MEG3. They revealed that MEG3 modulates the activity of TGF- β genes by binding to promotor-distal regulatory elements. MEG3 binding sites showed enrichment in GA-rich sequences which guide MEG3 to its target genes through formation of RNA–DNA triplex structures [38]. This parallelizes another study that states downregulation of MEG3 expression upon TGF- β 1 treatment in human hepatic stellate cells, in addition to the inhibition of MEG3 overexpression effects on cells [39]. Considering these data along with our observation, we suggest a probable feedback loop between miRNA-21, TGF- β and MEG3, through which DN progression occurs.

Our results, in accordance with other studies such as **Shilpa and Ayyali (2021)**, record FBG, HbA1c and urinary ACR levels significantly higher in diabetic and DN groups than in control with more accentuated levels in DN group. Also, urinary ACR and HbA1c positively correlate to miRNA-21 gene expression levels [40].

In conclusion, MEG-3 could impede the pathogenesis and the progression of DN, potentially through obturating miRNA-21 and TGF- β , with a potential negative feedback loop between elevated TGF- β and decreased MEG3 expression in DN.

Declarations

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Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

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Figures

Figure 1

Mean $\pm$ SD of the fold change of MEG3 and miRNA-21 expression among the studied groups. * denotes highly significant difference versus the control group. # denotes highly significant difference versus the diabetic group (p value < 0.001)

Figure 2

Mean $\pm$ SD of the protein expression levels of TGF- β 1 among the studied groups. * Denotes highly significant difference versus the control group. # Denotes highly significant difference versus the diabetic group (p value < 0.001).

Figure 3

correlations between MEG3, miRNA-21 and TGF- β 1. *r*: Correlation coefficient. *P*-value < 0.05 is considered statistically significant.

Figure 4

correlation between miRNA-21, MEG3 and HbA1c. *r*: Correlation coefficient. *P*-value < 0.05 is considered statistically significant.