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Original Article

MicroRNA-146b-3p regulates the development and progression of cerebral infarction with diabetes through RAF1/P38MAPK/COX-2 signaling pathway

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Abstract: Diabetes has been considered as an independent risk factor for cerebral infarction. However, the pathological mechanism of cerebral infarction with diabetes (DMCI) is still rarely known. In this study, we try to explore the relationship between microRNA-146b-3p (miR-146b-3p) and DMCI patients. The peripheral blood mononuclear cells were separated after the patients were selected from our hospital. Firstly, the content of IL-6 and COX-2 was detected by ELISA. Then, the total RNAs were extracted and analyzed by microRNA (miRNA) microarray. Moreover, the target genes of miR-146b-3p were predicted by online miRNA target prediction algorithms. Meanwhile, luciferase reporter system was used for assaying the target gene for miRNA-146b-3p. Simultaneously, RT-PCR assay was used for the miRNA expression detection. Furthermore, western blot was applied to determine the expression of the signal pathway involved proteins. Our results demonstrated that expression of IL-6 and COX-2 were remarkably up-regulated in peripheral blood of DMCI patients compared with that in normal control group. In addition, miRNA microarray data suggested that miR-146b-3p expression was significantly down-regulated in DMCI patients, with v-raf-1 expression negatively regulated. Moreover, miR-146b-3p regulated RAF1 expression was found to mediate P38MAPK signaling activation in thrombosis patients. The following research indicated that activation of RAF1 trough miR-146b-3p down-regulation contributed to activation of RAF/P38MAPK/COX-2 signaling pathway in vascular infarction. Our data have implied that altered expression of miR-146b-3p is closely related to the progression and development of DCMI mediating the RAF/P38MAPK/COX-2 signal transduction pathway.

Keywords: microRNA-146b-3p, RAF1, P38MAPK, COX-2, cerebral infarction with diabetes

Introduction

Stroke is an intricate clinical outcome of atherosclerotic vascular disease in patients with diabetes [1-3]. Cerebral infarction is a common disease in clinic, and also a leading disease that causes human death [4, 5]. At present, with the continuous improvement of quality of people's life, patients with cerebral thrombosis caused by diabetes have a larger and larger proportion in the cerebral thrombosis cases [6, 7]. The patients suffering from acute cerebral infarction in the presence of diabetes mellitus often have poor clinical outcomes when compared with those without diabetes mellitus [8]. Therefore, it is very important to pay more attention and perform further studies on cerebral infarction with diabetes (DMCI).

Inflammation is one of the serious pathological and physiological reactions after cerebral infarction [9]. At present, some researchers believe that diabetes can aggravate the acute inflammatory reaction after ischemic brain damage, which increased the inflammatory factors in the brain tissue [10, 11]. One of the inflammatory factors, Cyclooxygenase (COX), is divided into two categories: COX-1 and COX-2. In human brain tissue, COX-2 is usually expressed in the cortex, hippocampus, hypothalamus, and notochord central [12, 13]. The expression of COX-2 can be significantly increased under the stimulation of other inflammatory factors, such as inflammatory cytokine interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), angiotensin II (Ang II) and so on. IL-6 is proved to be pro-inflammatory when IL-6R can activate STAT3 pathway of aortic endothelial cells and promote the development of atherosclerosis [14, 15]. In recent years, the relationship between IL-6, COX-2 and arterial thrombosis has caused rising concerns.

In addition, studies indicated that microRNAs (miRNAs) played a critical role in numerous biological processes. Cao *et al.* have reported that miRNAs play a crucial regulatory role in the process of cell inflammation reaction, proliferation and migration of vascular endothelial cells, monocyte adhesion and differentiation, vascular integrity maintenance and cholesterol metabolism [16, 17]. So far, some miRNAs have been confirmed to play a key role in the pathophysiology of atherosclerosis. Therefore, the research of miRNAs as therapeutic targets for thrombosis diagnosis and treatment has become a research hotspot in recent years.

Human miR-146, one of the miRNAs, consists of 22 nucleotides. It works in two forms, including miR-146a and miR-146b. MiR-146b positions in the tenth human chromosome (10q24.32). Fulzele et al. have reported that miR-146b-3p is closely related to the progression and development of inflammation and arterial thrombosis [18]. The study of Yang et al. suggested that over-expression of miR-146a may be useful in the prevention and treatment of atherosclerosis [19]. Moreover, the study of Zhou et al. revealed that miR-146a contributes to cerebral ischemic injury, when negatively regulating the pro-apoptotic genes, Caspase7 and Bclaf1 in vitro [20]. Previous reports also demonstrated that impaired miR-146 expression links to subclinical inflammation and insulin resistance in type 2 diabetes [21, 22]. Moreover, the study also found a negative correlation between the expression of miR146 and IL-6 in circulating. Furthermore, microRNA (miRNA) is regulated by upstream signaling molecules, in which the signal transducer and activator of transcription 3 (STAT3) is one of the major regulatory molecules [23, 24]. JAK/STAT pathway is known to be one of the pivotal ways of cytokine signal transduction. Other research [25] have revealed that STAT3 can bind to the sequence of highly conserved miR-146b genes, which implies that the expression of miR-146b in arterial thrombosis was related to the regulation of STAT3. Moreover, MAPK is a potential target gene of multiple miRNAs, suggesting that MAPK may promote the formation of arterial thrombosis by regulating the function of miRNA in the arterial thrombosis. However, it is still not clear about if or how miR-146b participate in the pathology of cerebral infarction with diabetes.

Diabetes has been considered as an independent risk factor for cerebral infarction, which can aggravate the apoptosis of neural cells, and increase the disability rate and mortality rate of cerebral infarction [26]. The pathological mechanism of DMCI has been intensively studied in recent years. But little is known especially in signal transduction mechanisms of DMCI. In this study, we are committed to focus on the role of miR-146b in the development and progression of diabetes combined with cerebral infarction, and to clarify its molecular mechanism.

Experimental procedure

Sample

Selected objects: The cases were selected from patients with diabetes during April 2013 to December 2014 in our hospital. We performed a follow-up study of the incidence of thrombotic events.

Patient diagnostic criteria: Diagnostic criteria for diabetes according to the WHO criteria in 1999. Diagnosis of cerebral infarction was according to criteria developed by 2013 AHA/ASA, and confirmed by cranial computed tomography or magnetic resonance imaging, or both.

Exclusion criteria: Patients with liver, kidney or thyroid dysfunction were excluded; patients suffering from heart disease, autoimmune disease, cancer, infectious disease, cerebral hemorrhage as well as having a history of bleeding disorders; Patients with trauma or surgery history within one month.

Detection of IL-6 and COX-2 content by ELISA

Peripheral blood sample collection: Fasting venous blood 10 ml was extracted from the cases in the morning, and anticoagulant by 0.129 mmol/L sodium citrate in vitro, fully shake tube and stored at room temperature for inspection. Total serum IL-6 and COX-2 protein concentrations were detected by highly specific enzyme linked immunosorbent assays (ELISA) (R&B Systems). All samples were repeated at least three times. Standard curves were generated according to the operation instructions and the concentration of IL-6 and COX-2 in test samples was calculated.

Peripheral blood mononuclear cell separation

4 ml peripheral venous blood was extracted from the cases in morning, heparin anticoagu-

Table 1. Index of patients in each experimental group

Group	Patients (n = 15)	Normal (15)
Sex (Male/Female)	10/5	10/5
Age (year)	73.20±1.52	74.85±3.31
SP (mmHg)	131.70±1.43	121.70±1.23
DP (mmHg)	76.05±0.65	70.05±0.15
FBG (mmol/L)	5.48±0.19	5.18±0.09
2 h PG (mmol/L)	9.69±1.84	6.24±0.81
GHb (%)	6.14±1.04	5.93±0.58
PLT ($\times 10^9$/L)	200.40±3.33	206.40±5.31
ALT (IU/L)	22.38±0.12	23.68±0.92
AST (IU/L)	23.77±0.13	22.17±0.23
ALP (IU/L)	54.83±0.74	56.23±1.14
Urea nitrogen (mmol/L)	5.98±0.15	6.18±0.25
Cre (umol/L)	86.33±1.35	88.53±1.95
Uric acid (umol/L)	384.60±23.41	362.68±18.12
TG (mmol/L)	1.51±0.21	1.49±0.11
Chol (mmol/L)	4.33±0.19	4.13±0.09
HDL (mmol/L)	1.24±0.13	1.31±0.03
LDL (mmol/L)	2.44±0.06	2.37±0.07

lation, and low speed centrifugation for 15 minutes. Then, about 1 ml Tunica albuginea of intermediate layer was diluted by in volume of RPMI 1640 culture medium, mixed and slowly spread in separation liquid surface of 2 ml Ficoll lymphocyte. The mononuclear cells were isolated from the intermediate between separation fluid and RPMI 1640, and the cells were washed for three times and reserved.

RNA extraction and miRNA microarray analysis

Total RNA was isolated from PBMC using the miRNeasy Mini Kit (Qiagen). The quality of each RNA sample was further assessed by a NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies). miRNA microarray profile was performed using Agilent miRNA array 16.0 (Capital Bio Corporation) to identify the candidate miRNAs which differential expressed in cerebral infarction patients compared with normal control.

Quantitative real-time PCR analysis for miRNA and mRNA expression

For miRNA detection, we designed a stem-loop RT primer specifically hybridizing with 35 different miRNAs and RNU6B, respectively. RNU6B was used for normalization. The relative expression of the miRNAs was calculated using the -ΔΔCT method. For the mRNA detection we designed a specific RT primer specifically hybridizing with COX-2, RAF1, DNMT3B and other different miR-146b-3p target genes and GAPDH was regarded as normalization.

Luciferase reporter assay

The 3'-UTR sequences of RAF1 and DNMT3A were predicted to interact with miR-146b-3p or a mutated sequence within the predicted target sites was synthesized and inserted into the pMIR-REPORT Luciferase vector (Promega). pMIR-RAF1/DNMT3A-3'UTR-wt (wild-type) vector or 3'UTR-mt (mutant-type) vector were co-transfected with miR-146b-3p mimics or miRNA control using the Transfection Viafect (Promega) in PBMC cells. Then, reporter gene assays were performed using the Dual Luciferase Reporter Assay system (Promega) according to the operating directions. Renilla luciferase was co-transfected as a control. For each transfection, luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities.

Bioinformatics method

The target genes of miR-146b-3p were predicted by Target Scan Release 6.2 (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/home.do).

Western blot

The samples were collected and extracted by RIPA buffer (Thermo) on ice for 30 min. Total protein samples were separated through 8-10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Milipore). The primary antibodies were COX-2 (1:1000, Abcam, mouse), RAF1 (1:500, Abcam, rabbit), p-p38MAPK (1:1000, Cell signaling, rabbit), p38MAPK (1:500, Cell signaling, rabbit), P-STAT3 (1:5000, Abcam, mouse), STAT3 (1:1000, Abcam, rabbit) and GAPDH (1:1000, Cell signaling, rabbit). The membranes were incubated with primary antibody at 4°C for overnight. The next day they were incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. The bound antibody was detected with a chemo-fluorescence

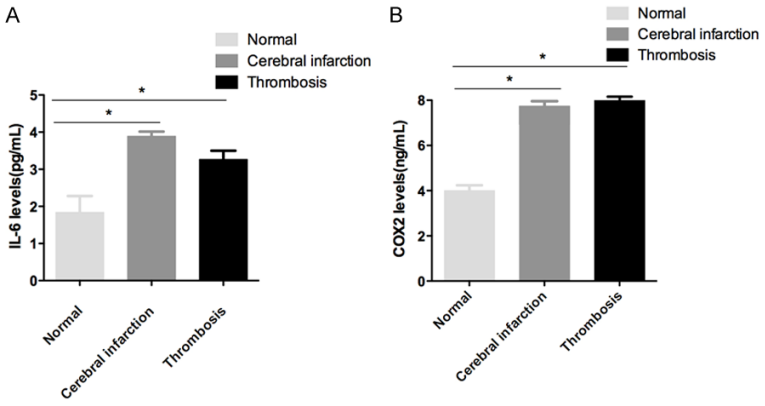

Figure 1. IL-6 and COX-2 expression were increased in peripheral blood of DMCI patients and thrombosis patients compared with normal control group. A. IL-6 levels in peripheral blood were detected by ELISA; B. COX-2 levels in peripheral blood were detected by ELISA. (n = 3, *indicates P<0.05).

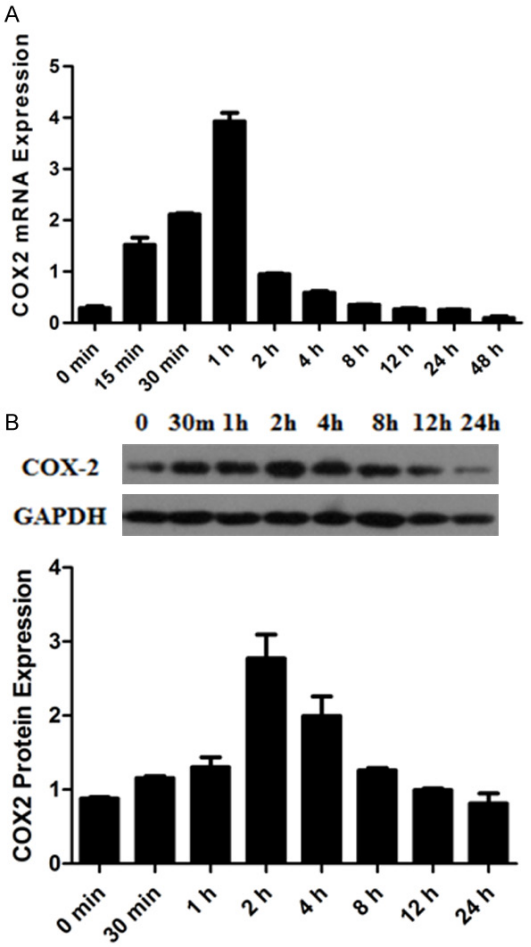

Figure 2. COX-2 expression was induced by IL-6 in peripheral blood mononuclear cells. A. RT-PCR assay was used to detected COX-2 mRNA expression; B. Representative images was selected to show COX-2 protein expression detected by western blot. And semi-quantative analysis was applied to compare the relative COX-2 protein expression. (n = 3).

detection kit (Amersham, Piscataway, NJ). The representative images were shown.

Statistical analysis

All experiments were performed at least in triplicate and data were presented as means ± SD. The statistical analyses were determined using Graphpad Prism version 5 software programs (Graphpad Software). Statistical analysis was adequately performed by ANOVA followed by Bonferroni multiple-comparison post hoc test. P<0.05 was considered as statistically significant difference and remarked as *, P<0.01 was remarked as **.

Results

The incidence of thrombotic events during follow-up

In the current study, 210 patients were followed up for 8-21months, with a median follow-up of 12 months. Totally 33 thrombotic events occurred, among which 15 cases were cerebral infarction (ten males, five females, mean age: 72.15±1.17 years, age range: 56-84 years old) and 18 cases were myocardial infarction. We also chose 15 cases as normal control group (ten males, five females, mean age: 74.85±3.31-year, age range 58-77 years old) (**Table 1**).

IL-6 and COX-2 expression in peripheral blood of DMCI patients and thrombosis patients compared with normal control

The data indicated that IL-6 and COX-2 expression were remarkably increased in peripheral blood of DMCI patientsnat there are some limitations t and thrombosis patients (IL-6 3.26±0.23 u/L and COX-2 14.67±0.53 u/L) compared with normal control group (IL-6 1.84±0.44 u/L and COX-2 4.0±0.24 u/L) (P<0.05, **Figure 1**).

COX-2 expression was induced by IL-6 in peripheral blood mononuclear cell (PBMC)

After 2 hours of IL-6 induction, the mRNA and protein expression of COX-2 were significantly increased. The mRNA and protein expression of

Table 2. miRNA microarray analysis of significantly up/down-regulated genes in Cerebral infarction patients compared with Normal control. Heat map displaying 35 miRNAs with 1.5-fold or more differential expression between the cerebral infarction patients versus normal control group, 12 miRNAs were up-regulated, and 23 miRNAs were down-regulated in cerebral infarction patients

Probe Set ID	Systematic Name	Fold change Cerebral infarction/Normal	*P*-value	Up/Down regulation
20500765	hsa-miR-125a-5p	3.03231	0.032406	Up regulation
20501278	hsa-miR-328-3p	2.00189	0.001142	Up regulation
20503794	hsa-miR-146b-3p	0.05327	0.010399	Down regulation
20518945	hsa-miR-4538	0.31076	0.010726	Down regulation
20500120	hsa-let-7d-3p	0.31125	0.041517	Down regulation
20500436	hsa-miR-7-1-3p	0.25872	0.005324	Down regulation
20500789	hsa-miR-186-5p	4.02126	0.025798	Up regulation
20500433	hsa-miR-139-3p	2.07618	0.030555	Up regulation
20504569	hsa-miR-1271-5p	1.69812	0.01526	Up regulation
20500164	hsa-miR-31-5p	0.37121	0.007469	Down regulation
20501291	hsa-miR-148b-3p	1.98423	0.031341	Up regulation
20504553	hsa-miR-671-3p	1.77911	0.043218	Up regulation
20500739	hsa-miR-133a-3p	0.21849	0.014382	Down regulation
20500714	hsa-let-7g-3p	2.01734	0.013407	Up regulation
20505746	hsa-miR-874-3p	0.203581	0.047385	Down regulation
20519702	hsa-miR-4800-3p	0.110589	0.010324	Down regulation
20517703	hsa-miR-4323	2.348962	0.007705	Up regulation
20525505	hsa-miR-6772-5p	0.380271	0.048961	Down regulation
20500385	hsa-miR-192-5p	0.293652	0.022549	Down regulation
20500730	hsa-miR-125b-5p	3.124150	0.047654	Up regulation
20504337	hsa-miR-598-3p	0.417821	0.024504	Down regulation
20500797	hsa-miR-194-5p	0.329815	0.044119	Down regulation
20503887	hsa-miR-505-3p	0.241522	0.038524	Down regulation
20501243	hsa-miR-378a-3p	0.293166	0.026236	Down regulation
20501312	hsa-miR-345-5p	2.396709	0.039404	Up regulation
20518936	hsa-miR-378i	0.442302	0.038002	Down regulation
20518842	hsa-miR-378h	0.312921	0.036864	Down regulation
20502122	hsa-miR-422a	0.401220	0.033778	Down regulation
20520351	hsa-miR-1273g-3p	0.120503	0.020185	Down regulation
20525707	hsa-miR-6873-5p	0.242396	0.049603	Down regulation
20515603	hsa-miR-3175	0.358972	0.027805	Down regulation
20528493	hsa-miR-7641	0.267331	0.020398	Down regulation
20525008	hsa-miR-6503-3p	0.255166	0.025419	Down regulation
20524036	hsa-miR-6126	3.626829	0.015622	Up regulation
20506837	hsa-miR-1246	0.121248	0.043795	Down regulation

COX-2 were significantly decreased following the increased IL-6. Among them, COX-2 mRNA expression reached the peak 60 minutes after IL-6 stimulation; COX-2 protein expression was slightly later than its mRNA by reaching the peak in 2 hours after IL-6 induction, then decreased gradually ($P<0.01$, **Figure 2**). Our data indicated that expression of COX-2 was regulated by IL-6.

The miR-146b-3p expression is significantly down-regulate in DMCI patients compared with control group

Hierarchical clustering analyses of miRNA expression showed significant changes (>1.5-fold difference) in expression levels for 35 out of 940 human miRNAs between DMCI patients and normal control group (**Table 2**). Of these

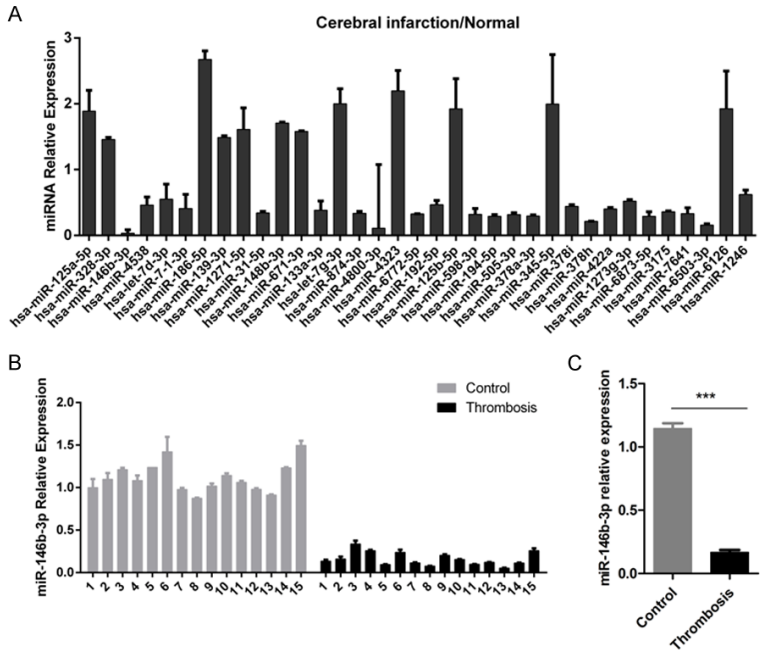

Figure 3. miR-146b-3p was significantly down-regulated in DMCI patients compared with control. A. RT-PCR verified 35 miRNAs differential expression in DMCI patients compared with control group. B, C. RT-PCR verified miR-146b-3p expression in 15 cases of thrombosis patients when compared to that of 15 cases of normal control. (***indicates P<0.001).

miRNAs, 12 miRNAs were up-regulated, while the other 23 miRNAs were down-regulated in DMCI patients. The data showed that miRNA-146b-3p was significantly down-regulate in thrombosis compared with normal control group (**Figure 3A**). We verified that expression of miRNA-146b-3p in 15 cases of DMCI patients and 15 cases of normal control. The average expression of miRNA-146b-3p was 0.16±0.07 in DMCI group while 1.12±0.16 in the normal group. The result was consistent with the miRNA microarray (**Figure 3**).

The miR-146b-3p expression is significantly down-regulate in DMCI patients and negatively regulates expression of RAF1

The miR-146b-3p target genes were predicted by miRNAs target prediction algorithms and miRanda. The data showed that 17 targets were predicted (**Table 3**). Our results illustrate that RAF1 and DNMT3A expression were dramatically increased in DMCI patients, and expression of other 15 target genes did not change significantly (**Figure 4A**). We further verified that expression of RAF1 and DNMT3A in 15 cases of DMCI patients and 15 cases of normal control, respectively (**Figure 4B-E**). The relative luciferase expression of RAF1-3'UTR in control group vs DMCI group was 1.00±0.19 vs 0.43±0.04; of RAF1-3'UTR-mutant group was 1.29±0.15 vs 1.19±0.14 (**Figure 4F**).

The miR-146b-3p targeted regulate RAF1 expression mediates P38MAPK signaling activation in DMCI patients compared with normal control

The data showed that expression of RAF1, phosphorylation of P38MAPK and COX-2 were significantly up-regulate in DMCI patients compared with the control group (**Figure 5A**). Infection with lentivirus expressing miR-146b-3p or transfection with miR-146b-3p mimics resulted in high expression of miR-146b-3p in PBMC (**Figure 5B**, **5C**). The corresponding is the expression of RAF1, phosphorylation of P38MAPK and COX-2 were apparently down-regulate in Lv-miR-146b-3p group compared with mock group, and Negative Control group.

IL-6 activated STAT3 and down regulated miR-146b-3p induced COX-2 expression in PBMC

The data showed that phosphorylation of STAT3 can be significantly activated in PBMC by treated with IL-6 for 5 minutes, p-STAT3 expression was reached maximum after induction of 15 minutes, then decreased gradually (P<0.01, **Figure 6A**). Furthermore, we also found that miR-146b-3p expression was evidently decreased in PBMC treated with IL-6 after 1 hour (**Figure 6B**). And the miR-146b-3p expression was significantly increased in IL-6+S3I-201 group (0.81±0.10) compared with IL-6 group (0.20±0.12) and control group (1.02±0.15, P<0.05, **Figure 6C**). We also found that mRNA and protein expression of COX-2 were significantly increased under IL-6 induction (4.98±0.69) and evidently decreased treated combined IL-6 with STAT3 specific inhibitor S3I-201 (1.46±0.23, P<0.05, **Figure 6D**, **6E**).

Table 3. miRNA target prediction algorithms were used to predict hsa-miR-146b-3p target genes

Systematic Name	Down regulation	Gene Symbol	
hsa-miR-146b-3p	Down	ESR1	Estrogen receptor 1
hsa-miR-146b-3p	Down	THRB	Thyroid hormone receptor, beta
hsa-miR-146b-3p	Down	PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B
hsa-miR-146b-3p	Down	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
hsa-miR-146b-3p	Down	IL2RB	Interleukin 2 receptor, beta
hsa-miR-146b-3p	Down	CAMK2A	Calcium/calmodulin-dependent protein kinase II alpha
hsa-miR-146b-3p	Down	KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
hsa-miR-146b-3p	Down	KCNJ14	Potassium inwardly-rectifying channel, subfamily J, member 14
hsa-miR-146b-3p	Down	KCNJ10	Potassium inwardly-rectifying channel, subfamily J, member 10
hsa-miR-146b-3p	Down	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
hsa-miR-146b-3p	Down	SLC46A1	Solute carrier family 46 (folate transporter), member 1
hsa-miR-146b-3p	Down	RAB3B	RAB3B, member RAS oncogene family
hsa-miR-146b-3p	Down	PIK3C2B	Phosphoinositide-3-kinase, class 2, beta polypeptide
hsa-miR-146b-3p	Down	GALT	Galactose-1-phosphate uridylyltransferase
hsa-miR-146b-3p	Down	SMC1A	Structural maintenance of chromosomes 1A
hsa-miR-146b-3p	Down	ANPEP	Alanyl (membrane) aminopeptidase
hsa-miR-146b-3p	Down	SSH3	Slingshot homolog 3 (Drosophila)

Discussion

Previous reports showed that COX-2 not only mediates the systemic inflammatory response, but also takes an important part in atherosclerotic lesions and regulated by a variety of inflammatory cytokines. In this study, compared with the normal control group, the expression of plasma IL-6 and COX-2 were increased significantly in DCMI patients. COX-2 is an important rate-limiting enzyme in the process of arachidonic acid metabolism in the formation of thrombosis, which affects the production of prostaglandin and TXA-2 [27]. COX-2 has a close correlation with the formation of arterial thrombosis. In recent years, more scholars tend to explore the role of COX-2 and its mechanism in the development of arterial thrombosis [28].

It has been suggested that miRNAs played a regulatory role in inflammatory reaction, aging, proliferation, migration of vascular smooth muscle cells, monocyte adhesion, and vascular endothelial cells differentiation, vascular integrity maintain, cholesterol metabolism, and most importantly in some pathophysiological process, including apoptosis after cerebral ischemia and ischemic stroke [29-32]. In our study, we verified differential expression of 35 miRNAs in thrombosis and normal control group by RT-PCR. miRNA-146b-3p was significantly down-regulated in thrombosis group. Then, we tried to confirm whether RAF1 or DNMT3A was a true target of miR-146b-3p. The luciferase reporter assay was used in PBMC transfected with pMIR-RAF1-3'UTR-wild type, pMIR-RAF1-3'UTR-mutant along with miR-146b-3p mimics or control RNA. A significantly decreased of luciferase activity was observed only in the cells transfected with pMIR-RAF1-3'UTR-wild type and miR-146b-3p mimics, but not in the cells transfected with pMIR-RAF1-3'UTR-mutant and miR-146b-3p mimics. On the contrary, no markedly decreased of luciferase activity was observed in the cells transfected with pMIR-DNMT3A-3'UTR-wild type and miR-146b-3p mimics (**Figure 4G**). These findings suggest that miR-146b-3p negatively regulates RAF1 expression by interacting with its 3'-UTR in DMCI development.

RAF1 and DNMT3A were potential targets for miR-146b-3p. Fulzele et al. [18] have reported that miR-146b-3p can regulate the smooth muscle cell function by transforming growth factor beta (TGF-β) signaling pathway. DNMT3A is one of RAF1 target genes, as an important transcription factors in bone morphogenetic protein signaling pathway. Moreover, DCMI development is a chronic inflammatory process of the vascular endothelial cells. Therefore,

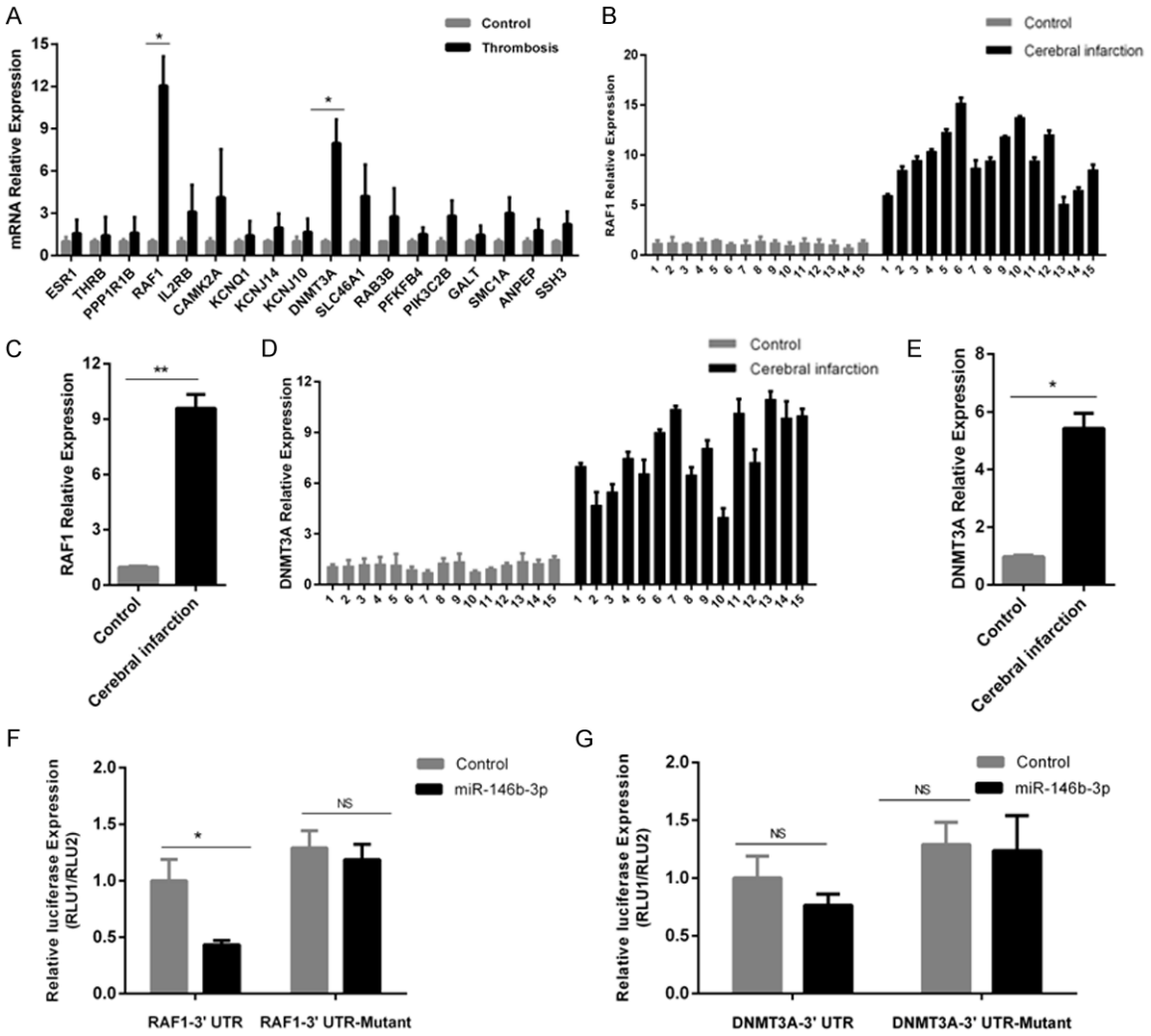

Figure 4. RAF1 was a functional target of miR-146b-3p. A. RT-PCR analysis of miR-146b-3p target genes expression in thrombosis patients compared with control group; B, C. RT-PCR verified RAF1 expression in 15 cases of thrombosis patients, compared to that of 15 cases of normal control; D, E. RT-PCR verified DNMT3A expression in 15 cases of thrombosis patients, compared to that of 15 cases of normal control; F. Verification of the miR-146b-3p cognate site in 3'-UTR of RAF1 by luciferase activity assay. G. Verification of the miR-146b-3p cognate site in 3'-UTR of DNMT3A by luciferase activity assay. (n = 3, *indicates P<0.05, **indicates P<0.01, NS: non-significant difference)

miR-146b-3p may play an important role in DCMI development by regulating the expression of its downstream target gene DNMT3A. Altogether, these data indicate that miR-146b-3p might be a potential biomarker for DMCI patients. Moreover, Riesco-Eizaguirre et al. have analyzed the molecular function of miR-146b-3p targeted genes by three aspects: biological process, cellular group and molecular function. The results have showed that miR-146b-3p is involved in the process of ion transport, positioning, development of the nervous system, cell adhesion, cell differentiation, cell proliferation [33]. Many of these biological processes are closely related to the biological characteristics of endothelial cells and the development of DCMI.

Our results showed that miR-146b-3p was evidently down-regulated in DMCI patients compared with normal control group. And we further verified that miR-146b-3p negatively regulates RAF1 expression by interacting with its 3'-UTR. Then, we attempted to find out whether miR-146b-3p regulated RAF1 expression mediates P38MAPK signaling activation. The results implied that the expression of miR-146b-3p was significantly down-regulated in

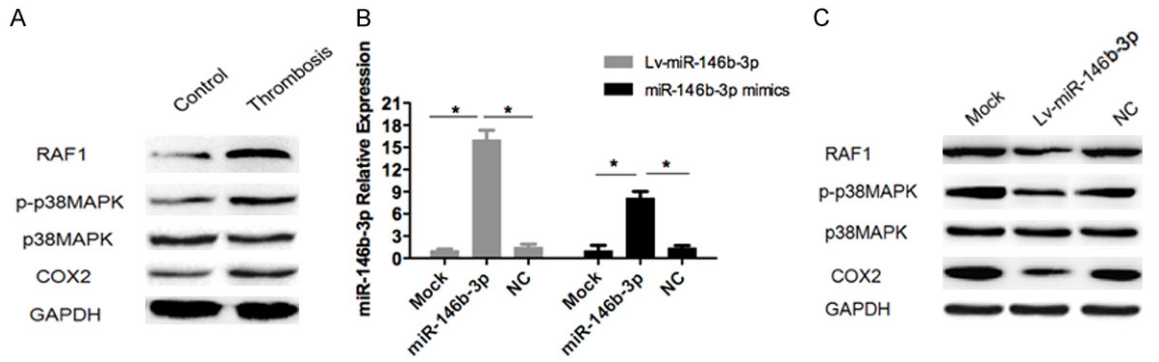

Figure 5. miR-146b-3p targeted regulate RAF1 expression mediates P38MAPK signaling activation in thrombosis patients compared with control. A. Expression of RAF1, phosphorylation of P38MAPK and COX-2 were assessed in thrombosis and control group. B. PBMC cells were infected with lentivirus at MOI of 10 or transfected with miRNA mimics at 200 nM, and measured miR-146b-3p expression levels at 24 h after transfection or infection. C. Expression of RAF1, phosphorylation of P38MAPK and COX-2 was assessed in Mock group, Lv-miR-146b-3p group and NC group. (n = 3, *indicates P<0.05).

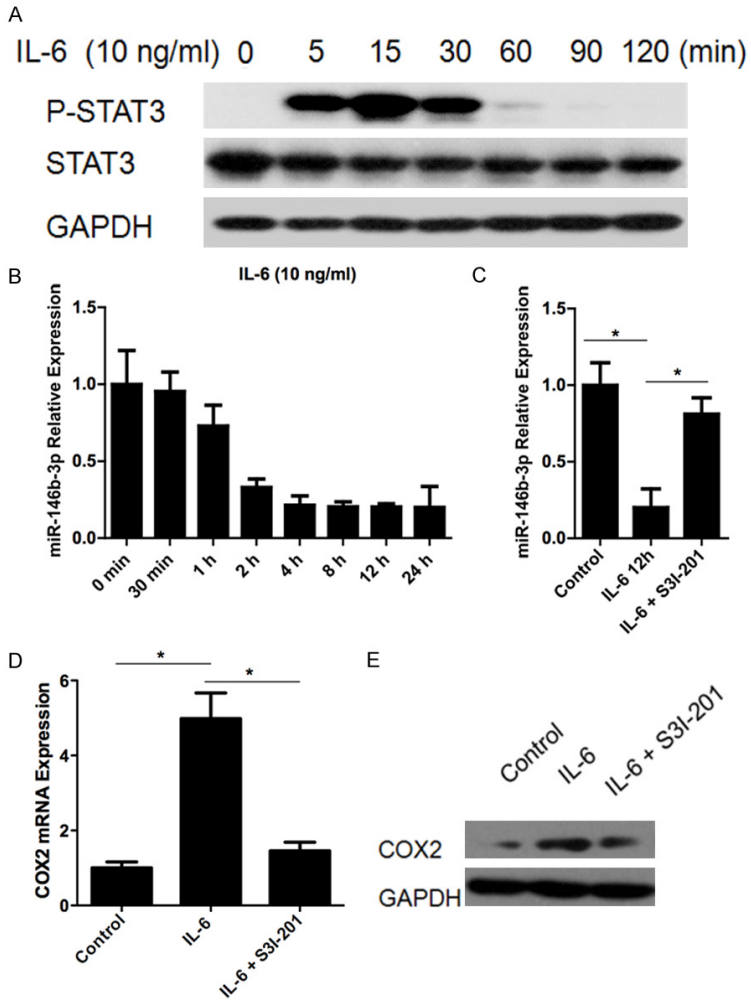

Figure 6. IL-6 activated STAT3 and down regulated miR-146b-3p induced COX2 expression in PBMC. A. Effect of IL-6 on the expression and phosphorylation of STAT3; B. Effect of IL-6 on the expression of miR-146b-3p; C. Expression of miR-146b-3p in Control group, IL-6 12 h group and IL-6+S3I-201 group; D, E. Expression of COX2 in Control group, IL-6 12 h group and IL-6+S3I-201 group. (n = 3, *indicates P<0.05)

DCMI patients, which induced the expression of RAF1. Then the p38MAPK signal pathway, which induced the up-regulation of COX-2, was activated, and ultimately promoted the formation of blood clots in the arteries.

In addition, IL-6 has been proved to stimulate the expression of STAT3 in monocytes, which is closely related to the STAT signaling pathway [33]. Riesco-Eizaguirre et al. have reported that the binding sites which recognize STAT3, existed in the miR-146b-3p [33]. To further understand the relationship between STAT3 and miRNA-146b-3p, PBMCs were treated by IL-6 combined with STAT3 specific inhibitor S3I-201. The results showed that the miR-146b-3p expression was significantly increased in combined group (IL-6+S3I-201). Due to the highly conserved STAT3 binding site sequence of miR-146b-3p gene, we speculated and verified that the expression of miR-146b-3p was closely related to the regulation of STAT3.

Moreover, we also demonstrated that STAT3 signaling path-

way was activated in the formation of arterial thrombosis, which was closely correlated to the up-regulation of IL-6. And the down-regulation of miRNA-146b-3p was induced by STAT3. Moreover, the regulated expression of COX-2 through MAPK signal transduction pathway was proved in the development of DCMI. Thus, by effectively targeting the intervention of miR-146b-3p/RAF1/P38MAPK/COX-2 signaling pathway, it will provide potential targets for DCMI prevention and treatment.

There are still some limitations in our study. Due to the design limitation, we only compared the diabetic patients with cerebral infarction (DMCI) with the normal population. However, diabetes patients with other complications or patients with ischemic stroke only were not included. Fulzele S et al. suggested that miR-146b-3p played a role in the regulation of retinal inflammation in diabetes by suppressing ADA2 [18], which suggested a relationship between miR-146b and diabetic microvascular complications. Others proved that miR-146a expression was down-regulated in diabetes mellitus patients with ischemic stroke, but not in patients with ischemic stroke only. Low plasma miR-146a expression is a risk factor for ischemic stroke in Chinese diabetes mellitus patients [34]. As we all know, the sequences of miR-146b are similar with miR-146a [19]. As the result of our study implied a relationship between miR-146b and DMCI, we might assume that miR-146 plays a role in the development and progression of cerebral infarction with diabetes when the signal pathway needs further research.

In summary, the major finding of this study is that the expression of miR-146b-3p is closely related to the development of DCMI through mediating the RAF/P38MAPK/COX-2 signaling pathway. Abnormal miR-146b-3p expression appears to be a critical determinant of DCMI development, which may serve as a new therapeutic target for detection and intervention of DMCI.

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Disclosure of conflict of interest

None.

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