European Review for Medical and Pharmacological Sciences 2019; 23: 3070-3079

MiR-92a contributes to the cardiovascular disease development in diabetes mellitus through NF-κB and downstream inflammatory pathways

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Abstract. – OBJECTIVE: To explore the role of microRNA-92a (miR-92a) during the development of cardiovascular disease (CAD) in diabetes mellitus (DM) patients, and to investigate its correlation with NF-κB and downstream inflammatory cytokines in diabetes mellitus-associated cardiovascular disease (DM-CAD).

PATIENTS AND METHODS: Expression of miR-92a in DM and DM-CAD patients was estimated by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Receiver operating characteristic (ROC) analysis was used to estimate the capability of miR-92a to discriminate between DM-CAD and DM patients. Nuclear factor-κB (NF-κB) p65 protein expression and serum concentrations of monocyte chemotactic protein-1 (MCP-1), endothelin-1 (ET-1) and intercellular adhesion molecule-1 (ICAM-1) were investigated. Correlations between miR-92a and NF-κB p65, inflammatory factors were assessed. Risk analysis based on miR-92a was performed for DM-CAD patients.

RESULTS: MiR-92a expression was increased in DM-CAD group compared with both DM and healthy groups (all $p<0.05$). The expression of miR-92a was associated with FIB and HbA1c of DM-CAD patients. MiR-92a could be used to distinguish DM-CAD patients from DM patients with an area under the ROC curve (AUC) of 0.866. Moreover, miR-92a was demonstrated to be a risk factor for DM-CAD onset. Expression levels of NF-κB p65, ET-1, MCP-1, and ICAM-1 were all elevated in DM-CAD patients and shown positive correlations with miR-92a.

CONCLUSIONS: Expression of miR-92a in DM-CAD patients is up-regulated, and serves as a potential marker to predict the CAD in DM patients. MiR-92a may contribute to the development of CAD through activation of NF-κB and downstream inflammatory pathways.

Key Words
MicroRNA-92a, NF-κB, Inflammatory cytokines, Cardiovascular disease, Diabetes mellitus.

Introduction

Cardiovascular disease (CAD) is a serious complication in the pathogenesis of type 2 diabetes mellitus (T2DM)[1]. According to the statistics, about 70% of T2DM patients die from the CAD, leading to an increasing attention on the development of diabetes mellitus-associated cardiovascular disease (DM-CAD)[2]. Emerging studies have reported that inflammation plays an important role in the progression of DM-CAD[3]. Various inflammatory factors have been demonstrated to be associated with the development of CAD and DM[4,5]. Nuclear factor-κB (NF-κB) signaling pathway is a critical regulator of inflammation. The activation of NF-κB induces the translation of diverse inflammatory factors, such as monocyte chemotactic protein-1 (MCP-1), endothelin-1 (ET-1) and intercellular adhesion molecule-1 (ICAM-1)[6]. There is compelling evidence that activations of NF-κB and downstream inflammatory signaling pathways are key events in DM and CAD[7]. Thus, we suspect that (the) NF-κB may be involved in the development of CAD in DM patients.

MicroRNAs (miRNAs), small non-coding RNA molecules, which regulate gene expression at the post-transcriptional level, have emerged as fundamental for many diseases[8-10]. MiRNAs have become one of the most encouraging and fruitful fields in biological researches, and have been implicated as new players in the pathogenesis of DM and DM-associated complications[11,12]. MicroRNA-92a (miR-92a) has been demonstrated to be associated with cardiovascular development and diseases[13]. In addition, some research found that miR-92a is involved in the development of diseases through the regulation of NF-κB[13,14]. However, the effects of miR-92a on the pathogenesis of DM and DM-associated complications, especially DM-CAD, remain elusive. To better understand the underlying molecular mech-

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anisms during the development of DM-CAD, this study examined the expression patterns of miR-92a, NF-κB p65 and downstream inflammatory factors in patients with DM-CAD. In addition, the association of miR-92a with NF-κB and downstream inflammatory factors, and risk factor analysis were performed in DM-CAD. Overall, this study provides evidence for a role of miR-92a-NF-κB signaling in the pathogenesis of T2DM and development of DM-CAD. If so, translation to the clinic may be feasible given current pharmacological strategies to lower miR-92a-NF-κB activity for DM-CAD treatment.

Patients and Methods

Patients

This investigation was approved by the Ethics Committee of Tianjin First Central Hospital, and each participant signed the informed consent. 186 patients were enrolled in this research and who were diagnosed with T2DM in Tianjin First Central Hospital from October 2014 to June 2015. These collected patients included 117 DM-CAD cases and 69 simple DM cases. The patients with following conditions were excluded: (1) acute coronary syndrome; (2) type 1 diabetes mellitus (T1DM), diabetic ketoacidosis acidosis, coma; (3) malignant tumor, hematological system diseases, immune system diseases; (4) acute inflammation, hemorrhage, pregnancy; (5) severe hepatic and renal dysfunctions, psychological problems. In addition, 68 healthy volunteers were recruited to act as negative control (NC) group. The clinicopathological characteristics of the participants, including age, gender, body mass index (BMI), low-density lipoprotein cholesterol (LDL-C), fibrinogen (FIB), HbA1c, hypertension, and smoking status, were recorded for the subsequent analyses. Self-reported T2DM status was confirmed by reviewing the medical records of the Tianjin First Central Hospital.

Samples Collection and RNA Extraction

A volume of 2 mL of whole blood was collected from the participants, and the serum samples were isolated from the blood through centrifugation. Total RNA was extracted from the serum using a commercial Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols and stored at −20°C. The concentration of the RNA was quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Single-stranded cDNA was obtained from the collected RNA using PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed to measure the expression levels of miR-92a using SYBR Premix Ex Taq TM II (TaKaRa, Otsu, Shiga, Japan) in a 7300 q-PCR system (Applied Biosystems, Foster City, CA, USA). The miRNA-specific primer sequences were designed based on the miRNA sequences obtained from the miRBase database (http://micro-rna.sanger.ac.uk/): miR-92a forward: 5'-TATTGCACTTGTCCCGGCCTGT-3', reverse: 5'-CTTTCTACACAGGTTGGGATCG-3'. The exogenous cel-miR-39 was used as the internal control of the reactions. Each amplification reaction was performed in the final volume of 20 μL containing 1 μL of the cDNA, 0.25 mM of each primer and 1× SYBR Green PCR Master mix, following the cycling conditions recommended by the supplier (40 cycles of 30 s at 90°C, 5 s at 95°C, and 31 s at 60°C). At the end of the PCR cycles, melting curve analyses as well as electrophoresis of the products on 3.0% agarose gels were performed to validate the specific generation of the expected PCR product. Each sample was run in duplicates for analysis. The expression levels of miR-92a were normalized to miR-39 and were calculated utilizing the $2^{-\Delta\Delta ct}$ method.

Western Blot Analysis

Western blot was performed as described elsewhere. In brief, the proteins in the serum samples were quantified by BCA protein quantification kit (Beyotime, Beijing, China), and were then separated by 15% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to membranes at 60 V for 4 h at 4°C. Membranes were blocked in 5% of nonfat dry milk in TBS, incubated with primary antibody of anti-NF-κB p65 (1:200; Proteintech, Wuhan, China) in TBST overnight at 4°C, and washed thrice with TBST. The membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase (1:5000; Proteintech, Wuhan, China) in TBST for 1 h at room temperature and then washed using TBST for thrice. Protein bands were visualized on an X-ray film using an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Proteins in the serum samples were quantified by BCA protein quantification kit (Beyotime, Bei-

Table I. Baseline characteristics of the participants.

Features	NC (n = 68)	DM (n = 69)	DM-CAD (n = 117)
Age (years)	62.98 ± 7.42	64.29 ± 3.77	64.73 ± 8.22
Male (%)	45 (66.18)	48 (69.57)	79 (67.52)
BMI (kg/m^2)	24.08 ± 2.42	25.61 ± 5.76	26.44 ± 3.31[a]
LDL-C (mM/L)	2.55 ± 0.83	3.24 ± 1.27	4.68 ± 1.70
FIB (g/L)	2.36 ± 0.37	4.29 ± 0.58[a]	4.41 ± 0.83[ab]
HbA1c (%)	5.29 ± 0.33	6.80 ± 2.41[a]	8.22 ± 2.64[ab]
Hypertension (%)	8 (11.76)	25 (36.23)[a]	48 (41.03)[a]
Smoking (%)	28 (41.18)	32 (46.38)	56 (47.86)

NC, negative control; DM, diabetes mellitus; DM-CAD, diabetes mellitus-associated cardiovascular disease; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; FIB, fibrinogen; [a], p<0.05 compared with NC group; [b], p<0.05 compared with DM group.

jing, China). The concentration of the pro-inflammatory cytokines: ET-1, MCP-1, ICAM-1, was estimated by ELISA (Biotang, MA, USA). Briefly, 100 µL of protein extract was added into sample wells and standard wells. For the blank, no protein extract or standard control protein was added. Wells were blended and covered with parafilm and incubated at 20-25°C for 90 min. 100 µL of ET-1, MCP-1 and ICAM-1 biotinylation anti-human IgG antibodies (Proteintech, Wuhan, China) were added into each well and incubated at 20-25°C for 60 min on an orbital shaker (100 rpm). Then, 100 µL of horse-radish peroxide (HRP)-conjugated detection antibodies (ProteinTech, Wuhan, China) were added into the wells and incubated at 20-25°C for 30 min. After the incubation, 100 µL of TMB substrate buffer solution was added into the wells and incubated at 20-25°C for 10-20 min away from light. Lastly, 100 µL of stop solution was added into each well to stop enzyme reaction. The absorbance can be read on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm within 2-15 min.

Statistical Analysis

Results are reported as means ± standard error values for the indicated number of experiments. Statistical significance was calculated using the Student's *t*-test for data pairs, and ANOVA analysis followed by the Dunnett test for multiple comparisons to control, and the Tukey test for multiple comparisons between groups. The association between miR-92a expression and clinicopathological features was assessed using the Chi-square test. The degree of dependency between variables was estimated by the Pearson correlation test. Logistic regression analysis was carried out to identify the risk factor for DM-CAD. SPSS (version 21.0) package was used for receiver operating characteristic (ROC) curve analysis. Significance was set at $p < 0.05$.

Results

Baseline Characteristics of Patients and Serum MiR-92a Expression

The general features of the patients were listed in Table I. No significant difference was found between DM-CAD, DM and NC groups at age and gender (all $p > 0.05$). The concentrations of FIB and HbA1c were higher in DM-CAD group than those in DM and NC groups (all $p < 0.05$).

According to qRT-PCR, we found that serum expression of miR-92a was elevated in DM group compared with the NC group ($p < 0.05$). Moreover, the highest miR-92a was detected in DM-CAD group when compared with DM and NC groups ($p < 0.05$, Figure 1).

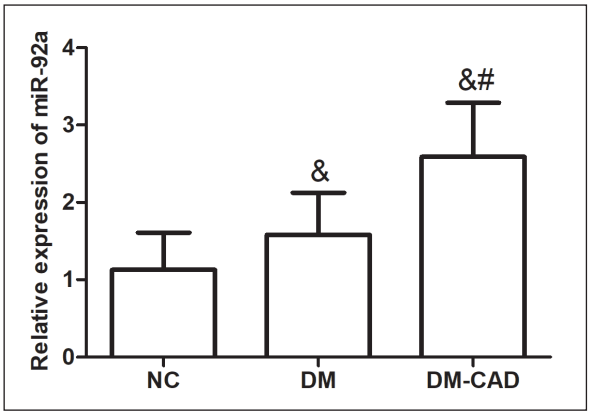

Figure 1. Expression of miR-92a in DM-CAD, DM and NC groups. The highest expression of miR-92a was detected in DM-CAD group compared with DM and NC groups. &, p<0.05 compared with NC group; #, p<0.05 compared with DM group.

Table II. Association of miR-92a expression with clinicopathological features of DM-CAD patients.

Features	Total No. n=117	miR-92a expression		*p*-values
		Low (n = 53)	High (n = 64)	
Age (years)				0.729
≤ 50	35	15	20	
> 50	82	38	44	
Gender				0.755
Female	38	18	20	
Male	79	35	44	
BMI				0.085
≤ 26	41	23	18	
> 26	76	30	46	
LDL-C (mM/L)				0.612
≤ 4.6	50	24	26	
> 4.6	67	29	38	
FIB (g/L)				0.007*
≤ 4.4	42	26	16	
> 4.4	75	27	48	
HbA1c (%)				0.042*
≤ 8.2	52	29	23	
> 8.2	65	24	41	
Hypertension				0.779
No	69	32	37	
Yes	48	21	27	
Smoking				0.379
No	61	30	31	
Yes	56	23	33	

DM-CAD, diabetes mellitus-associated cardiovascular disease; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; FIB, fibrinogen;*$p<0.05$.

Association of MiR-92a Expression and Clinicopathological Features of DM-CAD Patients

To investigate the role of miR-92a during the progression of CAD in DM patients, the relation between miR-92a expression and clinicopathological characteristics of DM-CAD patients was assessed in the current study. DM-CAD patients were divided into low miR-92a group (n = 53) and high miR-92a expression group (n = 64) based on the mean miR-92a expression value (2.587). The results of the Chi-square test analysis listed in Table II revealed that miR-92a was associated with FIB ($p = 0.007$) and HbA1c ($p = 0.042$). However, no significant relation was detected between miR-92a and age, gender, BMI, LDL-C, hypertension, and smoking (all $p > 0.05$). Moreover, a positive correlation between miR-92a expression and FIB was performed in DM-CAD group (R = 0.420 and $p < 0.001$, Figure 2A). However, this correlation was not found in DM group (R = 0.120 and $p = 0.327$, Figure 2B).

High Specificity and Sensitivity of MiR-92a for Determination of DM-CAD

According to the ROC analysis, we evaluated the capability of miR-92a to distinguish DM-CAD patients from DM and healthy individuals. The ROC curve constructed using the miR-92a expression in DM-CAD and healthy controls revealed that the area under the curve (AUC) was 0.958, with a sensitivity of 78.6% and specificity of 98.5% at the cutoff value of 2.035 (Figure 3A). In addition, miR-92a could be used to discriminate DM-CAD patients from DM patients, with the AUC value of 0.866 (Figure 3B). The cutoff value was 2.135, and the corresponding sensitivity and specificity were 76.9% and 88.4%, respectively.

Expression of NF-κB p65 Protein

As shown in Figure 4, the results of Western-blot revealed that the protein expression of NF-κB p65 was elevated in DM-CAD group compared with both DM and NC groups (all $p < 0.05$), and the lowest value was performed in NC group.

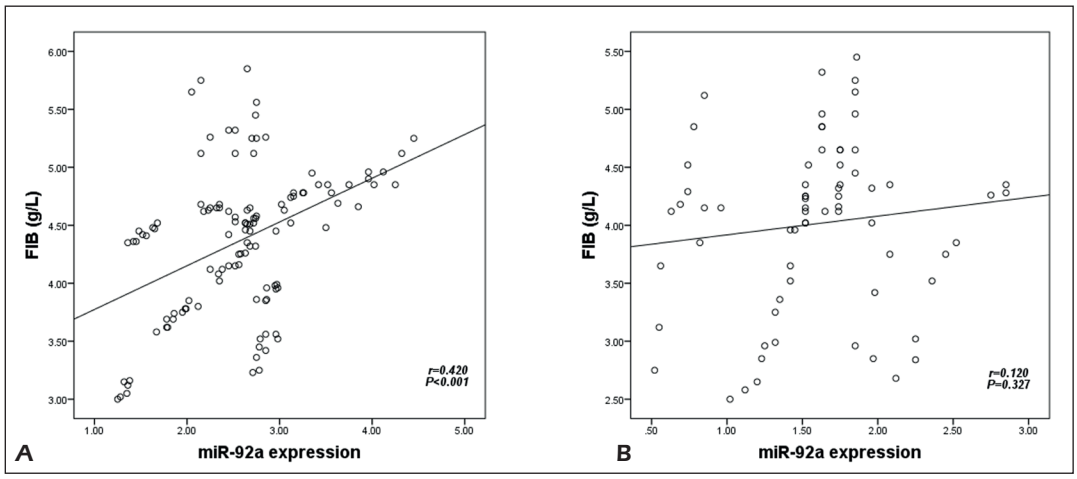

Figure 2. Correlation analysis between miR-92a expression and FIB concentration in DM-CAD patients and DM patients. **A**, Positive correlation was performed between miR-92a and FIB concentration in patients with DM-CAD (R=0.420, p<0.001). **B**, No significant correlation of miR-92a with FIB was found in sample DM patients (R=0.120, p=0.327).

Expression of MCP-1, ET-1, and ICAM-1

The serum concentration of pro-inflammatory cytokines, including MCP-1, ET-1, and ICAM-1, was evaluated by the ELISA analysis. The expression value listed in Table III indicated that the highest concentrations of MCP-1, ET-1, and ICAM-1 were all detected in DM-CAD group compared to that in the DM and NC groups (all $p < 0.001$).

Logistic Regression Analysis for DM-CAD Patients

To investigate the potential risk factors for CAD in DM patients, the Logistic analysis was conducted to analyze the effects of miR-92a, NF-κB p65, MCP-1, ET-1, ICAM-1 and other clinicopathological features on the occurrence of CAD in DM patients. The results shown in Table IV indicated that miR-92a (OR = 15.835, 95% CI = 6.307-39.754 and $p < 0.001$) and FIB (OR = 2.495, 95% CI = 1.109-5.614 and $p = 0.027$) were two independent risk factors for onset of CAD in DM patients.

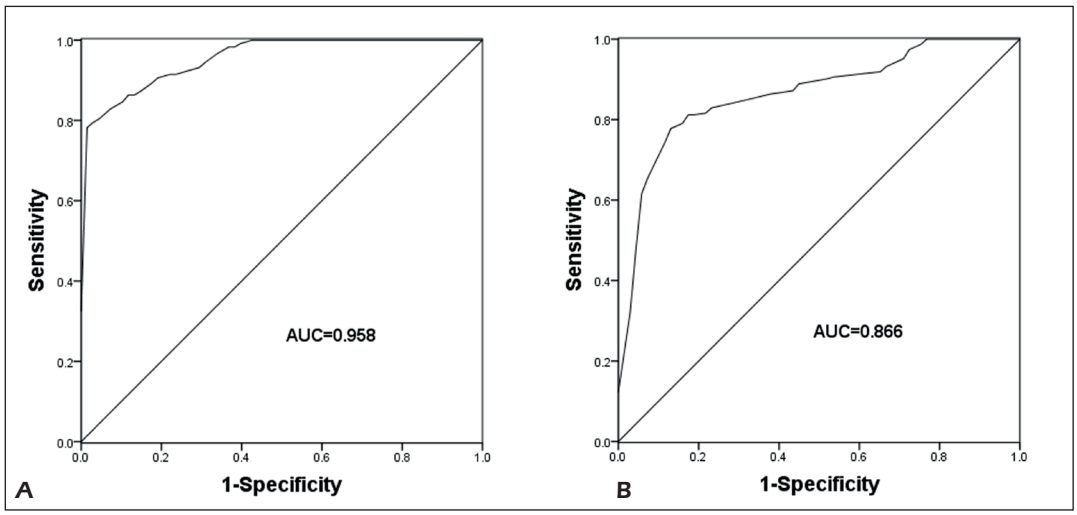

Figure 3. ROC analysis based on miR-92a expression in DM-CAD patients. **A**, MiR-92a could distinguish DM-CAD patients from NC controls with an AUC of 0.958. **B**, MiR-92a had the capability to discriminate between DM-CAD patients and DM patients with an AUC of 0.866.

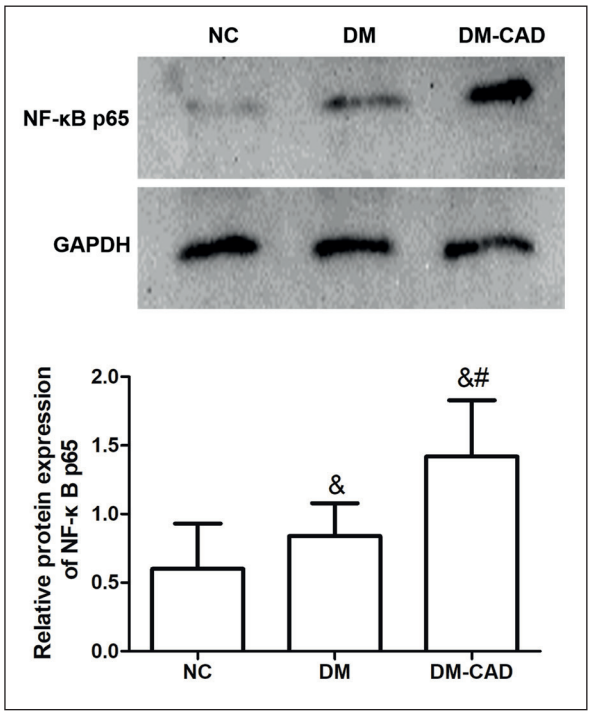

Figure 4. Protein expression of NF-κB p65 measured by Western blot. **A**, Results of Western blot analysis for NF-κB p65 expression. **B**, Expression of NF-κB p65 was elevated in DM-CAD group compared with DM and NC groups. &, $p<0.05$ compared with NC group; #, $p<0.05$ compared with DM group.

Correlation of MiR-92a with NF-κB p65, MCP-1, ET-1, and ICAM-1 in DM-CAD Patient

From the Pearson correlation test, we found a positive correlation between miR-92a and NF-κB p65 (R = 0.349 and p = 0.007, Figure 5A). Additionally, the miR-92a was also correlated with MCP-1 (R = 0.692 and p = 0.004, Figure 5B), ET-1 (R = 0.439 and p = 0.002, Figure 5C) and ICAM-1 (R = 0.785 and p = 0.005, Figure 5D).

Table IV. Logistic regression analysis for miR-92a and clinical factors in DM-CAD patients.

Variables	Logistic analysis		*p*-values
	OR	95% CI	
miR-92a	15.835	6.307-39.754	< 0.001*
Age	1.392	0.639-3.032	0.405
Gender	1.718	0.810-3.646	0.159
BMI	1.468	0.662-3.255	0.345
LDL-C	1.075	0.506-2.283	0.850
FIB	2.495	1.109-5.614	0.027*
HbA1c	1.614	0.758-3.434	0.214
Hypertension	1.339	0.634-2.827	0.444
Smoking	1.122	0.532-2.364	0.763
NF-κB p65	1.193	0.555-2.565	0.651
MCP-1	1.481	0.701-3.128	0.303
ET-1	1.231	0.590-2.567	0.579
ICAM-1	1.428	0.685-2.976	0.342

NF-κB, Nuclear factor-κB; MCP-1, monocyte chemotactic protein-1; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; FIB, fibrinogen; *$p<0.05$.

Discussion

According to the statistics, the incidence rate of DM is increasing rapidly, and this rate is expected to increase in the subsequent years. The vast array of long-term complications for DM contributes to the elevated mortality of this serious disease[14]. Among these complications, CAD accounts for most of the morbidity and mortality in the DM population[15]. It is reported that almost 80% of deaths related to DM occur due to heart diseases[16]. Thus, it is important to further understand the underlying molecular mechanisms during the development of CAD in patients with DM.

MiRNAs, a group of small non-coding RNAs with gene expression regulatory function, have been reported to be involved in various physio-

Table III. Serum concentration of MCP-1, ET-1 and ICAM-1.

Cytokines	NC	DM	DM-CAD
MCP-1 (pg/ml)	58.71 ± 16.23	64.24 ± 14.28[a]	293.15 ± 58.41[ab]
ET-1 (ng/ml)	38.35 ± 9.02	77.5 ± 11.22[a]	90.04 ± 22.46[ab]
ICAM-1 (μg/L)	205.3 ± 47.8	298.08 ± 65.32[a]	841.28 ± 125.45[ab]

MCP-1, monocyte chemotactic protein-1; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1; NC, negative control; DM, diabetes mellitus; DM-CAD, diabetes mellitus-associated cardiovascular disease; [a], $p<0.05$ compared with NC group; [b], $p<0.05$ compared with DM group.

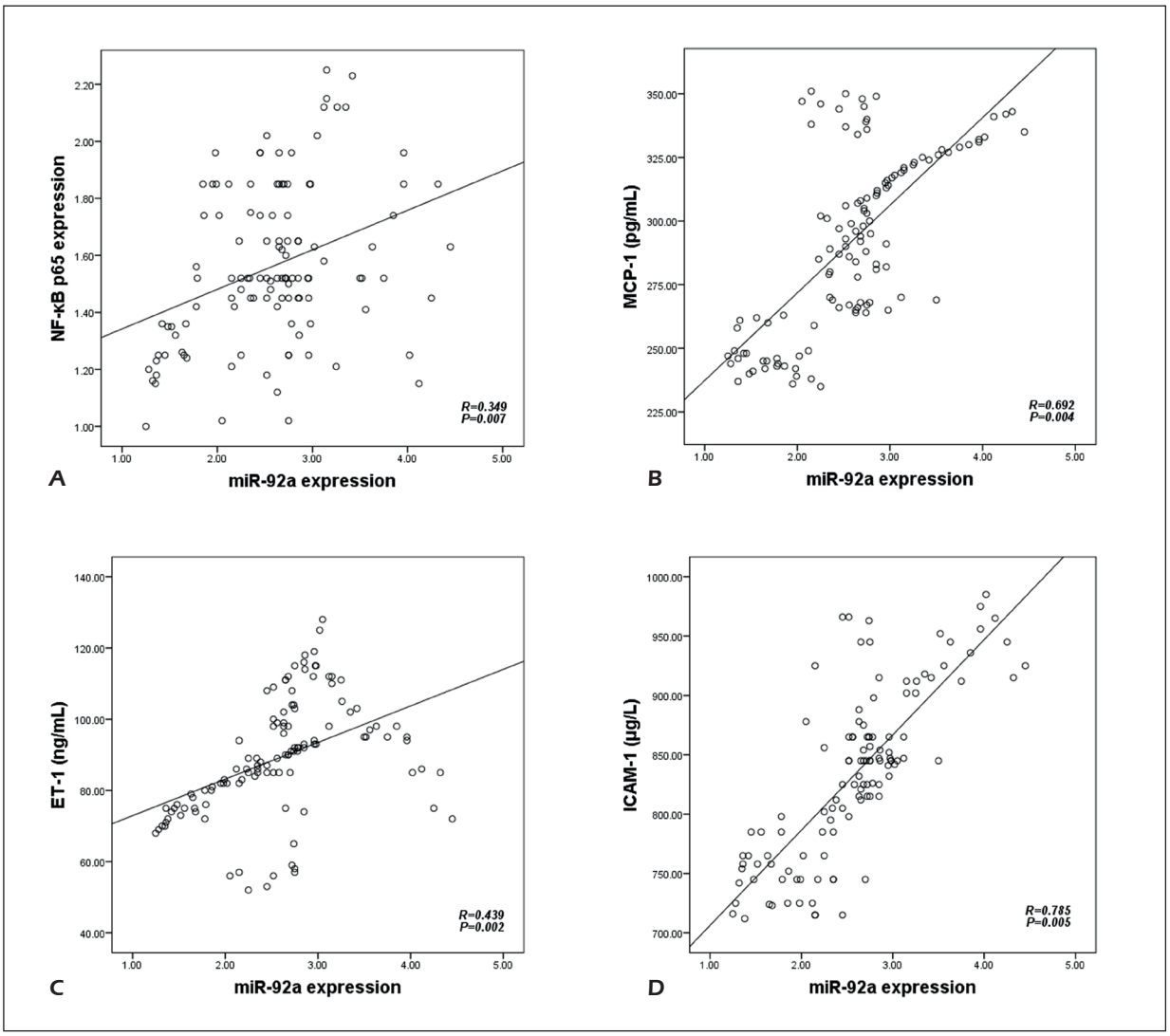

Figure 5. Correlations of miR-92a with NF-κB p65, MCP-1, ET-1, and ICAM-1 in DM-CAD patients. The expression of miR-92a was positively correlated with (**A**) NF-κB p65 (R=0.349 and p=0.007), (**B**) MCP-1 (R=0.692 and p=0.004), (**C**) ET-1 (R=0.439 and p=0.002) and (**D**) ICAM-1 (R=0.785 and p=0.005) in patients with DM-CAD.

logical and pathological processes[17]. Accumulated evidence revealed that miRNAs are associated with the diagnosis and prognosis of various human diseases[18]. Importantly, some miRNAs with abnormal expression patterns serve pivotal roles during the progression of DM. For example, a study scheduled by Zhang et al[19] revealed that circulating miR-126 could predict the onset of T2DM and acted as a non-invasive diagnostic biomarker. MiR-375, as another example, has been described as a key factor for glucose-regulated insulin secretion via regulation of MTPN and PDK1 and may serve as a candidate target for DM treatment[20]. Furthermore, miRNAs have also been demonstrated to be involved in the development of cardiovascular complications, such as angiogenesis, hypertrophy, endothelial dysfunction, and myocardial fibrosis[21-24]. Circulating miR-21-5p and miR-126a-3p have been reported as dynamic biomarkers for the prediction of major cardiovascular events in patients with T2DM[25]. MiR-92a is an extensively studied miRNA and plays important roles in various biological processes, such as cell proliferation, differentiation, migration, invasion, and apoptosis[26,27]. The relation between miR-92a and initiation and development of human diseases, including DM, has been reported in previous researches. For example, a previous study indicated that miR-92a could promote the treatment effects of $CD34^+$ cells for the

vascular repair in the cases with DM[28]. In the present study, we observed the increased serum expression of miR-92a in CAD-DM patients compared with DM patients. Thus, we suspected that miR-92a might be involved in the development of CAD in DM patients.

In the present study, miR-92a in DM-CAD, DM, and healthy individuals was investigated by qRT-PCR. The analysis results revealed that serum miR-92a was markedly elevated in DM-CAD patients compared with DM patients and NC. The expression of miR-92a in DM-CAD patients was found to be associated with patients' FIB and HbA1c, which respectively represent the risk factors of CAD and DM. Furthermore, the ROC analysis indicated that miR-92a had a high sensitivity and specificity to distinguish DM-CAD patients from DM patients. Additionally, the Logistic regression analysis indicated that miR-92a was an independent risk factor for DM-CAD patients. Given the above data, we considered that the aberrant expression of miR-92a might be involved in the development of DM-CAD, and could be used as an indicator to predict the DM-CAD onset.

NF-κB represents one of the most important transcription factors and is involved in the transcription of many genes[29]. It is a dimer comprised of p50 and p65. Under resting conditions, p65 forms a complex with the inhibitory subunit IκB, while p65 is released by the phosphorylation of IκB, leading to the translocation into the nucleus of p65 and the induction of genes transcription[30]. There is strong evidence in the previous literature that the NF-κB activation is an initial and crucial step in the evolution of T2DM, contributing to both hepatic insulin resistance and β-cell apoptosis, and providing a link between overnutrition, metabolic inflammation and impaired autophagy in the hypothalamus and the development of obesity[31-35]. In addition, the transcription of inflammatory mediators, such as MCP-1, ET-1, and ICAM-1, induced by the activation of NF-κB, is considered as the pathologic and physiologic basis of both DM and CAD[36]. Therefore, NF-κB and downstream inflammatory signaling pathways play critical roles in the development of CAD in DM individuals. In the current study, the protein expression of NF-κB p65 evaluated by Western blot was significantly upregulated in DM-CAD patients compared with both DM and NC groups. Moreover, the serum concentrations of inflammatory cytokines, including MCP-1, ET-1, and ICAM-1, were higher in DM-CAD group than those in DM and NC groups. These data were inconsistent with the previous studies, which indicated the crucial role of inflammation in the development of DM and DM-associated complications[37]. Furthermore, we found the positive correlations between miR-92a and NF-κB and the inflammatory cytokines levels, suggesting that miR-92a might be involved in the development of DM-CAD through the NF-κB and downstream inflammatory pathways. However, the precise molecular mechanisms between miR-92a and NF-κB remain unclear and warrant further studies.

Conclusions

We found that miR-92a is expressed at a high level in T2DM with CAD patients. The expression of NF-kB p65 as well as the downstream inflammatory cytokines, including ET-1, MCP-1 and ICAM-1, were significantly higher in DM-CAD group than those in DM and NC groups. The upregulated miR-92a is involved in the pathological changes of DM-CAD, and may contribute to the development of this disease through NF-κB and downstream inflammatory pathways. MiR-92a may serve as an efficient predictor of DM-CAD and could be applied in the DM-CAD screening.

Conflict of Interest

The authors have declared that they have no conflict of interests.

Acknowledgement

This work was supported by Tianjin Health Committee (2014KY13, 15KG132), National Natural Science Foundation of China (81602139), Tianjin Natural Science Foundation(16JCQNJC10100).

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