

## Original Article

# Diagnostic value of miR-133 in aortic dissection and its mechanism

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**Abstract:** Background: Acute aortic dissection (AD) is a life-threatening condition that remains challenging to diagnose and treat. MiR-133 plays important roles in aortic disease initiation and progression. However, its differential diagnostic value between acute myocardial infarction (AMI) and AD remains unclear. Methods: Serum samples were collected from healthy individuals, AMI and AD patients. Reverse transcription polymerase chain reaction (RT-PCR) was used to assess miR-133 expression. The differential diagnostic value values of miR-133 were analysed by statistical methods. Tissue samples were collected from AMI and AD patients, RT-PCR was used to test the expression of miR-133 and SOX4 mRNA, and Western blot and gelatin zymography were used to test the expression and activity of MMP-2, TIMP-1 and TIMP-2. *In vitro*, miR-133 mimics was transfected into VSMCs, and the biological function of miR-133 was evaluated by MTT assay, transwell assays, Western blot and gelatin zymography. A luciferase assay was used to explore whether SOX4-3'-UTR was a target gene of miR-133. Results: Compared with AMI patients, AD patients showed reduced serum miR-133 levels. In addition, ROC curve analysis shown that the sensitivity and specificity of serum miR-133 were 90.4% and 58.70% respectively for differential diagnoses between AD and AMI. *In vitro*, miR-133 up-regulation resulted in reduced VSMCs proliferation and invasion. In addition, an inverse correlation between miR-133 levels and SOX4 mRNA amounts was observed in AD tissues. More importantly, miR-133 upregulation resulted in reduced SOX4 expression, and the luciferase activity of the SOX4-3'-UTR plasmid was significantly suppressed by miR-133. Conclusion: MiR-133 has good application in differential diagnosis between AMI and AD. MiR-133 targets SOX4, thereby inhibiting VSMCs proliferation and migration and finally prevents the development of AD.

**Keywords:** Acute myocardial infarction, aortic dissection, miRNAs, differential diagnosis, SOX4

### Introduction

Acute aortic dissection (AD) is a life-threatening condition that remains challenging to diagnose and treat. Greater availability and increased use of advanced imaging modalities, particularly computed tomography (CT), have the potential to improve the diagnosis of AD [1]. Improvement in surgical and anesthetic techniques have led to improved survival of patients with type A dissection, whereas the expanded use of endovascular interventions is having a growing effect on management of type B dissection [2]. Over the past 2 decades, the exciting discovery of genetic mechanisms underlying thoracic aortic disease has begun to affect medical treatment.

Studies show that early diagnosis of AD is one of the most important factors that impact the prognosis of AD patients [3]. In recent years, media reports of the deaths of a number of celebrities from unrecognized AD have increased the public's awareness of what remains a very dangerous and unpredictable condition. Although severe, abrupt onset, chest or back pain is widely known as the classic presentation of AD, patients present as acutely ill with a variety of symptoms and signs, many of which can mimic more common conditions such as myocardial ischemia or pulmonary embolism [4]. According to Linda et al., about 30% AD patients were misdiagnosed as acute myocardial infarction (AMI) in the past

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**Table 1.** Clinical characteristics of AMI, AD patients and healthy controls

Parameters	Healthy control (n=46)	AMI (n=67)	AD (n=94)
Age (years)	49.1±6.4	62.9±8.7	51.5±9.1
Sex (Male)	37 (80.4%)	43 (64.2%)	79 (84.0%)
Hypertension	13 (28.3%)	42 (62.7%)	65 (69.1%)
Diabetes mellitus	4 (8.7%)	21 (31.3%)	16 (17.0%)
Smoking history	13 (28.3%)	30 (44.8%)	19 (20.2)
Family history of aortic diseases	1 (2.2%)	0	1 (1.1)
Mir-133	6.5±3.2	5.1±2.7	3.2±1.8

AD: Aortic dissection; AMI: Acute myocardial infarction.

17 years [5]. Thrombolytic or anticoagulant agents used for AMI may be disastrous for AD patients. So, founding a serum marker to differentiate AD from AMI is very important.

MicroRNAs (miRNAs) are small non-coding RNA molecules that bind to the 3'-untranslated region (UTR) of target mRNAs, resulting in translation repression or mRNA degradation [6]. Mounting evidence have revealed that deregulation of miRNAs expression is related with variety of human disease such as cardiovascular disease, metabolic disorders, and cancer [7]. Recently, some studies found that miRNAs could play important roles in aortic disease initiation and progression [8-11]. In this study, we aim to investigate the diagnostic value of miR-133 in aortic dissection and its potential mechanism.

### Materials and methods

#### Patients and sample collection

Preoperative venous blood samples and post-operative artery tissue specimens were collected from 46 AMI patients and 94 aortic dissection patients respectively, at The Second People's Hospital of Yunnan Province. Blood samples from 67 subjects receiving physical examination were also obtained (Table 1). Blood samples were centrifuged immediately for serum preparation; tissue specimens were confirmed pathologically, snap frozen in liquid nitrogen, and stored at -80°C. Written informed consent was obtained from each patient. The study was approved by the ethics committee of The Second People's Hospital of Yunnan Province.

#### Reagents

DMEM and trypsin were from Sigma-Aldrich (USA). Fetal bovine serum (FBS), restriction endonucleases BamHI and XhoI, T4 DNA ligase, and RNA extraction reagent were manufactured by Invitrogen Inc. (Carlsbad, CA, USA). Reverse transcription kit was from TaKaRa (China); SYBR Green fluorescent dye kit was a product of Roche (Switzerland). Rabbit or mouse anti-

human SM-actin, MMP-2, TIMP-1, TIMP-2, SOX4, and  $\beta$ -actin polyclonal antibodies were purchased from Abcam (USA). MTT assay kit was manufactured by Gefanbio (China). Horseradish peroxidase-labeled goat anti-rabbit or anti-mouse secondary antibodies and Western blot detection reagents were from Beyotime (China).

#### Vector construction and cell transfection

For the construction of over-expression plasmid, a miR-133 mimics was designed and synthesized by GenePharma company, Shanghai, China. The sequences were as follows: 5'-UUUGGUCCCUUCAACCAGCUA-3' and 3'-UUCUCCGAACGUGUCACGUTT-5'. We then searched the Sox4 gene sequence via PubMed, and designed Sox4 mRNA primers according to primer sequences in GenBank. SOX4 3'-UTR sequences were: 5'-CTTGACATGATTAGCTGGC-ATGATT-3' (forward) and 5'-CCTGTGCAATATGCCGTGTAGA-3' (reverse).

PCR products were cloned into the pcDNA3.0 vector (Addgene, MA, USA). All constructs were verified by sequencing.

For transfection, VSMCs were seeded into 6 well-plates at a density of  $5 \times 10^5$  per well. At 50-70% confluency, VSMCs were transiently transfected with miR-133 mimics or negative control, using Lipofectamine™ 2000 (Invitrogen, CA) according to the manufacturer's instructions.

#### Quantitative real-time PCR

Total RNA from clinical specimens and VSMCs was reverse transcribed into cDNA using Reverse transcription kit, in a reaction system of 20  $\mu$ L, at 16°C (30 min), 45°C (30 min), and

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85°C (5 min). SYBR Green was used to detect miR-133 and SOX4 mRNA amplification; RT-PCR was carried out for 40 cycles at 94°C (15 min), 94°C (30 s), 60°C (30 s), and 72°C (30 s), with a final extension at 72°C for 8 min. Data were normalized to the internal control  $\beta$ -Actin. Relative quantification of miR-133 and SOX4 mRNA expression levels was performed by the  $2^{-\Delta\Delta CT}$  method. All experiments were repeated three times.

### *Western blot*

Total protein was extracted from cells or tissue specimens. BCA Protein Assay Kit was used for protein quantitation. Equal amounts of protein were separated by SDS-PAGE and blotted onto PVDF membranes. After blocking for 1 h, the membranes were incubated with specific primary antibodies against MMP-2, TIMP-1, TIMP-2, SOX4, and  $\beta$ -actin overnight at 4°C, followed by addition of horseradish peroxidase-labeled goat anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature. Specific protein bands were visualized by enhanced chemiluminescence (ECL).

### *Gelatin zymography analysis*

Zymography was used to measure the activity of MMP-2 in the cells or tissue specimens. The supernatant was collected and mixed with 5 × SDS sample buffer without a reducing agent. Then, equal amounts (30 mg) of the sample were loaded onto the SDS-PAGE gel (8% polyacrylamide gel containing 0.1% gelatin) for electrophoresis. After that, the gels were washed for 30 min twice in 2.5% Triton X-100 at room temperature to remove the SDS and incubated in the renaturation buffer (pH 7.5, 50 mM Tris-HCl, 10 mM CaCl<sub>2</sub>, 0.02% Na<sub>3</sub>N) for 42 h at 37°C. 0.1% Coomassie blue R-250 in 10% glacial acetic acid/45% methanol were used to stain the gels. The gels were destained (50% methanol, 10% acetic acid, and 40% water solution) until clear bands of gelatinolysis appeared on a dark background, and then transferred to water for rehydration before acquiring images. Afterward, the images of the bands were analyzed by Quantity One 4.4 (BioRad, Hercules, CA, USA).

### *Primary culture and identification of VSMCs*

VSMCs were primarily cultured using tissue-sticking method for rat thoracic aorta as reported in our previous researches. We identified

VSMCs through immunofluorescence detection of SM-actin and ensured their purity through multiple fluorescent staining with DAPI and SM-actin antibody. Cells between 3 to 7 generations were used in the following experiments in DMEM containing 2.5% FBS.

### *Cell proliferation assay*

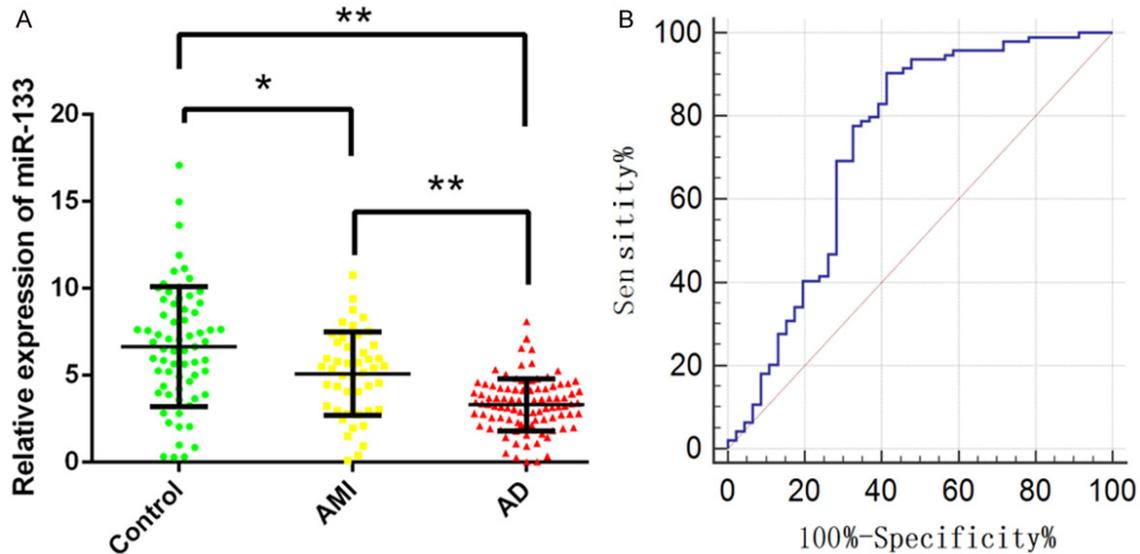
Cells were seeded into 96-well plates at a density of  $5 \times 10^3$  cells per well. Each group included 3 replicate wells; cells were cultured for 12, 24, 48, and 72 h. After incubation, 20  $\mu$ L of 5 mg/mL MTT reagent was added to each well, and further incubated for 4-6 h. Finally, 150  $\mu$ L DMSO was added to each well after culture medium removal, and absorbance was measured at 490 nm on a microtiter plate reader. Growth curves were generated for VSMCs cells. All experiments were performed three times.

### *Cell migration and invasion assay*

The 4-7 passage VSMCs were digested with 0.25% trypsin and suspended with DMEM containing 10% FBS and cells were seeded into 6-well plates. When cells grew to 80% confluence, they were cultured in serum-free medium containing hydroxyurea for 24 h to synchronize cells and suppress cells proliferation. We created wounds by manually scraping the cell monolayer with a 100  $\mu$ l pipet tip. After being washed to remove the isolated cells, corresponding intervention factor was added to each group. Multiple photographs of the wounds were then taken at 0 and 24 h post wounding under an inverted Nikon microscope (Nikon Corporation, Tokyo, Japan) at a × 200 magnification. The migration area was analyzed with Image-pro plus 6.0 (Media Cybernetics Inc., Bethesda, MD, USA) and the ratio of the cellular recovrage area to the whole wound area was used to evaluate cells migration.

Boyden chamber assays were used to assess cell invasion. The medium was supplemented with 1% heat-inactivated FBS, and cells were seeded at a density of  $5 \times 10^4$ /ml in the upper chamber; the lower chamber was filled with 500  $\mu$ l complete medium with 20% FBS. After incubation for 24 h at 37°C, non-invading VSMCs were gently removed from the upper chamber. Cells migrating to the lower compartment were fixed and stained with 0.1% crystal violet. Cells were washed 3 times with PBS, and

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**Figure 1.** Serum expression of miR-133 in healthy control and AD, AMI patients; B: ROC curve analysis of the differential diagnostic value of miR-133 between AMI and AD.

five random high power fields in each sample were analyzed by light microscopy.

### Luciferase reporter assay

Wild-type (WT) 3'-UTR of SOX4 and the mutated sequence were inserted into the pGL3 control vector (Promega, Madison, USA) to generate WT SOX4-3'-UTR and mutant SOX4-3'-UTR vectors, respectively. In the luciferase reporter assay, VSMCs were seeded into 48-well plates and allowed to adhere for 24 h before co-transfection with a mixture of 100 ng pGL3-WT SOX4-3'-UTR or pGL3-MUT SOX4-3'-UTR and 60 pmol miR-133 mimics or control sequence, using Lipofectamine 2000 reagent. Three independent experiments were performed. At 48 h post-transfection, firefly and Renilla luciferase activity was determined using the Dual-luciferase Reporter Assay System (Promega).

### Statistical analysis

Data are mean  $\pm$  standard deviation (SD). Receiver operating characteristic (ROC) curves were used to assess the diagnostic value of miR-133 in AD. Spearman correlation was used to analyze the relationship between miR-133 and SOX4 in the tissues. Differences between the groups were analyzed using Student's t-test.  $P < 0.05$  was considered statistically significant. Statistical analyses were performed using the SPSS 20.0 software, Medcalc and GraphPad Prism 6.0.

## Results

### *The expression of miR-133 in the serum of patients and its role for diagnosis of aortic dissection*

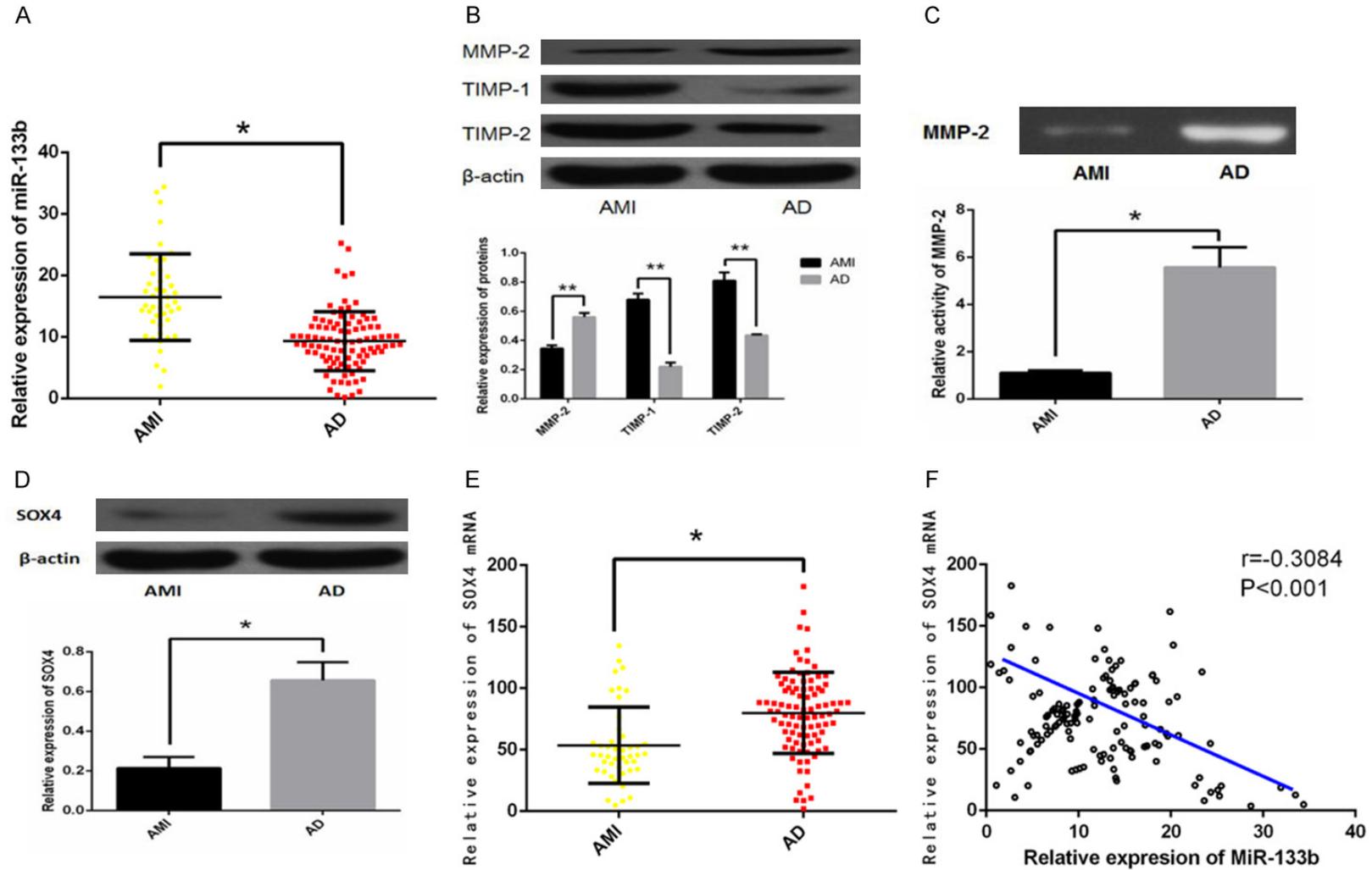
We tested the expression of miR-133 in the serums from 67 healthy volunteers, 46 AMI patients and 94 AD patients. The results showed that compared with the healthy volunteers, the expression of miR-133 was decreased both in the AMI and AD patients. In addition, compared with the AMI patients, the expression of miR-133 was further decreased in the serum of AD patients (**Figure 1A**).

To assess the value of serum miR-133 in the differential diagnosis between AMI and AD, ROC curve analysis was used. Interestingly, we found that the AUC value was 0.736 (95% CI, 0.655-0.807) ( $P < 0.01$ ); with a cut-off value of 4.917, sensitivity and specificity were 90.4% and 58.70%, respectively (**Figure 1B**), suggesting that miR-133 could be used as a biomarker for differential diagnosis between AMI and AD.

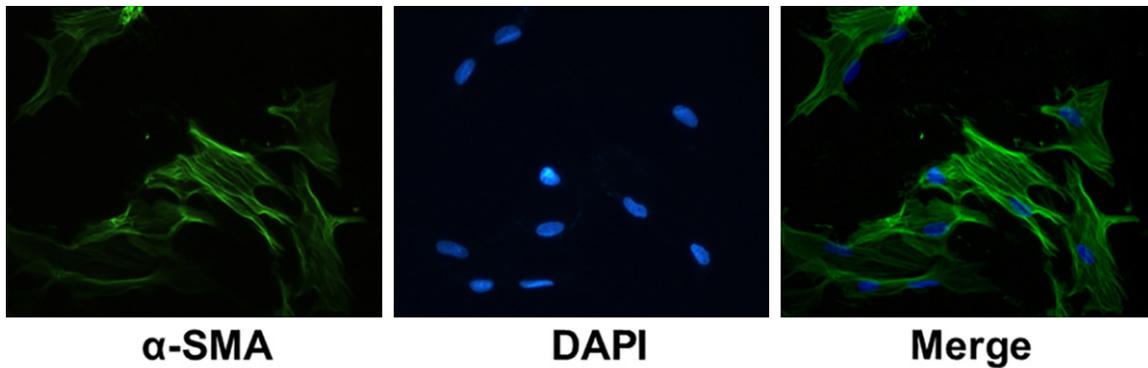
### *Gene expression levels of miR-133 and SOX4 in AMI and AD tissues*

Since the diagnosis role for miR-133 in clinic for AD was found, the underlying molecular mechanisms needs further investigated. Lower expression of miR-133 was found in AD tissues compared with the AMI ones,  $P < 0.05$

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**Figure 2.** Expression of miR-133, SOX4 mRNA and related proteins in AD and AMI tissues. A: The expression of miR-133 in AMI and AD tissues. U6 served as an internal reference. B: The expression of MMP-2, TIMP-1 and TIMP-2 in AMI and AD tissues. C: The activity of MMP-2 in AMI and AD tissues. D, E: The expression of SOX4 in AMI and AD tissues. F: miR-133 was negatively correlated with SOX4. \*P<0.05 compared with AMI tissues; \*\*P<0.01 compared with AMI tissues.



**Figure 3.** Identification of vascular smooth muscle cells.

(**Figure 2A**). Western blot analysis showed that the expression of MMP-2 was increased in the AD tissues compared with the AMI group and the expression of TIMP-1/2 decreased,  $P < 0.05$  (**Figure 2B**). In addition, gelatin zymography analysis revealed the higher activity of MMP-2 in the AD tissues,  $P < 0.05$  (**Figure 2C**). SOX4, a potential target of miR-133 found via software programs such as TargetScanS, miR and a and DIANA-microT, was detected in AMI and AD tissues by western blot and RT-PCR. Compared with the AMI tissues the expression of SOX4 was increased in the AD tissues,  $P < 0.05$  (**Figure 2D, 2E**). What's more, we found that the relative expression of miR-133 level was negatively correlated with SOX4 mRNA,  $P < 0.05$  (**Figure 2F**). These data reminded us the potential relationship between miR-133, SOX4 and AD.

#### *miR-133 suppressed VSMCs proliferation and migration*

*In vitro* experiments were carried out to further explore the biological function of miR-133 on thoracic aorta VSMCs. The primary culture of rat thoracic aorta VSMCs exhibited a typical spindle-shaped appearance, and immunofluorescence showed 98% positive SM $\alpha$ -actin staining (**Figure 3**). RT-PCR was used to detect miR-133 expression levels in VSMCs after transfection with miR-133 mimics or the corresponding negative control. Compared with miR-NC amounts, miR-133 expression in VSMCs transfected with miR-133 mimics was significantly increased,  $P < 0.01$  (**Figure 4A**). MTT assay were performed to explore the function of miR-133 on VSMCs proliferation. As shown in the **Figure 4B**, the cell proliferative ability was distinctly inhibited in the miR-133 mimics group compared with the other two groups,  $P < 0.05$ .

In addition, cell migration ability was inhibited by miR-133 as assessed by Wound healing assay and Transwell assay,  $P < 0.05$  (**Figure 4C, 4D**). What's more, western blot analysis showed that the expression of MMP-2 was decreased and the expressions of TIMP-1/2 were increased in the miR-133 mimics group,  $P < 0.05$  (**Figure 4E**). Taken together, these data indicated that miR-133 inhibited proliferation and migration in VSMCs.

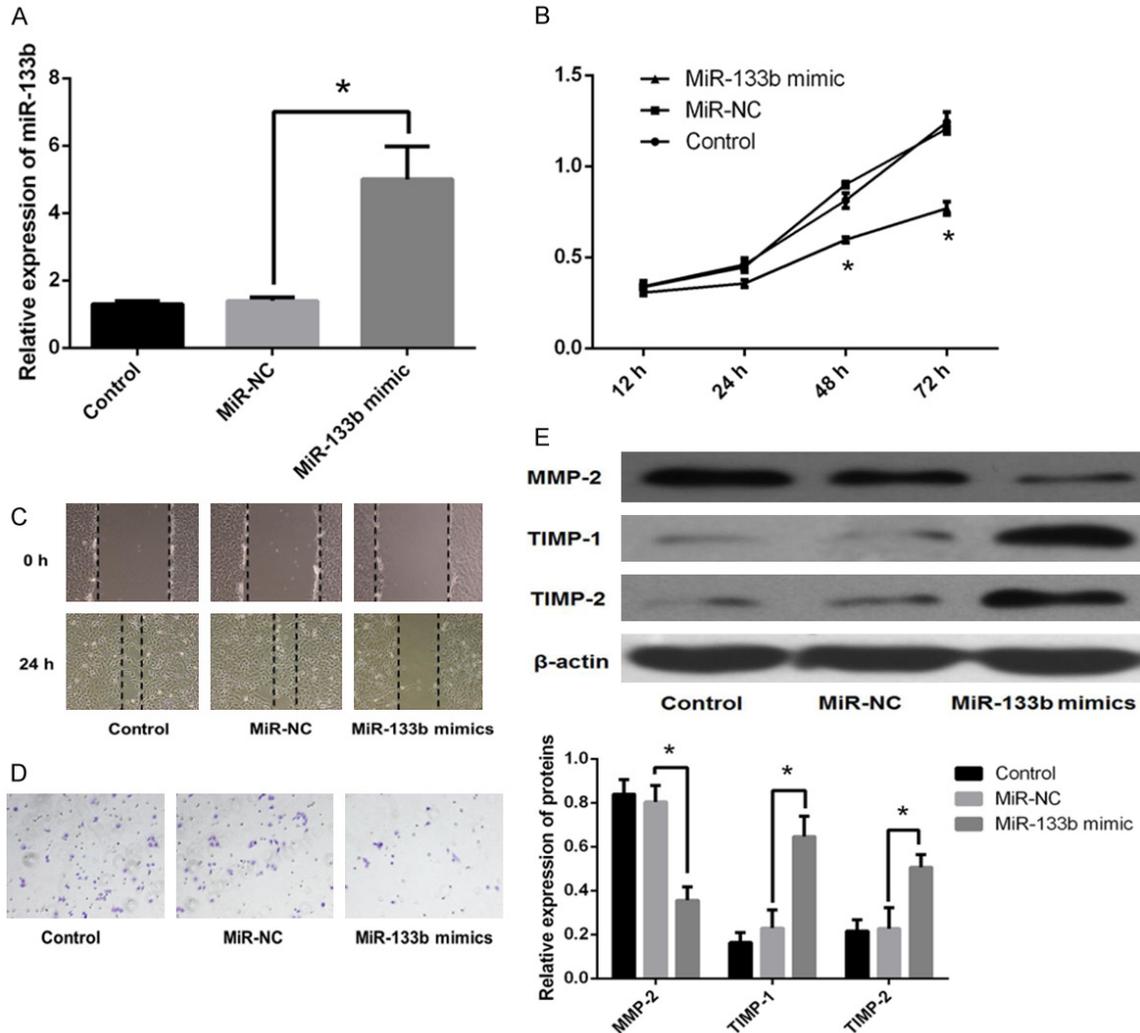
#### *SOX4 is a potential target of miR-133 in VSMCs*

To test the hypothesis that miR-133 modulated SOX4 expression, WB was used to assess protein expression of SOX4 in miR-133 mimics, miR-NC and control groups; interestingly, SOX4 levels were significantly reduced in the miR-133 mimics group compared with the other two groups,  $P < 0.05$  (**Figure 5A**). A putative miR-133-binding sequence in the 3'-UTR of SOX4 mRNA was suggested in **Figure 5B**. To further validate SOX4 as a target gene, luciferase reporter vectors with the putative SOX4 3'-UTR target site for miR-133 (pGL3-wt-SOX4, set as wild-type) and mutant version in the seed region (pGL3-mut-SOX4, set as mutanttype) were generated. Indeed, luciferase activity was suppressed by miR-133 in VSMCs harboring the WT plasmid ( $P < 0.05$ ), but not the MUT plasmid (**Figure 5C**). These findings suggested that miR-133 targeted the 3'UTR of SOX4 in VSMCs.

#### **Discussion**

The two major life-threatening differential diagnoses of acute excruciating chest pain in the emergency department are acute coronary syndrome, and aortic dissection [12]. Many patients with AD are diagnosed and treated as

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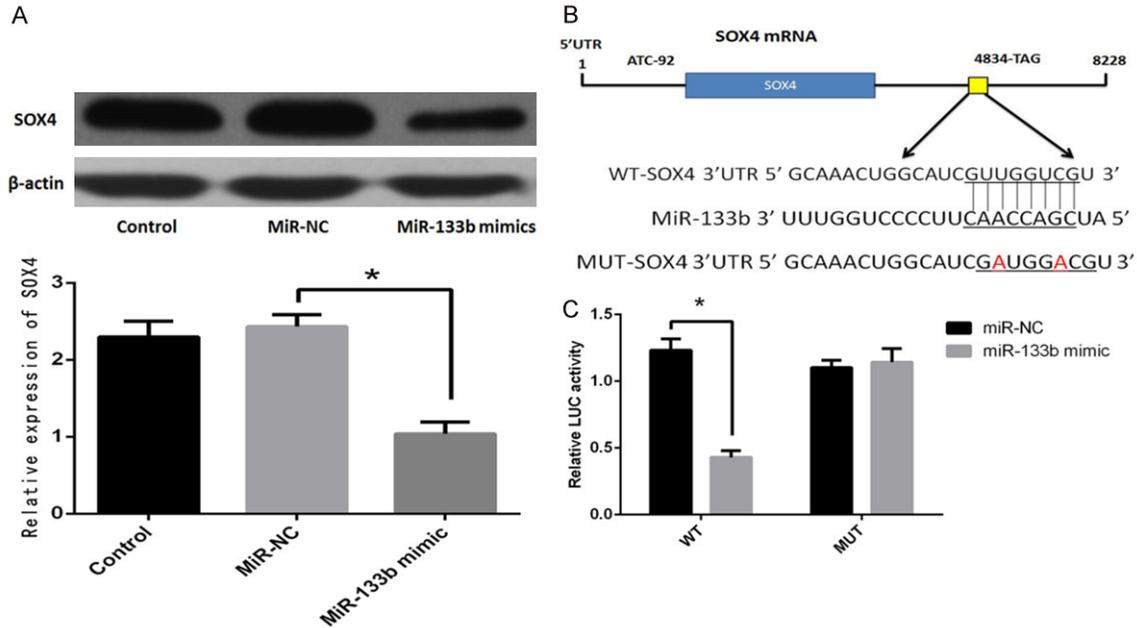
**Figure 4.** MiR-133 inhibited proliferation and invasion of VSMCs. A: miR-133 mimics were successfully transfected into VSMCs. B: MTT assay showed that miR-133 suppressed VSMCs proliferation. C, D: Wound healing assay and Transwell assay revealed that miR-133 inhibited VSMCs migration. E: MiR-133 decreased the expression of MMP-2 and increased the expression of TIMP-1/2. \* $P < 0.05$  compared with AMI tissues; \*\* $P < 0.01$  compared with AMI tissues.

having acute coronary syndrome, which is a much more frequent condition than AD [13]. Thrombolytic or anticoagulant agents used for AMI may be disastrous in aortic dissection. Although imaging, computed tomography, magnetic resonance imaging, and transesophageal echocardiography are widely used to diagnose dissection, in low- to intermediate-risk patients, a rapid test, preferably a simple biochemical blood test may help risk stratification [9, 14]. Development of such a test has been of paramount interest during last decade. In our study, we found that the serum level of miR-133 in the AD patients was decreased compared with the

AMI patients. In addition, ROC curve analysis shown that the sensitivity and specificity were 90.4% and 58.70% respectively for differential diagnoses between AD and AMI. These results indicated miR-133 could be used as a serum biomarker for differential diagnosis between AMI and AD.

AD usually results from a tear in the aortic intima, which allows a pressurized hematoma to form within the media between the inner two-thirds and outer one-third of the aorta. Pathological vascular remodeling plays a key role in the occurrence and development of AD [15,

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**Figure 5.** SOX4 is the direct target of miR-133 in VSMCs. A: Mir-133 inhibited the expression of SOX4 in VSMCs. B: The predictable binding site of miR-133 and SOX4 is shown. C: miR-133 inhibited luciferase activity in the SOX4 containing 3'UTR of WT binding sites. \* $P < 0.05$  compared with AMI tissues; \*\* $P < 0.01$  compared with AMI tissues.

16]. MMPs and TIMPs are thought to play important role in the subacute phase of dissection through degradation of extracellular matrix in the dissected media [17-19]. In our study, level of MMP-2 was increased in AD tissues compared with AMI. In addition, the levels of TIMPs were decreased in AD tissues. What's more, the activity of MMP-2 was much higher in the AD tissues. These results indicated that the balance between MMPs and TIMPs was broken in the aorta of AD patients, and inhibiting the expression and activity of MMPs could help prevent the development of AD.

Since the diagnosis role for serum miR-133 in clinic for AD was found, the underlying molecular mechanisms were further investigated. We tested the expression of miR-133 in AD and AMI tissues and found that the expression of miR-133 was decreased in the AD tissues. This result reminded us that miR-133 maybe not only a marker for AD, it participate in the development of AD. It is a dynamic process involving aortic VSMCs apoptosis, proliferation, migration, and corresponding events leading to medial wall thinning [20, 21]. It has been reported that VSMCs from AD tissues seem to proliferate and migrate faster than normal aorta tissues and the genes that participate in proli-

feration and migration showed an elevated level of expression [22]. Abnormal proliferation and migration of VSMCs can affect the stability of vascular media structure and function, which might be the main cause of pathologic vascular remodeling and vascular disease occurrence [20, 23, 24]. In vitro experiments, we found that miR-133 inhibited VSMCs proliferation and migration, and this maybe the mechanism by which miR-133 participated in the development of AD.

What's more, our results showed that the expression of SOX4 was increased in AD tissues. The SOX4 gene is a member of the SOX family, which has been shown to have important roles in the cell fate decision. Lots of studies showed that SOX4 was significantly elevated in high progressive and invasive cancers [25-28]. Hanieh et al. reported that down-regulation of SOX4 by aryl hydrocarbon receptor-microRNA-212/132 axis could suppresses cell proliferation in human breast cancer [27]. Additionally, Jin also found that MicroRNA-338-3p could function as tumor suppressor in breast cancer by down-regulation of SOX4 [29]. Inhibiting the SOX4 expression decreased the proliferation and migration ability of lots of cells. In our study, we found miR-133 inhibited the

expression of SOX4 in VSMCs and further validated that SOX4 was the target of miR-133 by luciferase reporter. So, we thought that SOX4 may be involved in miR-133 mediated AD development.

In summary, our study demonstrates that miR-133 has good application in differential diagnosis between AMI and AD. MiR-133 targets SOX4, there by inhibiting VSMCs proliferation and migration and finally prevents the development of AD. Our data provide new insight into the mechanism responsible for the development of AD, which may also be benefit for the development of miRNA-directed diagnostic and therapeutic against AD.

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### Disclosure of conflict of interest

None.

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