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Article

MiR-26b Suppresses the Development of Stanford Type A Aortic Dissection by Regulating HMGA2 and TGF- β /Smad3 Signaling Pathway

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Purpose: Stanford type A aortic dissection (TAAD) is one of the most dangerous cardiovascular diseases. MicroRNAs (miRNAs) have been considered as potential therapeutic targets for TAAD. In this present study, we aimed to investigate the functional role and regulatory mechanism of miR-26b in TAAD development.

Materials and Methods: MiR-26b mRNA expression was detected by real-time polymerase chain reaction (RT-PCR) and protein levels were measured by Western blot. Verifying the direct target of miR-26b was used by dual luciferase assay, RT-PCR, and Western blot. Cell Counting Kit-8 (CCK-8) and TUNEL staining assays were applied for detecting rat aortic vascular smooth muscle cells (VSMCs) viability and apoptosis, respectively.

Results: We found that miR-26b was under-expressed in TAAD patients and closely associated with the poor prognosis of TAAD patients. Re-expression of miR-26b facilitated while knockdown of miR-26b inhibited VSMC proliferation. However, miR-26b showed the opposite effect on cell apoptosis. More importantly, high-mobility group AT-hook 2 (HMGA2) was verified as the direct target of miR-26b. Furthermore, transforming growth factor beta (TGF- β)/Smad3 signaling pathway was involved in the development of TAAD modulated by miR-26b.

Conclusion: miR-26b impeded TAAD development by regulating HMGA2 and TGF- β /Smad3 signaling pathway, which provided a potential biomarker for TAAD treatment.

Keywords: miR-26b, Stanford type A aortic dissection, HMGA2, TGF- β /Smad3, 39 pathway

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Introduction

Aortic dissection, an acute macrovascular disease with a dangerous condition and a high mortality rate, can be divided into Stanford type A and Stanford type B depending on whether the dissection affects the ascending aorta.¹⁾ Acute Stanford type A aortic dissection (TAAD) has the characteristics of sudden onset, rapid progression, and high mortality.²⁾ In recent years, the use of advanced imaging technology makes TAAD clear diagnosis is no longer difficult, but there are still many questions about its pathogenesis and treatment options. Currently, it is believed that poor control of hypertension, mutations in extracellular matrix components, and associated

inflammation caused by endothelial cell damage might be the key link in the pathogenesis of TAAD.³⁾

MicroRNAs (miRNAs), single-stranded non-coding small molecular RNAs, are closely related to a variety of diseases and participate in various important biological processes.^{4,5)} Recently, mounting evidences have shown that miRNAs were involved in the development of many cardiovascular diseases, including aortic dissection.^{6–8)} Previous studies have confirmed that in vitro studies, miRNAs can participate in the regulation of vascular remodeling by regulating rat aortic vascular smooth muscle cell (VSMC) proliferation, migration, phenotypic changes, and other functions.⁹⁾ MiR-26b is a hotspot miRNA of current studies and plays an important role in various physiological and pathological processes. For instances, it was down-regulated in melanoma tissues¹⁰⁾ and showed the suppression effect on liver fibrosis and angiogenesis.¹¹⁾ Moreover, Xu *et al.* displayed that miR-26b was significantly decreased in TAAD and served as a biomarker for TAAD diagnosis, which might provide a new potential therapeutic target for TAAD.¹²⁾ Therefore, further understanding the molecular mechanism of miR-26b in the pathogenesis of TAAD is very essential.

MiRNAs were proved to play a huge role in cell differentiation, biological development, and disease progression via the regulation of their target mRNAs. Therefore, searching for the direct target of miR-26b was critical for understanding the potential mechanism underlying the pathogenesis of TAAD. High-mobility group AT-hook 2 (HMGA2), a member of the high-mobility group family, has been reported to take part in the proliferation and differentiation during embryonic development.¹³⁾ In addition, HMGA2 was discovered to be involved in the development and progression of many cardiovascular diseases, such as atherosclerosis, intravenous leiomyomatosis, and cardiac lipomas.^{14–16)} Moreover, Belge *et al.* found that HMGA2 was over-expressed in acute aortic dissection and associated with endothelial–mesenchymal transition.¹⁷⁾ However, the biological function of HMGA2 in TAAD pathogenesis and whether it was regulated by miR-26b has not been fully elucidated.

Transforming growth factor beta (TGF- β) signaling pathway exerts pleiotropic actions on cell physiology such as growth, survival, migration, cell fate specification, and differentiation. Previous studies have revealed that miR-26b mediates TGF- β signaling pathway in hepatocellular carcinoma progression.¹⁸⁾ Also, HMGA2 was involved in a variety of disease development via

TGF- β signaling pathway.^{19,20)} Here we investigated whether miR-26b/HMGA2 axis influenced the TGF- β signaling pathway in TAAD development.

In this present study, we aimed to explore the functional role and potential mechanism of miR-26b on the development of TAAD, which provided novel therapeutic targets for TAAD.

Materials and Methods

Clinical samples

In all, 25 patients admitted to the emergency department of the Affiliated Hospital of Southwest Medical University from October 2008 to December 2018 who met the diagnostic criteria of TAAD were collected. All patients with TAAD were divided into mild risk group (<20 scores, five cases), moderate risk group (20–30 scores, 11 cases), and severe high-risk group (>30 scores, nine cases) according to the risk assessment guidelines for acute aortic dissection issued by the United States in 2010²⁾ and EuroSCORE II.²¹⁾ Aortic wall tissues of ascending aorta of patients with TAAD were obtained during surgical operations and the aortic tissues of ascending aorta from donors for heart transplants were selected as the control group. Patients in the control group were excluded from Marfan syndrome, Ehlers-Danlos syndrome, familial thoracic abdominal aortic dissection, hyperkinetic inflammation, and other aortic diseases. A detailed description of the clinical characteristics of the study population is presented in **Table 1**. This study was approved by the medical ethics committee of the Affiliated Hospital of Southwest Medical University and was conducted in strict accordance with the experimental program. The informed consent was obtained from all subjects. Aortic wall tissues were fixed with neutral formalin, stored in the numbered enzyme-free EP tubes, and stored at -80°C for further analysis. Peripheral venous blood samples (5 mL) from subjects were collected and obtained the serum via centrifugation. The collected serum was stored in the numbered enzyme-free EP tubes, and stored at -80°C until use.

Cell culture and transfection

The rat aortic VSMCs were obtained from the cell bank of the Chinese Academy of Sciences (Beijing, China) and cultured in DMEM medium (Invitrogen) containing with 10% FBS (Invitrogen) and necessary antibiotic and then maintained at 37°C with 5% CO_2 atmosphere. MiR-26b mimic/inhibitor, HMGA2 siRNA, and the corresponding negative controls were synthesized by

Table 1 The clinical characteristics of acute aortic dissection and control groups

Characteristics	TAAD (n = 25)	Control (n = 20)
Age (years)	52.3 ± 12.1	53.4 ± 8.2
Male	24 (96%)	18 (90%)
Female	1(4%)	2 (10%)
Current smoking	10 (40%)	4 (20%)
Hypertension	20 (80%)*	0
Atherosclerosis	12 (48%)	4 (20%)
Aortic	1 (4%)	0
Hyperlipidemia	0	0
Diabetes mellitus	3 (12%)	0
Marfan syndrome	0	0

Data are presented as mean ± SD or n (%). *P <0.05. TAAD: type A aortic dissection

Genechem (Shanghai, China), which were used for increasing/decreasing miR-26b expression or inhibiting HMGA2 expression, respectively. They were transfected into VSMCs with the help of Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. After transfection for 48h, the VSMCs were collected for further use.

Real-time PCR

Trizol reagent (Invitrogen) was applied for extracting total RNA from aortic wall tissues and VSMCs following the manufacturer's instructions. The PrimerScript RT reagent kit (Takara, Dalian, China) was used for transcribing RNA samples. TaqMan miRNA assay kit and FAST SYBR-Green Master Mix were carried out for evaluating the relative expression of miRNA and target gene messenger RNA (mRNA), respectively. MiRNA and mRNA expression was normalized with U6 and GAPDH endogenous control, respectively. And their fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total proteins were extracted from aortic wall tissues and VSMCs using RIPA lysis buffer. The protein concentration was conducted by BCA kit. After the equal amounts of proteins loaded onto a 10%–15% SDS-PAGE gel, followed by electrotransferred onto polyvinylidene fluoride membranes, the membranes were blocked with 5% non-fat milk for 2 h. Then, the membranes were incubated with primary antibodies at 4°C overnight, subsequently the corresponding secondary antibodies at room temperature for 2 h. Finally, an enhanced chemiluminescence apparatus was used for visualizing the brands and Image Lab 4.0 imaging software was applied

for analyzing the gray value of proteins. These proteins were normalized to that of β -actin as the relative protein expression.

Cell Counting Kit-8 assay

VSMCs (5×10^3 /well) with different transfection were added into 48-well plates and cultured for 12, 24, 36, and 48 h, respectively. Then, Cell Counting Kit-8 (CCK-8) solution (10 μ L) was added and incubated at 37°C for another 2 h. Finally, the plates were read at a wavelength of 450 nm using the microplate reader (Bio-Tek, Winooski, USA) to measure the absorbance, which was used for evaluating cell viability.

TUNEL staining assay

One Step TUNEL Apoptosis Assay Kit was purchased from Beyotime (Shanghai, China). VSMC apoptosis was conducted following the manufacturer's instructions. A CoolSNAP photometric camera and a Nikon ECLIPSE Ti fluorescence microscope were applied for photographing cells and counting TUNEL-positive cells, respectively. The relative apoptosis rate (%) was calculated as the percentage of TUNEL-positive cells in total number of cells in 12 random fields for each sample.

Dual luciferase reporter assay

The wild type (WT) or mutated (MuT) of HMGA2 3'UTR, named as HMGA2 3'UTR-WT or HMGA2 3'UTR-MuT, containing the binding sites of miR-26b were inserted into psiCHECK-2 vector (Promega, Madison, USA). Then, the luciferase reporter vectors and miR-26b mimic or inhibitor were co-transfected into VSMCs with the aid of Lipofectamine 2000 following the manufacturer's instructions. After transfection for 24 h, the relative luciferase activities were measured by a luciferase reporter assay system.

Statistical analysis

All experiments were repeated in triplicates. The values were represented as mean ± SD. All experimental data were analyzed by GraphPad Prism 6.0 software (San Diego, CA, USA). P values between different groups were calculated by student t-test or Tukey's multiple range post-hoc test after one-way analysis of variance (ANOVA). The correlations between miR-26b and HMGA2 or aortic dissection risk score were analyzed by Spearman's rank test. The log-rank test and Kaplan–Meier analysis were applied for calculating the relevance between miR-26b expression and the postoperative

survival time of patients with TAAD. $P < 0.05$ was considered to indicate a significant difference.

Results

Patient characteristics

In all, 25 patients with TAAD and 20 controls were enrolled in this study. The clinical characteristics of the patients are listed in **Table 1**. Age and gender were comparable between the two groups. There were no statistically significant differences in clinical characteristics between the TAAD and control groups, except for hypertension. Most TAAD patients (80%) had concomitant hypertension and almost half of patients (48%) had atherosclerosis.

Down-regulation of miR-26b was associated with the poor prognosis of TAAD

To investigate the significance and mechanism of miR-26b in the pathogenesis of TAAD, we examined the expression of miR-26b in the aortic wall tissues and peripheral serum of patients with acute TAAD. As **Fig. 1A** and **1B** presented, miR-26b levels were significantly reduced in the aortic wall tissues and serum in TAAD group in comparison with control group. Moreover, the expressional level of miR-26b was obviously lower in the moderate risk and severe risk groups than the mild risk group both in the aortic wall tissues (**Fig. 1C**) and serum (**Fig. 1D**) in TAAD group. Furthermore, we evaluated the association between miR-26b level and the risk severity of TAAD patients. Results displayed that they were negatively correlated in the aortic wall tissues and serum of TAAD patients (**Fig. 1E** and **1F**). Importantly, Kaplan–Meier analysis showed that lower miR-26b expression was closely related to shorter overall survival time of TAAD patients (**Fig. 1G**). These results suggested that dysregulation of miR-26b might be associated with the prognosis of TAAD and the severity of this disease.

MiR-26b promoted VSMC proliferation and inhibited VSMC apoptosis

To investigate miR-26b role in the development of acute TAAD, we detected the effect of miR-26b on VSMC proliferation and apoptosis. MiR-26b expression was first measured in VSMCs after transfected with miR-26b mimic or inhibitor by real-time polymerase chain reaction (RT-PCR). Results showed that

miR-26b was significantly increased in miR-26b mimic VSMCs, while decreased in miR-26b inhibitor VSMCs (**Fig. 2A**). Then, CCK-8 assay was applied for detecting VSMC viability after over-expression of miR-26b or silence miR-26b. The findings displayed that over-expression of miR-26b promoted, while silence miR-26b inhibited VSMC viability (**Fig. 2B**). Proliferating cell nuclear antigen (PCNA), a good indicator of cell proliferation, was also used to test miR-26b effect on VSMC proliferation. As we saw in **Fig. 2C**, PCNA was significantly elevated by miR-26b mimic, whereas reduced by miR-26b inhibitor. In addition, Western blot and TUNEL staining assays were used to measure miR-26b effect on VSMC apoptosis. As shown in **Fig. 2D**, re-expression of miR-26b facilitated Bcl-2 level and suppressed Bax and cleaved-caspase-3 level. However, inhibiting miR-26b displayed the opposite effect on the protein levels of Bcl-2, Bax, and cleaved-caspase-3. Moreover, re-expression of miR-26b impeded VSMC apoptosis rate markedly, whereas knockdown of miR-26b significantly enhanced VSMC apoptosis rate (**Fig. 2E**). All these findings indicated that miR-26b might suppress the development of acute TAAD.

MiR-26b modulated HMGA2 expression by binding to its 3'UTR

To explore whether miR-26b regulated HMGA2 expression in the development of acute TAAD, HMGA2 expression in aortic wall tissues of patients with acute TAAD was first determined by RT-PCR. As **Fig. 3A** displayed, HMGA2 was significantly increased in the aortic wall tissues of TAAD patients compared to control tissues. Moreover, repression analysis results showed that miR-26b was negatively correlated with HMGA2 in the aortic wall tissues of TAAD patients (**Fig. 3B**). Next, Western blot and RT-PCR were performed to measure the effect of miR-26b on HMGA2 expression in VSMCs. As we see in **Fig. 3C** and **3D**, miR-26b up-regulation repressed the expression of HMGA2, while miR-26b down-regulation facilitated HMGA2 expression both in protein and mRNA level. These data displayed that miR-26b modulated HMGA2 expression negatively. To explore how miR-26b regulated HMGA2 expression, we applied TargetScan to predict the putative binding sites of miR-26b with HMGA2. As **Fig. 3E** shown, HMGA2 was one of the candidate targets of miR-26b. Moreover, luciferase reporter assay was performed to further determine whether HMGA2 was the direct target of miR-26b. The findings showed that the relative luciferase activity

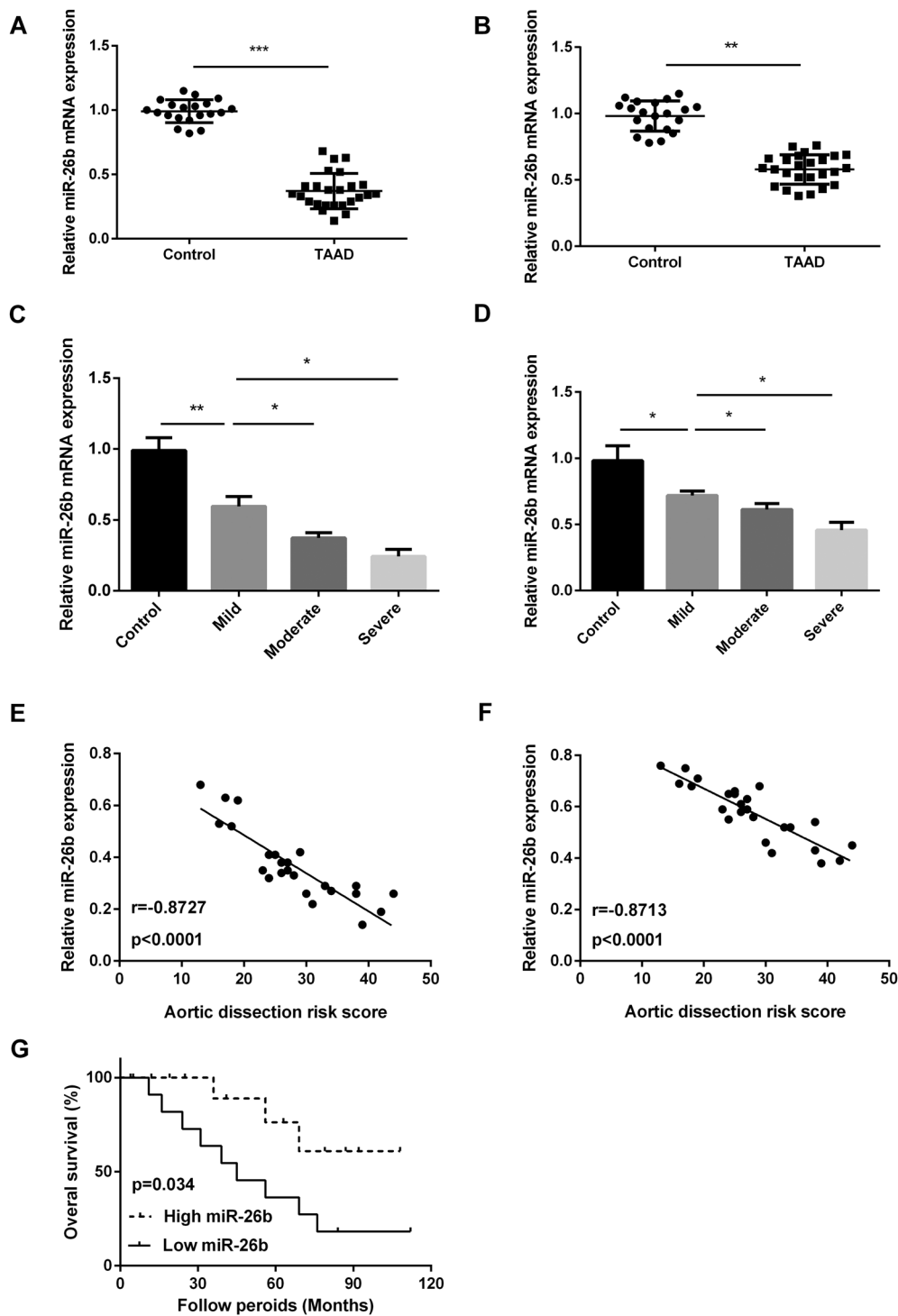


Fig. 1 Down-regulation of miR-26b was associated with the prognosis of TAAD. (A) Relative expression of miR-26b measured in the aortic wall tissues and (B) peripheral serum of TAAD patients. (C) Relative expression of miR-26b detected in the mild, moderate, and severe risk groups of TAAD patients in the aortic wall tissues and (D) serum. (E) The relationship between miR-26b level and the risk severity of TAAD patients in the aortic wall tissues and (F) serum. (G) The relationship between miR-26b level and the overall survival time of TAAD patients. *P < 0.05, **P < 0.01, ***P < 0.001. TAAD: type A aortic dissection

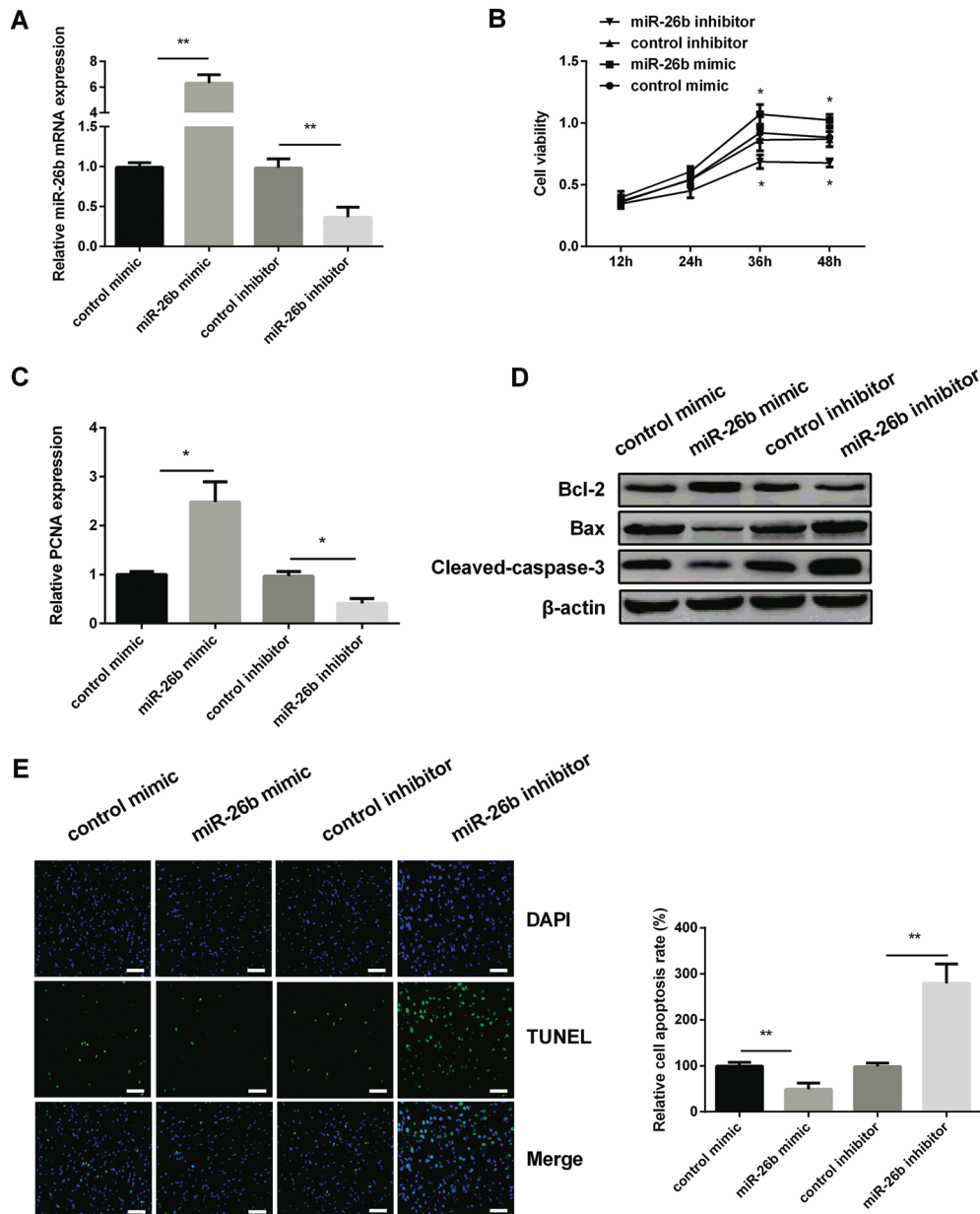


Fig. 2 MiR-26b facilitated VSMC proliferation and impeded VSMC apoptosis. (A) Relative expression of miR-26b in VSMCs after treated with miR-26b mimic or inhibitor. (B) CCK-8 analysis of cell viability; (C) RT-PCR analysis of PCNA expression; (D) Western blot analysis of Bcl-2, Bax, and cleaved-caspase-3 level; and (E) TUNEL staining analysis of cell apoptosis rate in VSMCs transfected with miR-26b mimic or inhibitor. Scale bars = 50μm in E. *P <0.05, **P <0.01. CCK-8: Cell Counting Kit-8; PCNA: proliferating cell nuclear antigen; RT-PCR: real-time polymerase chain reaction; VSMC: vascular smooth muscle cell

in VSMCs co-transfected with HMGA2 3'UTR-WT reporter and miR-26b mimic was dramatically decreased while increased by miR-26b inhibitor. However, the relative luciferase activity in HMGA2 3'UTR-MuT reporter cells has no changes (Fig. 3F). These results demonstrated that miR-26b regulated HMGA2 expression negatively by binding to its 3'UTR.

Knockdown of HMGA2 facilitated VSMC proliferation and repressed VSMC apoptosis

To investigate HMGA2 biological function on the development of acute TAAD, we measured HMGA2 effect on VSMC proliferation and apoptosis as well. HMGA2 expression was first detected in VSMCs after transfected with HMGA2 siRNA by RT-PCR. The findings

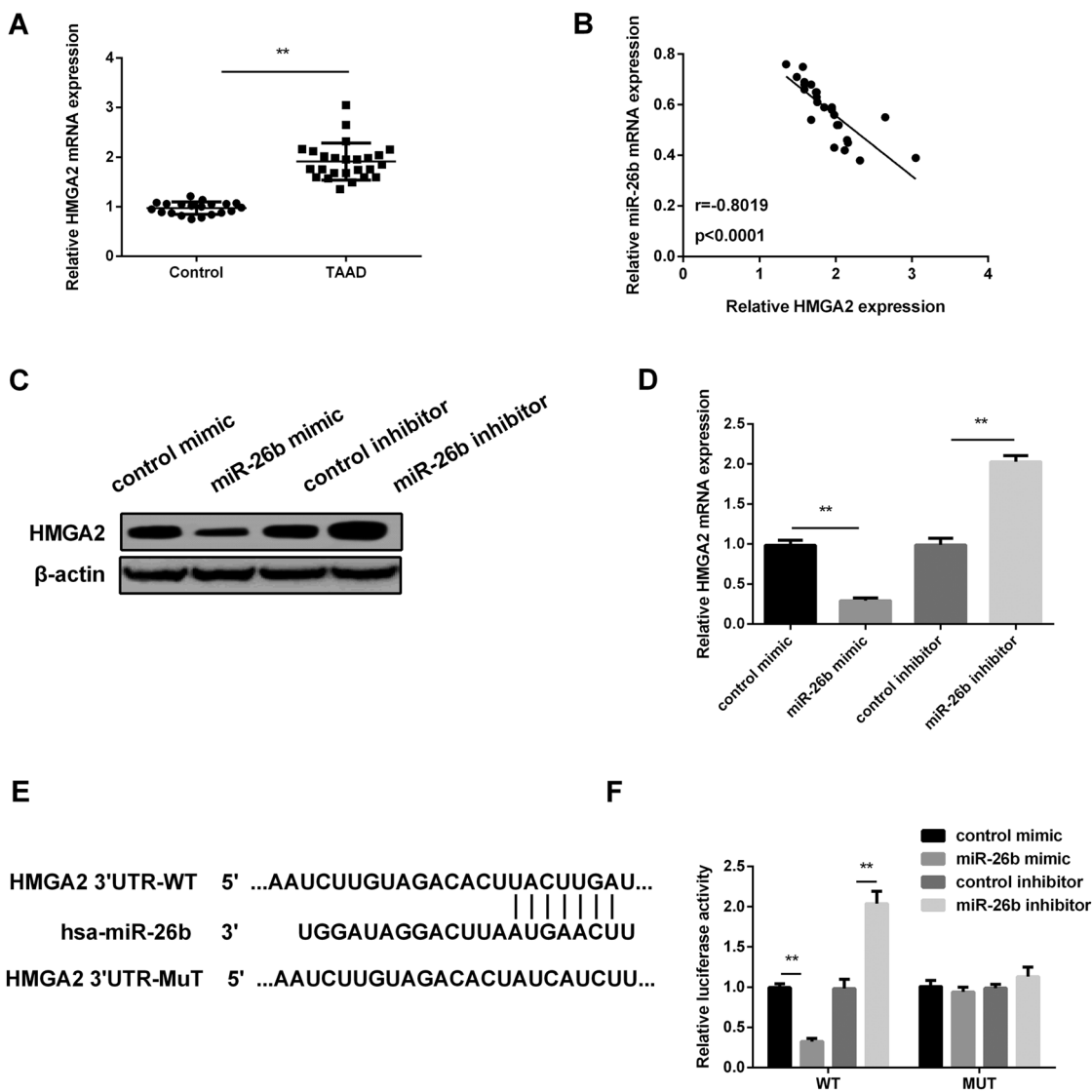


Fig. 3 HMGA2 was the target of miR-26b. (A) Relative expression of HMGA2 in the aortic wall tissues of TAAD patients. (B) The correlation of miR-26b expression with HMGA2 expression in the aortic wall tissues of TAAD patients. (C) The protein level of HMGA2 and (D) mRNA expression of HMGA2 in VSMCs treated with miR-26b mimic or inhibitor. (E) The putative binding sites of miR-26b with HMGA2 3'UTR. (F) Relative luciferase activity in VSMCs co-transfected with HMGA2 3'UTR-WT or -MuT and miR-26b mimic or inhibitor. **P < 0.01. HMGA2: high-mobility group AT-hook 2; MuT: mutated; TAAD: type A aortic dissection; VSMC: vascular smooth muscle cell; WT: wild type

displayed that HMGA2 was significantly decreased in HMGA2 siRNA VSMCs in comparison with control group (Fig. 4A). CCK-8 assay results displayed that knockdown of HMGA2 significantly enhanced VSMC viability (Fig. 4B) and RT-PCR results showed that PCNA expression was significantly elevated by inhibiting HMGA2 (Fig. 4C). More strikingly, knockdown of HMGA2 increased Bcl-2 level and decreased Bax and cleaved-caspase-3 level (Fig. 4D). Moreover, decreasing HMGA2 impeded VSMC apoptosis rate markedly

(Fig. 4E). Correctively, all these data indicated that knockdown of HMGA2 showed the suppression effect on the development of acute TAAD.

MiR-26b/HMGA2 axis facilitated the activation of TGF-β/Smad3 signaling pathway in VSMCs

To understand the potential mechanism of miR-26b in the pathogenesis of TAAD, we examined whether TGF-β/Smad3 signaling pathway was involved in the development of acute TAAD modulated by miR-26b. Western

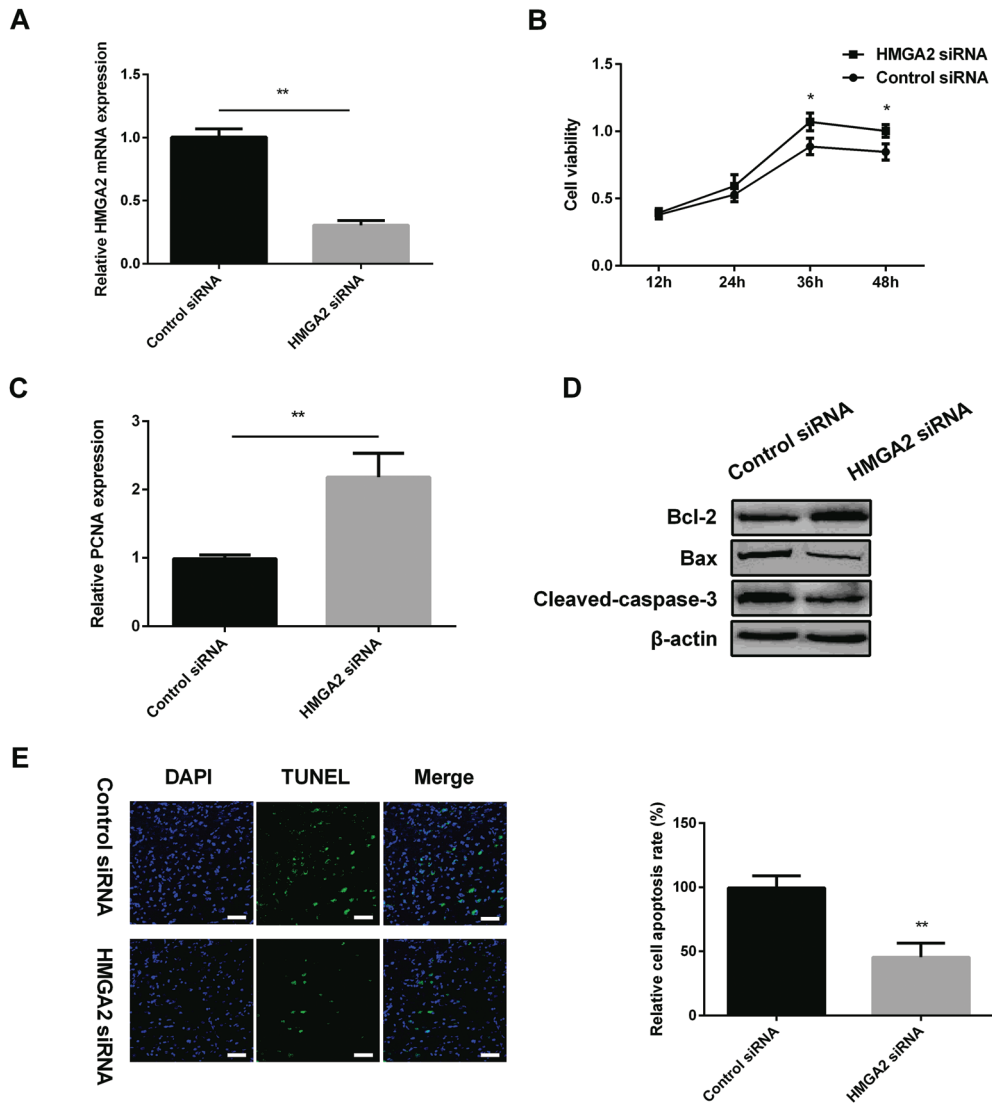


Fig. 4 Knockdown of HMGA2 enhanced VSMC proliferation and repressed VSMC apoptosis. **(A)** Relative expression of HMGA2 in VSMCs after silence HMGA2. **(B)** CCK-8 analysis of cell viability; **(C)** RT-PCR analysis of PCNA expression; **(D)** Western blot analysis of Bcl-2, Bax, and cleaved-caspase-3 level; and **(E)** TUNEL staining analysis of cell apoptosis rate in VSMCs transfected with HMGA2 siRNA. Scale bars = 50 μ m in E. *P < 0.05, **P < 0.01. CCK-8: Cell Counting Kit-8; HMGA2: high-mobility group AT-hook 2; PCNA: proliferating cell nuclear antigen; RT-PCR: real-time polymerase chain reaction; VSMC: vascular smooth muscle cell

blot assay was applied to detect the downstream genes of TGF- β /Smad3 pathway in VSMCs after treated with miR-26b mimic, inhibitor or HMGA2 siRNA. As **Fig. 5A** shown, the levels of TGF- β and p-Smad3 were enhanced by miR-26b restoration. Moreover, knockdown of HMGA2 increased their levels as well. However, inhibiting miR-26b repressed the expressional level of TGF- β and p-Smad3. Furthermore, the quantitation of TGF- β level and p-Smad3/Smad3 level revealed that miR-26b mimic and HMGA2 siRNA showed facilitating effect, while

miR-26b inhibitor showed suppression effect on TGF- β /Smad3 signaling pathway (**Fig. 5B** and **5C**).

Discussion

TAAD is a critical cardiovascular disease with high mortality, and timely diagnosis and treatment is the key to the prognosis of patients with TAAD. Currently, plasma biological markers such as d-dimer, smooth muscle myosin heavy chain (sm-mhc), and creatine kinase BB

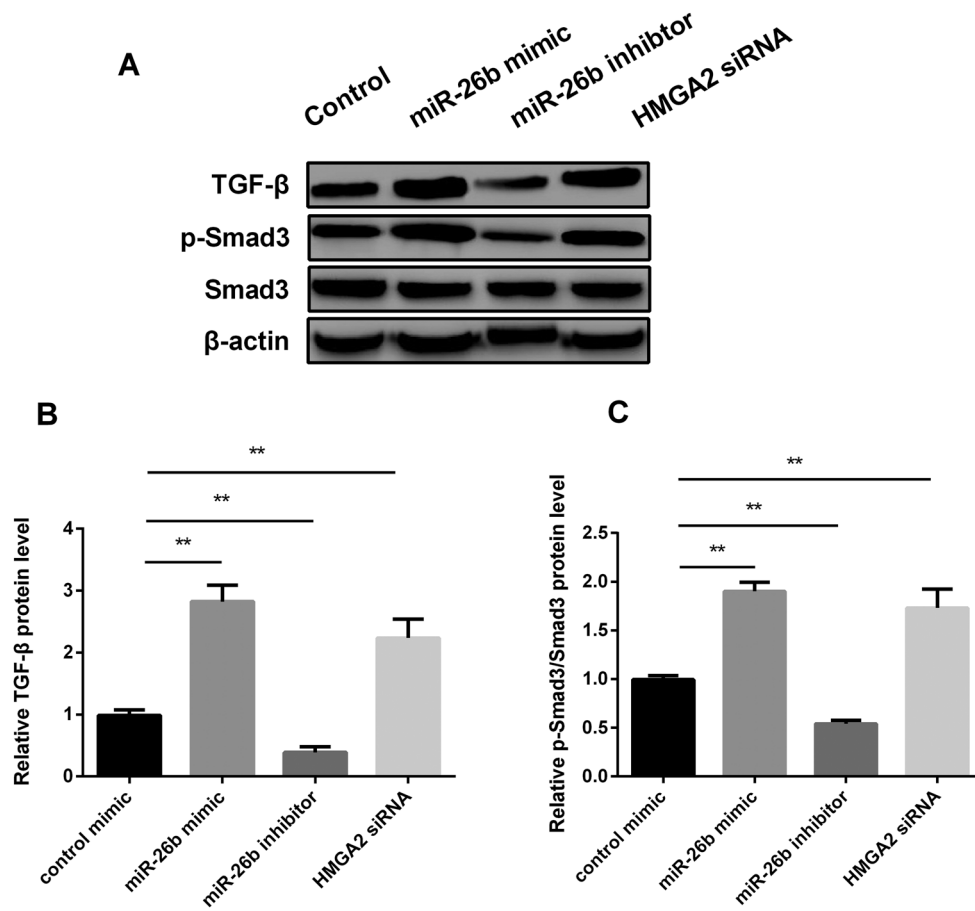


Fig. 5 MiR-26b promoted the activation of TGF-β/Smad3 signaling pathway. (A) Western blot analysis of TGF-β, p-Smad3, and Smad3 level. (B) Quantitation of TGF-β and (C) p-Smad3/Smad3 in VSMCs treated with miR-26b mimic, inhibitor or HMGA2 siRNA. **P < 0.01. HMGA2: high-mobility group AT-hook 2; TGF-β: transforming growth factor beta; VSMC: vascular smooth muscle cell

(ck-bb) have certain indicative significance in the diagnosis of aortic dissection aneurysms, but often lack specificity and sensitivity. Emerging evidences have showed that miRNAs can participate in the development of various diseases by promoting or inhibiting the expression of target genes and served as biomarkers for the prognosis of these diseases. Previous studies have showed that miR-26b served as a novel diagnostic biomarker for degenerative myelopathy.²²⁾ Moreover, by regulating DEPDC1 gene, miR-26b acted as a tumor suppressor in triple negative breast cancer.²³⁾ Ovchinnikova *et al.* found that miR-26b was associated with increasing acuity of heart failure.²⁴⁾ Also, miR-26b was discovered to participate in the pathophysiology of many cardiovascular diseases, which can improve myocardial remodeling in myocardial infarction.²⁵⁾ It is the first time Xu *et al.*¹²⁾ found that miR-26b was down-regulated in TAAD.

However, there is no research on the mechanism and clinical significance of miR-26b on aortic dissection. In this study, RT-PCR was performed to detect the expression of miR-26b in the aortic wall tissues and peripheral serum of patients with TAAD and control groups. We found that miR-26b is closely related to the lesion of aortic dissection. MiR-26b expression in aortic wall tissues and peripheral serum was significantly down-regulated compared with the control group and its down-regulation predicted the shorter survival time of TAAD patients. Thus, miR-26b might become a new molecular biomarker for TAAD diagnosis. Moreover, we found that miR-26b was inversely associated with TAAD risk severity. Therefore, it is necessary to screen the level of miR-26b in the high-risk patients in the early stage. If the miR-26b is significantly decreased, it indicates that the risk of dissection is high, which has certain clinical

significance for selecting the timing of surgery and evaluating the prognosis.

Presently, due to the molecular mechanism of miR-26b in the development of TAAD is not fully clarified, Target-Scan target gene prediction software was applied for predicting its target genes, and the results displayed that HMGA2 is one of the candidate target genes of miR-26b. HMGA2 belongs to the family of high-mobility group with AT-hook DNA-binding domain.²⁶⁾ Previous researches have confirmed that a variety of tumor diseases such as extrahepatic cholangiocarcinoma and pancreatic cancer are associated with excessive activation of HMGA2.^{27,28)} Moreover, the current studies showed that HMGA2 was associated with atherosclerosis, autoimmune inflammation, and other vascular diseases,²⁹⁾ which might be associated with the development of aortic dissection. Here we displayed that HMGA2 was significantly increased in TAAD patients, which was consistent with the results that HMGA2 was up-regulated in acute aortic dissection.¹⁷⁾ Moreover, we also found that knockdown of HMGA2 enhanced VSMCs proliferation and impeded VSMCs apoptosis, which showed the opposite effect of miR-26b inhibitor. More importantly, miR-26b modulated HMGA2 expression negatively by binding to its 3'UTR and regulated TGF- β /Smad3 signaling pathway in VSMCs.

Conclusion

MiR-26b regulated the development of TAAD by regulating HMGA2 and TGF- β /Smad3 signaling pathway. This provided a theoretical basis for gene therapy and predicted the risk of aortic dissection, which has certain clinical significance for selecting the timing of surgery and evaluating prognosis.

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Disclosure Statement

The authors declare that they have no competing interests.

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