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Upregulation of Long Non-coding RNA MEG3 and miR-181b in Type 2 Diabetic Patients with Cerebral Stroke

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Abstract

Several studies have demonstrated that long non-coding RNA (lncRNA) and microRNA (miRNA) molecules were closely related to the pathogenesis of diabetes mellitus (DM) and its complications. This study aimed to explore the possible relationship of lncRNA Maternally Expressed Gene 3 (MEG3) and miR-181b with the development of ischemic cerebral stroke (CS) as a complication of type 2 DM. The study involved 20 diabetic patients (DM), 20 diabetic patients with CS (DM+CS), 17 CS patients and 10 apparently healthy subjects. The expression levels of MEG3 and miR-181b were analyzed in serum samples using real time PCR. The expression of MEG3 was significantly higher in both DM+CS and CS groups as compared to DM or control group. In addition, miR-181b was significantly upregulated in DM+CS and CS compared to control group. In conclusion, this study suggested that MEG3 and miR-181b may play a role in the progression of CS in diabetic patients.

Keywords: Cerebral stroke; MEG3; miR-181b; type-2 diabetes mellitus

1. Introduction

Diabetes mellitus (DM) is a disorder of metabolism characterized by persistent elevation of blood glucose level, which may be fatal if not managed appropriately [1]. The persistent elevation of blood glucose is caused by hypo-insulinemia due to pancreatic β-cell failure (type 1 DM) and/or insulin insensitivity due to periphery insulin resistance (type 2 DM), diabetes may be resulted from both genetic and environmental factors [2]. The global prevalence of DM was 10.5% (536.6 million people) in 2021 and estimated to be raised to 12.2% (783.2 million) in 2045 while in Egypt, the prevalence was 20.9% in 2021 and estimated to be 23.4% in 2045 [3].

Diabetes mellitus is usually accompanied by vascular complications including both the macrovascular (cardiovascular disease) and microvascular system (diabetic kidney, retinopathy, and neuropathy diseases) and these complications are the leading cause of both morbidity and mortality in individuals with diabetes [4].

Diabetes is accompanied with higher risk of cerebral stroke (CS) as hyperglycemia may induce damage of the macrovascular system, including the cerebrovascular and coronary arteries which may lead to death in individuals with diabetes [4]. Both diabetes and stroke have substantial economic and community-based consequences around the world. As a result, gaining a better knowledge of stroke in diabetes can aid in the prevention of occurrences and the development of new therapeutic targets, both of which are top priority in stroke research [5].

Cerebral stroke is characterized by insufficient supply of blood, oxygen, and nutrients to the brain due to cerebrovascular rupture or blockage [6] . There are three types of strokes, ischemic (due to vascular blockage), transient ischemic attack (due to temporary vascular blockage) and hemorrhagic (due to vascular rupture). As a result of bleeding or inadequate blood supply to the brain, a death of neuronal cells and rapid loss of the main functions of the brain perhaps be developed, which may be permanent [7]. Common comorbidities caused by DM including atrial

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fibrillation, hypercholesterolemia, atherosclerosis, hypertension and obesity, all of these comorbidities contribute to high risk of CS independently [8].

Diabetes increases the risk of CS through a gathering of different factors including low high-density lipoprotein cholesterol (HDL-C), Glycated hemoglobin A1C (HbA1c) and microvascular complications [9]. Several possible mechanisms in which diabetes may lead to CS include; dysfunction of vascular endothelial cells, systemic inflammation, increased early-age arterial stiffness, and thickening of the capillary walls [10].

Non-coding ribonucleic nucleic acids (RNAs) are recognized as RNA molecules that regulate cellular homeostasis during growth and involved in the development and pathogenesis of diseases [11]. According to the size, non-coding RNA are classified into long non-coding RNA (lncRNA) which is >200 nucleotides in length and microRNA (miRNA) which is 20~22 nucleotides [12]. lncRNAs are thought to regulate homeostasis of cells during pathogenesis of diseases by inhibition of miRNA function [13].

Human Maternally Expressed Gene 3 (MEG3) is an imprinted gene that is located on the chromosome 14q32.3 in human genome, encodes a noncoding RNA and expressed in many normal tissues [14]. MEG3 is an antiproliferative gene in cancer and is considered as a tumor suppressor [15]. Inhibition of MEG3 expression leads to formation of various types of cancers, while upregulation of MEG3 leads to inhibition of them [16]. Several results revealed that MEG3 is involved in biological processes of CS [14, 17]. Upregulation of cerebral MEG3 was observed following ischemia and thought to mediate ischemic neuronal death via activating of tumor protein p53 (p53) [18]. Downregulation of MEG3 was shown to improve neurobehavioral outcomes and protect against ischemic damage [19, 20].

Different miRNAs were studied for their role in early detection of DM and its complications. Yan et al., construct a multi-parameter model for the early detection of type 2 DM (T2DM) consisting of miR-148b, miR-223, miR-130a, and miR-19a [21]. Elalawi et al., reported that miR-377 might be a useful promising biomarker for prediction the development of nephropathy in T2DM patients.[22]. Moreover, hsa-miR-novel-chr5_15976, hsa-let-7a-5p, and hsa-miR-28-3p were reported to had great role as biomarkers for detection of early stage T2DM with retinopathy [23]

Several studies have shown that particular miRNAs play a role in neurogenesis, neuronal differentiation, neural cell specification, and neuronal function development. [24, 25]. In the etiology of stroke, miRNAs have distinct expression patterns that modulate pathogenic processes, including hyperlipidemia, atherosclerosis, hypertension, and plaque rupture [26].

Platelet activation is one of the underlying mechanisms for initiation and progression of atherosclerosis and thrombogenicity which are common complications of T2DM that frequently lead to ischemia at later stages of the disease [27]. Platelets are one of major sources of circulating miRNAs as they contain large amounts of miRNAs that regulate molecular targets in atherosclerosis [28]. Some of miRNAs shows antithrombogenic activity in diabetes, for example miR-126 reduces the primary initiator of blood coagulation released from blood vessels that is called tissue factor (TF) and hence exhibits antithrombotic function [29]. miR-19a also reduces the expression of TF and strongly associated with miR-126 to work in a cooperative manner to protect against thrombogenicity in diabetic patients [30]. Tissue factor was also found to be posttranscriptional regulated by miR-181b that play an important role in improvement of diabetes related thrombogenicity and coagulability [31].

miR-181b was demonstrated to play a crucial role in alteration of circulating low-density lipoprotein cholesterol (LDL-C) levels [32] furthermore, downregulation of miR-181b was shown to protect against neural ischemic stroke (IS) [33, 34].

This study aimed to evaluate the possible relationship of MEG3 and miR-181b with the development of ischemic cerebral stroke as a complication of type 2 DM.

2. Patients and Methods

Total 67 Egyptian participants were included in this study, 57 patients, and 10 healthy volunteers. Patients were selected from the Tropical Medicine Department of Kasr El-Aini hospital, Cairo University, Egypt, and included 26 males and 31 females with an average age of 61.5 years (range 41 – 75 years). Patients were divided into three groups. A group of 20 patients with T2DM without CS, group of 20 patients with T2DM+CS and a group of 17 patients with CS without T2DM. Patients with T2DM were diagnosed according to HbA1c levels >=6.5%, while CS cases were ischemic and diagnosed via CT scanning.

Ten healthy volunteers, 4 males and 6 females, with an average age of 62.8 years, were chosen as the control group (Range 47 – 78 years). Diabetic patients with any complications other than CS, patients with either acute or chronic infection, autoimmune diseases, systemic inflammation, cancer, and other diseases were excluded from the study.

The Declaration of Helsinki (DOH) was followed during the research. Medical study involving human beings was conducted in accordance with all ethical

norms. Throughout the study, the confidentiality and rights of the human subjects were respected. Each participant was given a signed informed consent form to sign.

2.1. Biochemical parameters

An aliquot of 5 ml venous blood specimens was collected from each subject, 3 ml was placed in Gold-top serum separator tubes (Gold top-SST), left to stand at room temperature for 10 minutes, and subsequently centrifuged at (3000 rpm) for 15 minutes to separate serum for measuring lipid profile and total RNA extraction. The other 2 ml was placed in grey-top vacutainer (Sodium fluoride containing tubes) for determination of fasting and postprandial blood glucose.

Fasting blood sugar (FBS), 2 hours post prandial plasma glucose (2HPP) (Code: 11503), Serum triacylglycerols (TAGs) (Code: 11529), total cholesterol (TC) (Code: 11505), HDL-C (Code: 11648) and LDL-C precipitating reagent (Code: 11579) were measured according to manufacture instructions using Biosystem Chemistry Autoanalyzer A15. Kits were obtained from BioSystems (S.A., Barcelona Spain). Atherogenic index (AI) for each sample was calculated from the following equation: AI = (Total cholesterol – HDL-cholesterol)/ HDL-cholesterol [35].

Long non-coding RNA and miRNA assay:

Total RNA was extracted from serum by miRNeasy extraction kit (Qiagen, Valenica, CA) using QIAzol lysis reagent as directed by manufacturer's instructions. Quality of RNA was determined using NanoDrop2000 (Thermo Fisher Scientific, USA). Reverse transcription (RT) was carried out on 60 ng of total RNA in a final volume 20 µl RT reactions using RT2 first strand Kit (Qiagen, Valenica, CA) according to the manufacturer's instructions.

The RT products were diluted with 50 µl RNAase-free water before quantitative real-time PCR (qPCR). Serum expression levels of the MEG3 and miR-181b were evaluated using GAPDH and SNORD 68 as internal control respectively using customized primers provided by Qiagen (Qiagen, Valenica, CA) and Maxima SYBR Green PCR kit (Thermo, USA) according to the manufacturer's protocol. The primer sequences used for qPCR were as follow:

MEG3 forward, CTGCCCATCTACACCTCACG;
MEG3 reverse, CTCTCCGCCGTCTGCGCTAGGG;
GAPDH forward, GTCAACGGATTTGGTCTGTATT;
GAPDH reverse, AGTCTTCTGGGTGGCAGTGAT

Miscript primer assay for Hs-miR-181b-5p catalogue number (MS00006699). The fold change in MEG3 and miR-181b-5p and expression levels were calculated using the equation $2^{-\Delta\Delta Ct.}$

2.2. Statistical analysis

All descriptive statistics, data analyses, and graphics were conducted using GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA). All data were subjected to Shapiro-Wilk Test of Normality. For data that passed the normality test, One-Way ANOVA test followed by Tukey's analysis method (post-hoc analysis) was used for comparison between means. While Kruskal–Wallis followed by Dunn's multiple comparison test was used for Data that didn't pass. Outliers were identified and removed using ROUT method. P value < 0.05 was set as the significant level.

3. Results

All data were subjected to Shapiro-Wilk Test of Normality. Outliers were identified and removed using ROUT method.

3.1. Biochemical measures

Table 1 summarized changes in the studied biochemical parameters. FBS and 2HPP levels were significantly higher in DM group (145 ± 46.5 mg%, 236 ± 68.8 mg% respectively) than in control group (86.4 ± 7.01 mg%, 124 ± 10.2 mg%) and CS group (86.8 ± 9.89 mg%, 129 ± 13.9 mg%). while their levels in patients with DM+CS (163 ± 37.2 mg%, 303 ±87.0 mg%) were significantly increased than in control and in patients with CS only. TAGs were increased significantly in DM patients (152 ± 68.3 mg%) than in patients with CS (102 ± 30.4 mg%). HDL-C levels were significantly lower in patients with CS (34.1 ± 9.18 mg%) and patients with DM+CS (29.1 ± 10.8 mg%) than in control group (44.5 ± 3.60 mg%). For the calculated AI, it was significantly higher in DM, CS and DM+CS groups than in control (Table 1).

3.2. MEG 3 expression

Expression of MEG3 was significantly higher in both CS and DM+CS groups than in healthy control and DM group ($P<0.05$). MEG3 expression was higher in DM+CS group than in DM group but the difference was not statistically significant ($P<0.05$) (Table 2, Figure 1).

3.3. miR-181b expression

miR-181b was significantly highly expressed in both CS and DM+CS groups than in DM group and healthy control. While its expression in CS group was highly significant than in DM group ($P<0.05$) (Table 2, Figure 2).

Table 1. comparison of the clinical characteristics between the studied groups

	Control n = 10	DM n = 20	CS n = 20	DM+CS n = 17
Males/females	4/6	6/14	13/7	7/10
Age (years)	62.8 ± 9.72	58.5 ± 11. 2	59.9 ± 8.04	66.1 ± 5.72
FBS (mg%)	86.4 ± 7.01	145 ± 46.5 [a]	86.8 ± 9.89 [b]	163 ± 37.2 [ac]
2HPP (mg%)	124 ± 10.2	236 ± 68.8 [a]	129 ± 13.9 [b]	303 ±87.0 [abc]
TAGs (mg%)	110 ± 18.9	152 ± 68.3	102 ± 30.4 [b]	125 ± 63.1
TC (mg%)	167 ± 22.6	189 ± 45.2	162 ± 47.8	157 ± 56.4
HDL-C (mg%)	44.5 ± 3.60	35.1 ± 9.89	34.1 ± 9.18 [a]	29.1 ± 10.8 [a]
LDL-C (mg%)	74.2 ± 10.5	86.0 ± 17.1	105 ± 36.6	101 ± 51.3
AI	2.21 ± 0.69	4.72 ± 1.76 [a]	4.08 ± 1.59 [a]	4.31 ± 1.68 [a]

Values are expressed as means ± SD; Significant different is considered if P value < 0.05 using One-way ANOVA followed by Tukey's multiple comparison test.

TAGs: Triacylglycerols; TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; AI: Atherogenic index FBS: Fasting blood sugar; 2HPP: 2 hours postprandial blood sugar; DM: Diabetes Mellitus; CS: Cerebral Stroke

[a] significantly different from control group

[b] significantly different from DM group

[c] significantly different from CS group

Table 2. relative expression of MEG3 and miR-181b in the studied groups.

	Control	DM	CS	DM+CS
MEG3	1	1.21 (0.56-2.22) [0.10-3.50]	5.09 (2.58-23.4) [1.73-28.8] [ab]	7.01 (5.09-9.15) [4.28-11.5] [ab]
miR181b	1	8.70 (2.56-15.3) [0.41-36.4]	27.9 (7.65-54.8) [4.14-89.2] [ab]	14.4 (4.50-72.8) [2.91-99.0][a]

Data are expressed as median, (IQR) and [range]. Significant different is considered if P value < 0.05 using Kruskal–Wallis followed by Dunn's multiple comparison test

[a] significantly different from control group [b] significantly different from DM group [c] significantly different from CS group

4. Discussion

According to the International Diabetes Federation, DM is a chronic metabolic disease that affects 536.6 million adults in 2021 and will reach 783.2 million by 2045 [3]. As a chronic disease, many complications of diabetes may be induced including either microvascular as neuropathy, nephropathy, and retinopathy, or macrovascular complications including cardiovascular disease, CS, and peripheral artery disease [36].

lncRNA and miRNAs are key regulators for controlling cell functions and can be applied as early diagnostic and/or therapeutic markers in conditions accompanied by insulin resistance [37].

MEG3 is a lncRNA located on chromosome 14q32.3 in human genome, was identified early with function of tumor suppressor [15]. Mounting evidence has also revealed that MEG3 has a role in T2DM and insulin resistance in the pancreas [38]. The role of MEG 3 was studied in complications of diabetes including diabetic retinopathy [39, 40], diabetic nephropathy [41-43] and diabetic neuropathy [44].

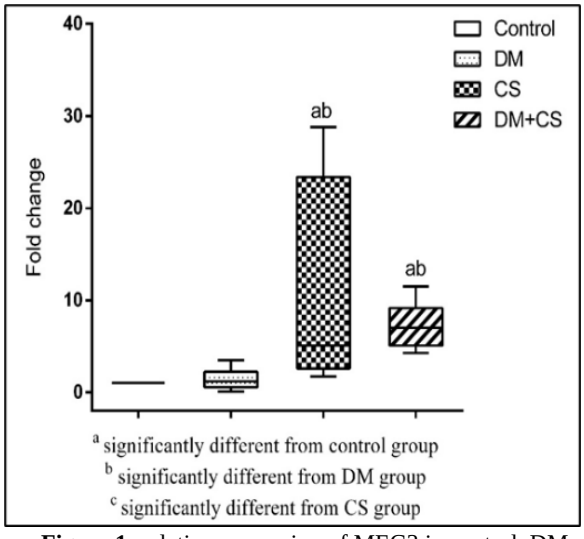

Figure 1: relative expression of MEG3 in control, DM, CS and DM+CS groups

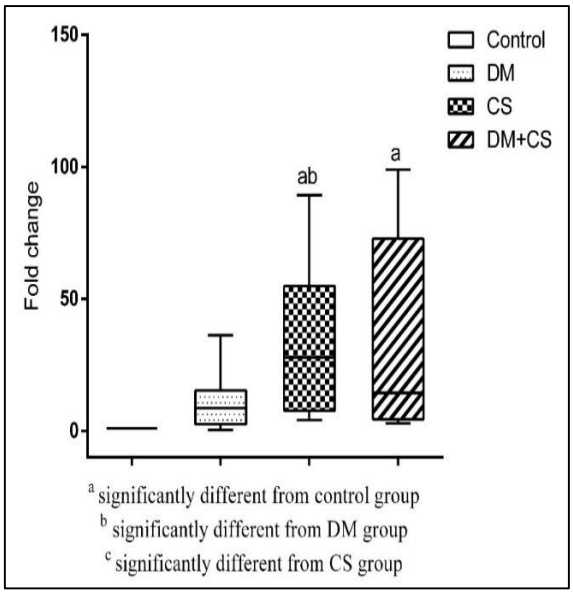

Figure 2: relative expression of miR-181b in control, DM, CS and DM+CS groups.

For the best knowledge, it is the first time to estimate the expression of MEG3 in diabetic patients with CS as a macrovascular complication. Results of this study showed that MEG3 was significantly upregulated in CS (5 folds) and in D+CS patients (7 folds) than in healthy control and diabetic patients. This finding agrees with another study carried out by Chang and his colleagues in which MEG3 expression was measured in diabetic patients with vascular complications. They found that MEG3 expression was significantly increased in diabetic patients with vascular complications more than in diabetic and control group [45]. Results also showed that MEG3 expression in DM group was higher than in normal, but the difference was not statistically significant, a similar finding was observed by Change et al.

In other diabetic complications, MEG3 was also dysregulated. For example, in diabetic nephropathy MEG3 was upregulated and could promote cell fibrosis and inflammatory response [43]. Li et al. showed that MEG3 was significantly upregulated in the serum of diabetic nephropathy patients than in healthy persons, and found MEG3 level was highly expressed in different stages of diabetic nephropathy, implying that MEG3 was concerned with the severity of diabetic nephropathy [42].

MEG3 is documented to be an important regulator in controlling neuronal cell death during the ischemic pathological process and its expression is upregulated and can aggravate the injury induced by ischemia through acting as competing endogenous RNAs of some miRNAs [46].

After CS, angiogenesis occurs in the border of the ischemic core and periphery within 4 to 7 days as neuroprotective mechanism [47]. Liu and his colleagues discovered that vascular endothelial cells could promote angiogenesis by proliferating, migrating, and forming tubes when MEG3 levels are low [20]. Another study showed that MEG3 upregulation significantly suppressed the proliferation and in vitro angiogenesis in vascular endothelial cells [48]. MEG3 was also found to function as a cell death promoter in cerebral ischemia through interaction with p53 to mediate ischemic damage [17].

In a study conducted by Wang et al., middle cerebral artery occlusion (MCAO) in mice was performed to stimulate an in-vivo IS model and oxygen–glucose deprivation (OGD) in human brain microvascular endothelial cells (hBMECs) were performed to simulate an in-vitro IS model. Wang and his team revealed that In-vivo study showed that MEG3 increased significantly in the IS group and the survival time of the mouse with the high MEG3 group was significantly lower than that with the low MEG3 group. In-vitro study showed that OGD treatment upregulated expressions of MEG3, Bax, and cleaved caspase-3 and promoted apoptosis of hBMECs, while silencing MEG3 blocked these effects [49]. Inhibition of MEG3 could protect from the death of neuronal cells, decrease the infarct area, promote neurogenesis and accelerate recovery of neurological function in brain [47]. The previous data suggest that upregulation of MEG3 may participate in the development and progression of diabetic complications including CS and may differentiate between diabetic patients without and those with CS.

In the present study, miR-181b was significantly highly expressed in both CS and DM+CS groups than in control group, suggesting that miR-181b may play a role in the development and prognosis of CS in diabetic patients. Another study showed that miR-181b was highly expressed in diabetic complications, Zhu et al. measured the expression of miR-181b in diabetic patients complicated with nephropathy and found that miR-181b was highly expressed more than in control suggesting that miR-181b may be a useful marker for prognosis of diabetic nephropathy [50].

Embolism of cerebral blood vessels by stenotic atherosclerotic plaques in the aortic arch or intracranial vessels is important cause of ischemic CS [51]. Several studies revealed that miR-181b is atheroprotective. A study carried out by An et al., showed that serum miR-181b levels were decreased in acute stroke patients with atherosclerotic plaque [52].

Sun et al., showed that miR-181b decreases vascular inflammation and atherosclerosis through targeting and hence inhibition of nuclear factor-κB in the intima [53].

In the present study, the atherogenic index was significantly higher in DM, CS and DM+CS groups than in control group, indicating a high risk of atherosclerosis in these groups. Another study showed that miR-181b is proatherogenic and that is supporting results from this study. Di Gregoli et al., reported an upregulation of miR-181b in symptomatic atherosclerotic patients with a correlated decrease in vascular smooth muscle cells (VSMCs) elastin which is essential for maintenance of atherosclerotic plaques, hence the upregulation of miR-181b promotes instability of the plaques which may rupture causing acute events of CS [54].

Another study supports these results was carried out by Casey et al., who studied the change in circulating 6 miRNAs in Hypoxic ischemic encephalopathy and found that miR-181b was significant upregulated rapidly after 1 hour of post hypoxic ischemia in moderate and severe cases considering it as early biomarkers of hypoxic ischemia [55]

Peng et al., studied the expression of miR-181b in mouse brain following MCAO as model for induced cerebral ischemic injury in vivo and in OGD - treated N2A cells as a model for ischemic injury in vitro. In this study, the expression of miR-181b significantly decreased in mouse brain following MCAO and in OGD-treated N2A cells and upregulation of miR-181b promoted OGD-induced N2A cell death, while its repression inhibited the cell death induced by OGD. Additionally, continuous delivering of the miR-181b antimir to mouse brain effectively reduced neural cell loss and improved neurological function of mice after MCAO model of IS [33].

In other hands, VSMCs of mature blood vessels are predominantly in quiescent state “known as contractile phenotype” which is responsible for regulation of blood vessels diameter, blood pressure and flow. In a case of blood vessels injury, VSMCs are switched from the contractile phenotype to proliferative and migratory phenotype to repair tissues in response to injury [56]. Li et al., found that miR-181b was significantly increased after balloon catheter injury in rats’ carotid artery, revealing that miR-181b promotes the migration and proliferation of VSMCs through activation of phosphoinositide 3 kinase and mitogen activated protein kinase pathways [57]. Otherwise, the Wnt signaling pathway has an important role in the process of neurogenesis after cerebral ischemia through interaction with many inflammatory and anti-apoptotic process to regulate the proliferation, migration, and differentiation of neural stem cells and neural progenitor cells [58] hyperactivation of Wnt signaling was shown to increase the abundance of neuron precursors and proliferating cells [59]. Wnt signaling was shown to activate the expression of miR-181b in hepatocellular carcinoma [60] and it suggested to have a similar activity in CS.

Studies carried out by Di Gregoli et al., Peng et al. and Li et al., may explain the cause of miR-181b upregulation in the present study supporting that this upregulation contributes to the development and prognosis of CS.

Conclusion

In conclusion, MEG3 was significantly upregulated in DM+CS than in DM and healthy control groups, indicating that MEG3 upregulation may serve as a marker for prognosis of DM and a new strategy for treatment of diabetic related CS complication. Findings also showed that upregulation of miR-181b may be a molecular factor for progression of CS in diabetic patients.

5. Conclusions

In conclusion, MEG3 was significantly upregulated in DM+CS than in DM and healthy control groups, indicating that MEG3 upregulation may serve as a marker for prognosis of DM and a new strategy for treatment of diabetic related CS complication. Findings also showed that upregulation of miR-181b may be a molecular factor for progression of CS in diabetic patients.

6. Conflicts of interest

There are no conflicts to declare.

7. Formatting of funding sources

None.

8. Acknowledgments

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9. References

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