EXPERIMENTAL STUDY

MicroRNA-665 Regulates Cell Proliferation and Apoptosis of Vascular Smooth Muscle Cells by Targeting TGFBR1

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Summary
Coronary artery disease (CAD) is one of the heavy health burdens worldwide. Aberrant proliferation of vascular smooth muscle cells (VSMCs) contributes to the occurrence and development of CAD. This study aimed at exploring differentially expressed microRNAs (miRNAs) and their regulatory mechanisms in the development of CAD.

The miRNA expression profile of GSE28858 was obtained from the Gene Expression Omnibus database. Differentially expressed miRNAs (DEmiRNAs) between CAD and healthy control samples were analyzed using limma package in R. Target genes of DEmiRNAs were predicted, and a miRNA-target gene network was constructed. The relationship between miR-665 and transforming growth factor beta receptor 1 (TGFBR1) was selected for further analysis. The interaction between miR-665 and TGFBR1 was confirmed by dual luciferase reporter assay. Effects of miR-665 on cell viability and apoptosis of VSMCs were evaluated by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Besides, western blot assays for BCL2L11 and caspase 3 were also conducted.

A total of 38 upregulated miRNAs and 28 downregulated miRNAs were identified. The expression level of miR-665 was significantly downregulated in patients with CAD. TGFBR1 was proved to be a target gene of miR-665. Besides, ectopic expression of miR-665 obviously inhibited VSMC growth and promoted VSMC apoptosis. TGFBR1 overexpression in VSMCs transfected with miR-665 mimic could restore the effect of miR-665 on the proliferation and apoptosis of VSMCs.

MiR-665 might participate in the proliferation and apoptosis of VSMCs by targeting TGFBR1.

Key words: Coronary artery disease, Transforming growth factor, Target genes

Coronary artery disease (CAD) is one of the leading health threats worldwide.1 It is the most common cardiovascular disease whose clinical symptoms include angina pectoris, myocardial infarction, and sudden cardiac death.2 Atherosclerosis or atherosclerotic occlusion of the coronary artery is the main pathological change that results in CAD.3,4 Vascular smooth muscle cells (VSMCs) are key constituent cells of normal blood vessel walls and are associated with the pathogenesis of atherosclerosis. In the normal condition, VSMCs display quiescent status with low rate of proliferation and migration.5,6 Under certain circumstances, aberrant proliferation of VSMCs triggered by vascular injury or chemoattractants, such as platelet-derived growth factor-beta (PDGF-bb), leads to the initiation of atherosclerosis, which further results in the occurrence and progression of CAD.7 Therefore, in-depth research of the potential mechanism of VSMC proliferation and migration will help to explore more therapeutic strategies for CAD.

MicroRNAs (miRNAs) are a class of small-strand, non-coding RNA with length of 18-23 nt. MiRNAs modulate gene expression by affecting the transcription of target miRNAs by binding to their seed sequences within 3’ untranslated regions (3’UTRs).8 Numerous studies showed that a variety of miRNAs are involved in almost all biological functions, including cell apoptosis, proliferation, differentiation, and carcinogenesis.9 Growing number of evidence suggested that miRNAs are associated with CAD and that miRNAs act as biomarkers for CAD diagnosis.10,11 Additionally, a variety of miRNAs, such as miR-541, miR-574-5p, and miR-214, have been proved to play regulatory roles in the proliferation of VSMCs, which may further affect the development of CAD.12,13 Considering the prospect of miRNAs in clinical treatment of diseases, exploring more miRNAs that are related to CAD and VMSC will help find more effective therapeutic tar-
gets.

In this study, differential expression analysis for CAD and control samples was conducted to screen differentially expressed miRNAs (DEmiRNAs). Afterward, the downregulated miR-665 was chosen for functional study, and the mechanism of miR-665 in VSMCs was explored.

**Methods**

**Bioinformatics analysis:** The miRNA expression profile of GSE28858, including 12 samples from patients with premature CAD and 12 samples from age- and sex-matched healthy control, was obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database. This dataset was based on the platform of GPL 8179. Illumina Human v2 miRNA expression beadchip. According to the platform annotation information of GPL 8179, probes were converted to the same gene symbols. For different probes mapped to the same gene symbol, the mean value of probes was taken as expression value. Differential analysis was conducted by limma package in R software (version 3.10.3), and the obtained P value was adjusted using Benjamini and Hochberg’s method of obtaining adj.P value. DEmiRNAs were selected based on the criteria of log2 fold change (FC) ≥ 0.3 and adj.P value < 0.05. miRWalk3.0, which integrates the results from miRdb, TargetScan, and miRTarBase, was adopted to predict the target genes of DEmiRNAs. Relationships between DE-miRNAs and target genes were visualized using the Cytoscape software. The functional enrichment analysis tool Database for Annotation, Visualization and Integrated Discovery (version 6.7) was used to analyze the Gene Ontology (GO) functional annotations and KEGG pathway of the target genes of DEmiRNAs.

**Patients:** A total of 48 patients with CAD who underwent coronary angiography in our hospital were enrolled in this study. The inclusion criteria were as follow: 1) the patient was diagnosed with stenosis with lumen narrowing of ≥ 50% in at least one coronary artery by coronary angiography; 2) the patient was newly diagnosed and did not receive any treatment before; and 3) the patient has no history of liver or kidney disease, blood disease, or infectious disease. The control group included age- and gender-matched healthy controls who underwent regular physical examination. These participants were free of CAD confirmed by clinical examination. Patients with vascular disease, heart disease, or malignancy were excluded. Supplemental Table I shows the baseline characteristics of all included participants. Peripheral blood (6 mL) was collected from the enrolled subjects, and serum was isolated and stored at −80°C. All protocols were approved by the Clinical Research Ethics Committee of Renmin Hospital of Wuhan University, and all participants had signed the informed consent forms.

**Cell culture and transfection:** Human VSMCs were obtained from Chinese Type Culture Collection (Chinese Academy of Sciences, Shanghai, China) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (HyClone, UT, USA) and 1% penicillin-streptomycin (Invitrogen, CA, USA). All cells were incubated in a humidified air containing 5% CO2 at 37°C.

The miR-665 mimic, mimic control (NC control), miR-665 inhibitor, inhibitor control (IN control), pcDNA 3.1(+) vector, or pcDNA3.1-transforming growth factor beta receptor 1 (TGFBRI) were synthesized from GenePharma (Shanghai, China). Lipofectamine™ 2000 (Invitrogen, CA, USA) reagent was applied for transient transfection according to the commercial instructions.

**Quantitative reverse transcription PCR (qRT-PCR):** The TRIzol reagent (Invitrogen, CA, USA) was used to isolate total RNA according to the user’s manual. Reverse transcription of miRNA and mRNA was conducted using PrimeScript™ RT Master Mix (Takara, Japan) and AMV Reverse Transcriptase Kit (Thermo Fisher Scientific, MA, USA), respectively. The target molecule was quantitatively detected using SYBR Green Master Mix (Roche, Basel, CH). Primer sequences were displayed in Supplemental Table II. GAPDH and U6 were used as internal control for miRNA and mRNA quantification. Expression of miRNAs and mRNAs were analyzed using the 2−ΔΔCt method.

**Cell viability assay:** Experimental cells at density of 6 × 104 cells/well were seeded in 96-well plate and were harvested at 12, 24, 48, and 72 hours post-seeding for cell counting kit-8 (CCK-8) assay. After removing the supernatant, the harvested cells were incubated with 100 μL DMEM containing 10 μL CCK-8 reagent (Beyotime, Shanghai, China) at 37°C for 2 hours, followed by the record of OD550 on a microplate reader (Bio-Rad, VT, USA).

**Cell apoptosis assay:** The apoptosis rate of VSMCs was analyzed using Annexin V-FITC/propidium iodide (AV/PI) Apoptosis Detection Kit (YEASEN, Shanghai, China) according to the commercial instruction. Briefly, the resuspended VSMCs were incubated with mixture containing Annexin V-FITC and PI staining solution for 15 minutes in the dark. With the addition of 400 μL of 1 × binding buffer, all tubes were gently mixed and placed on ice, and flow cytometry detection was performed within 1 hour.

**Dual luciferase reporter assay:** The wild-type (WT) and mutant (MUT) fragment of TGFBRI 3’ UTR were individually reconstituted into pGL3-basic reporter vector (Promega, WI, USA). At 48 hours post transfection, luciferase activity was quantified by Dual Luciferase Reporter Assay System (Promega, WI, USA).

**Western blotting:** Protein was fractionated by 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Boston, MA, USA) for target protein detection. The membranes were incubated overnight with the corresponding primary antibodies at 4°C. Antibody against BCL2L11, caspase 3, and GAPDH were brought from Abcam (Cambridge, UK). Then, the membranes were reacted with HRP-conjugated secondary antibodies for 2 hours at room temperature. Protein extracts were visualized by ECL Plus Western Blotting Detection System (Bio-Rad, CA, USA).

**Statistical analysis:** The software package of GraphPad Prism 6.0 (GraphPad Software, Inc., CA, USA) was used for statistical analysis. Quantitative variables were presented as mean ± standard deviation, and qualitative data were represented as percentages. Differences of qualitative and quantitative data were compared using X2 test or Students’ t test, respectively. One-way analysis of variance was used to calculate differences among groups (>2
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Figure 1. MiRNA screening and target gene function analysis. A: Volcano map of DEmiRNAs; B: Heat map of top 10 (sorted according to log2 FC descending order) DEmiRNAs. Red and green indicated upregulated and downregulated miRNAs, respectively.

Results

Differential expression analysis: According to the screening threshold (logFC ≥ 0.3 and adj.P.value < 0.05), 66 DEmiRNAs, including 38 upregulated miRNAs and 28 downregulated miRNAs, were identified (Figure 1A). The clustering heat map of the DEmiRNAs suggested that the DEmiRNAs could distinguish the samples with different disease statuses (Figure 1B).

To investigate the functions of DEmiRNAs, miRWalk 3.0, which integrates data from miRdb, TargetScan, and miRTarBase, was used to predict corresponding target genes of DEmiRNAs. A miRNA regulatory network that includes 194 relationships among 6 DEmiRNAs and 187 target genes was built by Cytoscape (Figure 2). Among the six DEmiRNAs, hsa-miRNA-199a-5p, hsa-miRNA-299-5p, hsa-miRNA-193a-3p, and hsa-miR-665 were the nodes with higher degrees.

The GO function and KEGG pathway enrichment analysis showed that a total of 25 biological processes (BP), 3 cellular components (CC), 2 molecular functions, and 2 KEGG pathways (P < 0.05) were significantly enriched by the target genes. As shown in Supplemental Table III, the target genes of miRNAs were involved in GO-BP term of “positive regulation of cell motion” and KEGG pathways of “MAPK signaling pathway” and “Focal adhesion.” Particularly, BP term of “heart development” (ACVR2B, ACTC1, ERBB4, TGFBR1, TEAD1, SMYD1, NFATC4, PROX1, and SRF), KEGG pathway of “MAPK signaling pathway” (DUSP4, TAOK1, CACNG8, TGFBR1, PDGFRB, CACNB2, NFATC4, STMN1, SRF, and AKT3), and several genes, such as ACTC1, ERBB4, and TGFBR1, were reported to play critical role in heart development.24-26)

MiR-665 is downregulated in patients with CAD: To confirm the bioinformatic analysis results, the abundance of miR-665, miR-199a-5p, miR-299-5p, and miR-193a-3p was examined using qRT-PCR assay. As shown in Figure 3A, the expression levels of miR-665 and miR-199a-5p in patients with CAD were significantly lower than those in control group (P < 0.05). Especially, the difference of miR-665 between patients with CAD and control group reached to an extremely significant level (P < 0.001). Besides, the expression of TGFBR1 was significantly higher in patients with CAD compared with that in control group (P < 0.05, Figure 3B). As TGFBR1, one of the target genes of miR-665, was reported to play a critical role in heart development, we selected the miR-665/TGFBR1 axis for further analysis.

As shown in Figure 3C and Supplemental Figure A, the incubation of PDGF-bb (20 ng/mL) accelerated VSMC proliferation. The expression of miR-665 was reduced in a time-dependent manner, while the expression of TGFBR1 was increased along with the treatment time of PDGF-bb (Figure 3D). PDGF-bb is one of the commonly used growth factors for VSMCs. This result showed that the amount of miR-665 negatively correlated with the treatment time of PDGF-bb, which profoundly indicated that the expression of miR-665 was downregulated in VSMCs.

MiR-665 binds to TGFBR1: TGFBR1 is involved in cell proliferation of VSMCs27) and heart development. In the miRNA regulatory network, TGFBR1 was predicted as one of the potential targets of miR-665 (Figures 2, 4A). To determine the association between miR-665 and TGFBR1, luciferase reporter assay was performed. MiR-665 overexpression reduced the luciferase activity of cells transfected with WT-3’UTR TGFBR1 but did not affect the TGFBR1 MUT-3’UTR transfection group (Figure 4B). In addition, the mRNA and protein abundance of TGFBR1 was significantly reduced by miR-665 mimic transfection in VSMCs but were significantly increased in miR-665 inhibitor treatment group (Figure 4C and D). These results suggested that there was interaction between TGFBR1 and miR-665 in VSMCs.

MiR-665 inhibits VSMC proliferation: MiR-665 mimic
or miR-665 inhibitor was delivered into VSMCs to study the functional effect exerted by miR-665 in VSMCs. Transfection of miR-665 mimic and inhibitor effectively affected intracellular level of miR-665 (Figure 5A). The cell viability assay revealed that, compared with control, transfection of miR-665 mimic greatly repressed cell proliferation, whereas inhibition of miR-665 promoted VSMC growth (Figure 5B and Supplemental Figure B). Notably, the alteration of cell growth was not caused by chemical oligomer transfection (Figure 5B).

**MiR-665 promotes the apoptosis rate of VSMCs:** Subsequently, we explored the role of miR-665 on the apoptosis of VSMCs. As shown in Figure 6A, the apoptosis rate of VSMCs with miR-665 mimic transfection was obviously increased, while the suppression of miR-665 reduced the apoptosis rate of VSMCs. The protein level of two apoptosis-related factors, BCL2L11 and caspase 3, were upregulated by miR-665 mimic transfection but were downregulated by miR-665 inhibitor addition (Figure 6B), implying that miR-665 could promote VSMC apoptosis.

**MiR-665 affected the proliferation and apoptosis of VSMCs through inhibiting TGFBR1 expression:** TGFBR1 was identified as the target gene of miR-665 in VSMCs. To investigate whether miR-665 regulate the proliferation and apoptosis of VSMCs through targeting TGFBR1, we ectopically delivered the construction carrying open reading frame of TGFRB1 into VSMC cells. As shown in Figure 7A and Supplemental Figure C, the CCK-8 assay revealed that overexpression of TGFRB1 could restore the effect of miR-665 on cell growth of VSMCs. Similarly, TGFRB1 transfection partly suppressed VSMC apoptosis when compared with control group (Figure 7B), which was consistent with the protein detection results (Figure 7C).

**Discussion**

In the present study, through DEmiRNA screening and qRT-PCR detection, the level of miR-665 was found to be greatly downregulated in patients with CAD. Functional experiments found that ectopic expression of miR-665 inhibited VSMC proliferation and promoted VSMC apoptosis. Based on the online bioinformatic analysis, TGFB1 was speculated to be one target of miR-665 in VSMCs, which was verified by dual luciferase assay and western blot assay. Additionally, TGFB1 overexpression in VSMCs transfected with miR-665 mimic could restore the effect of miR-665 on VSMC proliferation and apoptosis. Taken together, these results implied that miR-665 regulates the proliferation and apoptosis of VSMCs in CAD by targeting TGFB1.

To accurately screen and select the functional molecules, the DEmiRNAs in patients with CAD and their target genes were further analyzed in miRWalk3.0. To improve the accuracy of miRNA-mRNA prediction, only miRNAs and their target genes appearing in all three databases of miRdb, TargetScan and miRTarBase were included in subsequent verification experiments. Consequently, miR-665, miR-199a-5p, miR-299-5p, and miR-193a-3p were selected for qRT-PCR detection. The results
showed that the level of miR-665 and miR-199a-5p was significantly reduced in patients with CAD (Figures 1C, 2).

MiR-665 has been reported to participate in various diseases, including cancer and heart failure. However, its expression in heart disease is controversial. Fan et al. found significant increase of miR-665 in endothelial cells of human heart with heart failure. In a recent study, Liu et al. found that miR-665 was upregulated in ischemia/reperfusion rat model. In the other hand, Mohnle et al.
Figure 4. MiR-665 binds to the 3’UTR of TGFBR1. A: Sequences of wild-type and mutant 3’UTR of TGFBR1. B: Dual luciferase reporter assay was conducted to investigate the association between TGFBR1 and miR-665. C: Effect of miR-665 on TGFBR1 expression was analyzed by qRT-PCR. D: Effect of miR-665 on TGFBR1 was analyzed using western blot assay. E: Quantitative analysis of western blot result. Data were representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, compared with control.

Figure 5. MiR-665 inhibits the proliferation of VSMCs. A: Transfection of miR-665 mimic and inhibitor was determined by qRT-PCR. B: CCK-8 assay was conducted to measure the proliferation of VSMCs. Data were representative of three independent experiments. ***P < 0.001, compared with control; ****P < 0.001, compared with inhibitor NC; ####P < 0.001, compared with mimic NC.
Figure 6. MiR-665 promotes the apoptosis of VSMCs. A: Effect of miR-665 on the apoptosis of VSMCs was determined by flow cytometry analysis. Quantification result was displayed in the right panel. B: Effect of miR-665 on intracellular expression of apoptosis-related factors including BCL2L11 and caspase 3 in VSMCs were analyzed using western blot. C: Quantitative analysis of western blot result. Data were representative of three independent experiments. **P < 0.01; ***P < 0.001, compared with control.
Figure 7. MiR-665 inhibits the proliferation and promotes the apoptosis of VSMCs by targeting TGFBR1. A: CCK-8 assay was conducted to measure the proliferation of VSMCs. B: Effect of miR-665 and TGFBR1 on the apoptosis of VSMCs was determined by flow cytometry analysis. C: Effect of miR-665 on intracellular expression of apoptosis-related factors including BCL2L11 and caspase 3 in VSMCs were detected by western blot experiments. D: Quantitative analysis of western blot result. Data were representative of three independent experiments. ***P < 0.001, compared with control; ###P < 0.001, compared with miR-665 mimic + pcDNA3.1.

demonstrated that miR-665 is downregulated in patients with heart failure.\(^{32}\) Li suggested that miR-665 was downregulated by PDGF-bb in the VSMC proliferation.\(^{33}\) In this study, we found that miR-665 was decreased in patients with CAD in the miRNA array of GSE28858 and in our own cohort of patients. Besides, our study supported the result of Li that miR-665 was downregulated by PDGF-bb. We speculated that the body is a complex or-
ganism and that the level of miRNAs can be modulated by other type of non-coding RNAs, such as long non-coding RNA (lncRNA) and circular RNA (circRNA). lncRNAs are linear RNA with lengths exceeding 200 nucleotides, and circRNAs are covalently closed RNAs. Growing evidence proved that both lncRNAs and circRNAs can function as sponges of miRNAs to regulate the abundance of miRNAs and miRNA-targeting genes. For example, circABC2, lncRNA HIF1A-AS2, and lncRNA BCAR4 were found to affect the cellular level of miR-665 and interact with miR-665, implying the potential lncRNA/circRNA-miR-665 regulatory axis. However, further studies on the role of miR-665 in CAD or other disease are still warranted.

miR-199a-5p has been reported as a potent regulator of arteriogenesis, which is an adaptive response to transient coronary artery occlusion and is associated with lower incidence of myocardial infarction. Additionally, miR-199a-5p was found to target WNT2 signaling pathway, resulting in the alteration of cell proliferation.39) TGFB signaling pathway is involved in the progression of CAD through targeting TGFBR1. TGFBR1 is a membrane-bound protein that behaves as a receptor for TGFB and its family members. The current research only validated the role of miR-199a-5p in heart disease also deserved to be further investigated.

The integrated results from miRdb, TargetScan and miRTarBase predicted that TGFBR1 was one target gene of miR-665. Besides, the KEGG analysis and GO enrichment analysis showed that TGFBR1 was associated with “MAPK signaling pathway” and “heart development.” Previous study proved that TGFBR1 could be considered as a new candidate prognostic biomarker after acute myocardial infarction, indicating the relation between TGFBR1 and cardiovascular disorders. Combined with the quantification results, we speculated that miR-665 may be involved in the progression of CAD through targeting TGFBR1. TGFBR1 is a membrane-bound protein that belongs to TGFBR subfamily. TGF signaling pathway is the key intercellular pathway and modulates multiple types of physiological processes, including cell apoptosis, proliferation, differentiation, and extracellular matrix production. The interaction between TGFBR1 and TGFBR1 also affects cell proliferation of VSMCs. Previous study demonstrated that the inhibition of TGFBR1 expression could accelerate cell growth of VSMCs, suggesting that TGFBR1 and its regulatory manner were critical for sustaining the normal condition of VSMCs.27) With dual luciferase assay and functional experiments, the present study proved that miR-665 regulates the proliferation and apoptosis of VSMCs through targeting TGFBR1. TGFBR1 exerts a critical role in the initiation of a variety of signaling pathways including Smad2/Smad3, ERK, and PI3 K/Akt. The current research only validated the role of miR-665-TGFBR1 axis in VSMC proliferation and apoptosis, but the downstream signaling pathway involved in the process remains unknown, which needs to be further investigated.

Conclusion

The above results proved that miR-665 participated in regulating the proliferation and apoptosis process of VSMCs through binding to the seed sequences within TGFBR1. The present investigation exhibited novel therapeutic strategies and clinical targets and for CAD treatment. Nevertheless, the roles of these genes and miRNA in CAD should be further confirmed in in vivo experiments.

Disclosure

Conflicts of interest: The authors declare that they have no conflicts of interests.

References


Supplemental Files
Supplemental Tables I-III
Supplemental Figure
Please see supplemental files; https://doi.org/10.1536/ihj.20-016