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Original Article

Regulation of apoptosis by long non-coding RNA HIF1A-AS1 in VSMCs: implications for TAA pathogenesis

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Abstract: Objective: Long non-coding RNAs (lncRNAs) play important roles in diverse biological processes, such as transcriptional regulation, cell growth and tumorigenesis. However, little was known about whether lncRNA HIF 1 alpha-antisense RNA 1 (HIF1a-AS1) in regulating the proliferation and apoptosis of VSMCs in vitro and the expression of HIF1a-AS1 in serum of TAA patients. Methods: The cell viability was detected by the CCK8 assay. The cell apoptosis was assessed by annexin V-PI double-labeling staining. Expression of genes and proteins were analyzed by real-time PCR and western blotting respectively. Cells were transfected with siRNAs as a gene silencing methods. Results: In serum of TAA patients, the expression of HIF1a-AS1 was significantly increased (superior to 6 folds) compared to the normal control. Moreover, PA induced cell apoptosis in VSMCs in a time- and dose-dependent manner, and the proportion of the apoptotic cells had gained as compared to untreatment group. PA also induced upregulation expression of HIF1a-AS1. We also found that transfection of cells with HIF1a-AS1 siRNA decreased the expression of caspase3 and caspase8 and increased the expression of Bcl2, and protected PA-induced cell apoptosis in VSMCs. Conclusions: HIF1a-AS1 was overexpressed in the thoracoabdominal aorta aneurysm and the interaction between HIF1a-AS1 and apoptotic proteins plays a key role in the proliferation and apoptosis of VSMCs in vitro, which may contribute to the pathogenesis of thoracoabdominal aorta aneurysm.

Keywords: Thoracoabdominal aorta aneurysm, HIF 1alpha-antisense RNA 1, vascular smooth muscle cells, long non-coding RNA

Introduction

Aneurysm of the thoracoabdominal aorta (TAA) is relatively uncommon in the spectrum of aneurysmal disease, accounting for only 3% of diagnosed aneurysms in the United States [1]. Currently, the incidence and operations of thoracoabdominal aortic aneurysms have significantly increased. The indications for repair are considered to be a diameter of 6 cm or more and 5.5 cm for patient groups with increased risk of rupture [2]. Complex open surgical repair is associated with significant mortality and complication rates. The endovascular approach has evolved to be a good and predominant alternative to open repair of these aneurysms for older and high-risk patients as well as for aneurysms with optimal morphological suitability [3]. Nevertheless, the advances in effective therapy for TAA have been limited because the pathological mechanisms causing tumor are not known. Therefore, revealing the molecular mechanism for the TAA is indispensable for developing effective treatment.

The aortic media is mainly composed of vascular smooth muscle cells (VSMCs), which are the main source of extracellular matrix proteins such as collagen and elastin. VSMCs associated with the extracellular matrix largely determine the biomechanical properties of the aortic wall [4]. Increased apoptosis of VSMCs observed in the aortic wall of patients with TAAs is considered to be an important cause for TAA [5]. Functional research shows that Ang II induces calpain-1 expression in the aortic walls in vivo and ex vivo and VSMC in vitro. The Ang II mediated, age-associated increased MMP2 activity and migration in VSMC. Over-expression of calpain-1 in young VSMC results in cleavage of intact vimentin, and an increased migratory capacity mimicking that of old VSMC [6]. The

Table 1. Disease-related LncRNA in human

Symbol	Cancers	Chromosome location	Start	End	Strand	Species	Alias	NCBI No.	
7SK	Cancer	chr6	52860418	52860749	+	Human	RN7SK; 7SK	NR_001445.2	22377309
BCAR4	Breast cancer	chr16	11913687	11922689	-	Human	BCAR4	NR_024049.1	21506106
BCYRN1	Breast cancer	chr2	47562454	47562653	+	Human	BCYRN1; BC200; BC200a; LINC00004; NCRNA00004	NR_001568.1	15240511
BOK-AS1	Testicular cancer	chr2	242483799	242498558	-	Human	BOK-AS1; BOKAS; NAToB; BOK-AS; NCRNA00151	NR_033346.1	19287972
C1QTNF9B-AS1	Prostate cancer	chr13	24463028	24466242	+	Human	C1QTNF9B-AS1; PCOTH	BC073902	15930275
CASC2	Endometrial cancer	chr10	119806332	119969665	+	Human	CASC2; C10orf5	NR_026939.1	15024726
CBR3-AS1	Prostate cancer	chr21	37504065	37528606	-	Human	CBR3-AS1; PIncRNA-1	NR_038892.1	22264502
CCAT1	Colorectal cancer	chr8	128219629	128231724	-	Human	CCAT1	XR_108886.3	23416875
CDKN2B-AS1	Breast cancer	chr9	21994790	22121096	+	Human	CDKN2B-AS1; ANRIL; p15AS; CDKN2BAS; CDKN2B-AS; NCRNA00089; RP11-145E5.21	NR_003529.20	17440112
DNM3OS	Ovarian cancer	chr1	172107724	172113975	-	Human	DNM3OS; DNM3-AS1; MIR199A2HG	NR_038397.1	20400975
DSCAM-AS1	Breast cancer	chr21	41755010	41757285	+	Human	DSCAM-AS1; M41	NR_038896.1	12177779
EPB41L4A-AS1	Cancer	chr5	111496223	111498198	+	Human	EPB41L4A-AS1; TIGA1; C5orf26; NCRNA00219	NR_015370.2	16973895
GAS5	Breast cancer	chr1	173833039	173837125	-	Human	GAS5; SNHG2; NCRNA00035	NR_002578.7	20673990
H19	Bladder cancer	chr11	2016406	2019065	-	Human	H19; ASM; BWS; WT2; ASM1; PRO2605; D11S813E; LINC00008; NCRNA00012	NR_002196.5	11193051
HIF1A-AS1	Kidney cancer	chr14	62147759	62162536	-	Human	HIF1A-AS1; 5'aHIF-1A		21897117
HOTAIR	Breast cancer	chr12	54356096	54362515	-	Human	HOTAIR; HOXAS; HOXC-AS4; HOXC11-AS1; NCRNA00086	NR_047517.15	19182780
IGF2-AS	Prostate cancer	chr11	2161758	2169896	+	Human	IGF2-AS; PEG8; IGF2AS; IGF2-AS1	NR_028044.1	19767753
KCNQ1OT1	Colorectal cancer	chr11	2661768	2721228	-	Human	"KCNQ1OT1; LIT1; KvDMR1; KCNQ10T1; KCNQ1-AS2; KvLQT1-AS; NCRNA00016		16965397
lincRNAp21	Lung cancer	N/A	N/A	N/A	N/A	Human	N/A	N/A	22535282
LSINCT5	Breast cancer	N/A	N/A	N/A	N/A	Human	N/A	N/A	21532345
MALAT1	Cancer	chr11	65265233	65273940	+	Human	MALAT1; HCN; NEAT2; MALAT-1; PRO2853; LINC00047; NCRNA00051	NR_002819.6	20711585
MEG3	Bladder cancer	chr14	101292445	101327363	+	Human	MEG3; GTL2; FP504; prebp1; PRO0518; PRO2160; LINC00023; NCRNA00028	NR_002766.7	14602737
MIR31HG	Breast cancer	chr9	21454267	21559697	-	Human	MIR31HG	NR_027054.1	22289355
PCA3	Prostate cancer	chr9	79379354	79402465	+	Human	PCA3; DD3; NCRNA00030	NR_015342.12	18602209
PCAT1	Prostate cancer	chr8	128025399	128033259	+	Human	PCAT1; PCAT-1	NR_045262.1	21804560
PCGEM1	Prostate cancer	chr2	193614571	193641625	+	Human	PCGEM1; LINC00071; NCRNA00072	NR_002769.2	16515751
PCNCR1	Prostate cancer	N/A	N/A	N/A	N/A	Human	N/A	N/A	22535282
PVT1	Breast cancer	chr8	128806779	129113499	+	Human	PVT1; LINC00079; NCRNA00083	NR_003367.6	17908964
RRP1B	Cancer	chr21	45079432	45115960	+	Human	RRP1B; Nnp1; RRP1; NNP1L; KIAA0179	NM_015056.2	19710015
SRA1	Breast cancer	chr5	139929653	139937678	-	Human	SRA1; SRA; SRAP; STRAA1; pp7687	NM_001035235.6	20079837
TDRG1	Testicular cancer	chr6	40346163	40347631	+	Human	TDRG1; LINC00532	NR_024015.1	21243750
UCA1	Bladder cancer	chr19	15939757	15946230	+	Human	UCA1; CUDR; LINC00178; NCRNA00178	NR_015379.3	20117985
WRAP53	Cancer	chr17	7589389	7606820	+	Human	WRAP53; DKCB3; TCAB1; WDR79	NM_001143990.1	21441950
XIST	Breast cancer	chrX	73040495	73072588	-	Human	XIST; SXI1; swd66; DXS1089; DXS399E; LINC00001; NCRNA00002	NR_001564.3	17545591
Yiya	Cancer	chr1	214098092	214099997	+	Human	LINC00538; Yiya	NR_046189.1	22258142
ZNFX1-AS1	Breast cancer	chr20	47894715	47905797	+	Human	ZNFX1-AS1; HSUP1; HSUP2; ZFAS1; C20orf199; NCRNA00275	NR_003604.2	21460236

Table 2. PCR primers used in this study

Symbol	Forward	Reverse
7SK	AAACAAGCTCTCAAGGTC	CCTCATTTGGATGTGTCT
BCAR4	GGACTCATTGTTGTTCTAC	ACCTATGGCTATCATTGTT
BCYRN1	CTGGGCAATATAGCGAGAC	TGCTTTGAGGGAAGTTACG
BOK-AS1	CTTGGCAGTTCTGATTGTG	TTGTCCGCTGTGGATAAG
C1QTNF9B-AS1	AGACACCTGAACATTCCT	CTGAGCAAGTTTCCTTCTT
CASC2	CTATTCCGAGTAAGAAGTG	TCTGTGTTGATGTTGATT
CBR3-AS1	CTTCTGGTTACAATGATTCTC	CACTTACTGCCTACATTAGA
CDKN2B-AS1	TCATCATCATCATCATCATC	TGCTTCTGTCTCTTCATA
DNM3OS	ATAGAGCAAGTCTGGATT	GGATGAGGCAATAACATT
DSCAM-AS1	ACTAGCACAGATGGCATTC	CAGGAAGCATCGTGAACA
EPB41L4A-AS1	TAAGACAGTGAGGATGTGAAT	ATTATGGTGACAGCAGTGA
GAS5	CACAGGCATTAGACAGAA	AGGAGCAGAACCATTAAG
H19	CTCCACGACTCTGTTTCC	TCTCCACAACTCCAACCA
HIF1A-AS1	AATGTGTTCCTTGCTCTT	GTATGTCTCAGTTATCTTCCT
HOTAIR	AATAGACATAGGAGAACACTT	AATCTTAATAGCAGGAGGAA
IGF2-AS	CGCCACTGTGTTACCATT	TTGCCCATCCCAGATAGAA
KCNQ1OT1	GCATATCTGTCTTCCGTAT	CCTCTTCCTTCGTTCAAT
LSINCT5	TAGACAACTTACTTAACCTCAT	TCCTTATCCACCTTATCCA
MALAT1	CCGCTGCTATTAGAATGC	CTTCAACAATCACTACTCCAA
MEG3	TGGCATAGAGGAGGTGAT	AGACAAGTAAGACAAGCAAGA
MIR31HG	ACTTCCACGATAGCAATG	GAATGAATCCTCTGTCCTC
PCA3	AATCATACTGGTCACTTATCT	TTAACAACTGGTCCTGAG
PCAT1	TAGAGCCTTGAAGATGAG	TCGTGTAGTTGTAAGATGA
PCGEM1	TAGTTAAGCAGATTCATAGA	GATGTCATAGTCCTCTTC
PVT1	CTTGAGAACTGTCCTTACG	CAGATGAACCAGGTGAAC
RRP1B	CAGTATATCTCAACTCAGT	TTCTTCTTCCTTCTTCTC
SRA1	TTACAGAGATTAGAACCACATT	GGCAAGTCAGAGTTACAAT
TDRG1	GATTCGTCTGGTTCCTTA	TTCCTCTTGACTGATTCTAA
UCA1	TTCCTTATTATCTCTTCTG	TCCATCATACGAATAGTA
WRAP53	CAATAGTGCTGATAACAT	CAGTAATCATAGATGGTAT
XIST	GAACCACCTACACTTGAG	TGCTATGCGTTATCTGAG
Yiya	TATCCTATTCTTAGCAACTG	ACATACCTGGCATATAGT
ZNFX1-AS1	CCAGTTCCACAAGGTTAC	GCAGGTAGGCAGTTAGAA
U6	CTCGCTTTGGCAGCACA	AACGCTTCACGAATTTGCGT

above research results show that the functional dysfunction of VSMCs may be correlated with cardiovascular diseases and cancers.

Eukaryotic genomes encode numerous long non coding RNAs (LncRNAs), which is defined as endogenous cellular RNAs with length longer than 200 nucleotides, but lack open reading frames of significant length [7]. Within 4 years, the number of identified lncRNA genes increase more than 8000 [8]. Although the function of most lncRNAs is still unknown, their increasing numbers and the accumulating evidence for their involvement in many biologic processes provide compelling arguments in support of the dysregulation of lncRNAs has been correlated to cancer development, invasion and metastasis in the malignant cell [8-10] (**Table 1**). To date, the underlying mechanisms for lcnRNAs regulation VSMCs proliferation and apoptosis are quite limited.

In this study, we performed a hierarchical cluster analysis of the differentially expressed Lnc-RNA in the serum of TAA patients to identify the lncRNA HIF 1 alpha-antisense RNA 1 (HIF1A-AS1) that associated with Lnc-RNA expression. We also investigated the role of HIF1A-AS1 in vitro in regulating the proliferation and apoptosis of aortic VSMCs.

Materials and methods

Serum samples and cell culture

Human serum samples were obtained with written informed consent from The Fourth Hospital of Hebei Medical University. The study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University. 50 serum samples of TAA patients and 50 cases of normal control group were collected between 02/2010 and 12/2014.

The vascular smooth muscle cells (VSMCs) were maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of palmitic acid (Sigma, USA).

Cell viability detection by CCK8

VSMCs (5.0×10^3/well) were plated and treated in 96-well plates (three wells per group) with various concentrations of palmitic acid (0, 0.2, 0.4 or 0.8 mM) for 24 h, 48 h or 72 h respec-

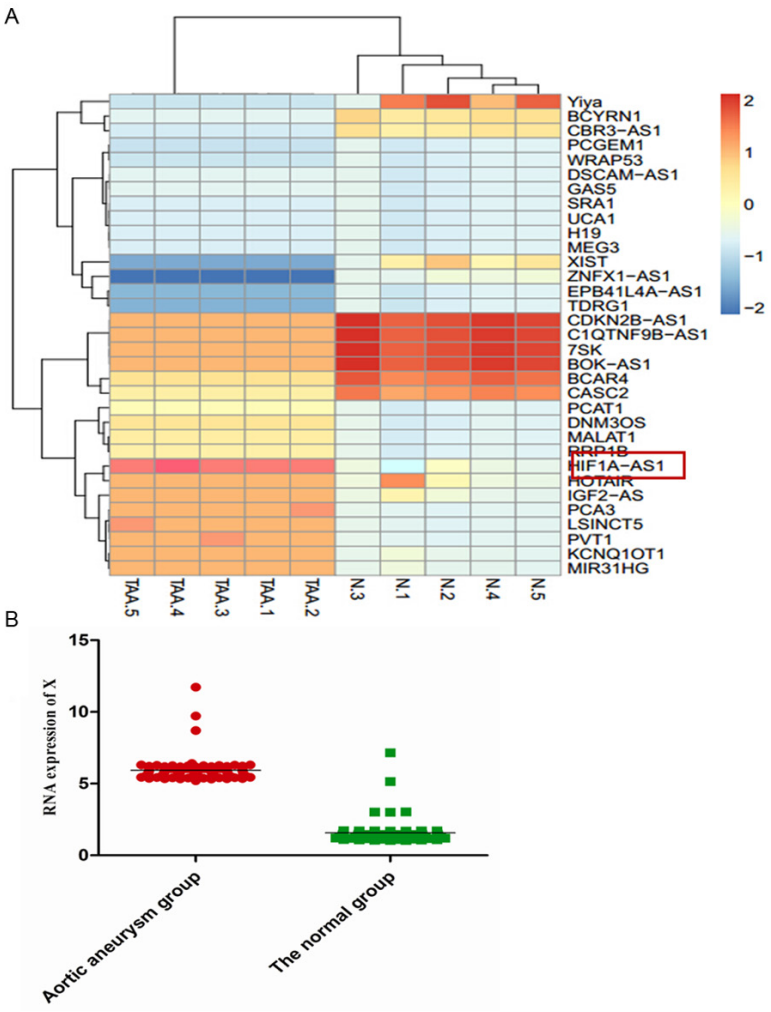

Figure 1. Hierarchical cluster analysis of the differentially expressed long non-coding RNAs (LncRNA) and sHIF1a-AS1 expression in serum of TAA patients. The figure is drawn by MeV software (version 4.2.6). A. Differentially expressed LncRNAs chosen from lncRNA and disease database. Correlation similarity matrix and average linkage algorithms are used in the cluster analysis. Each row represents an individual LncRNA, and each column represents a sample. The dendrogram at the left side and the top displays similarity of expression among LncRNAs and samples individually. The color legend at the right represents the level of mRNA expression, with red indicating high expression levels and blue indicating low expression levels; B. The expression of HIF 1alpha-antisense RNA 1 (HIF1a-AS1) in serum of TAA patients is measured by Quantitative real-time PCR, 50 serum samples of TAA patients and 50 cases of normal control group were collected. Values are expressed as mean ± SEM, n=50 in each group.

tively. 10 μL of CCK8 (Beyotime, China) was added to the cells, and the OD value of the cells was measured at 450 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. VSMCs (5.0×10^5/well, 1 ml) were plated and treated in 6-well plates (three wells per group). After treatment, cells were washed twice with PBS (pH=7.4), and re-suspended in staining buffer containing 10 μl PI and 5 μl annexin V-FITC. Double-labeling was performed at room temperature for 15 min in the dark before the flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson). Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with flow cytometer.

Quantitative real-time PCR

VSMCs (5.0×10^5/well) were plated and treated in 6-well plates (three wells per group) after 24 h with treatment for 48 h. The VSMCs RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Switzerland) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). The cycling conditions

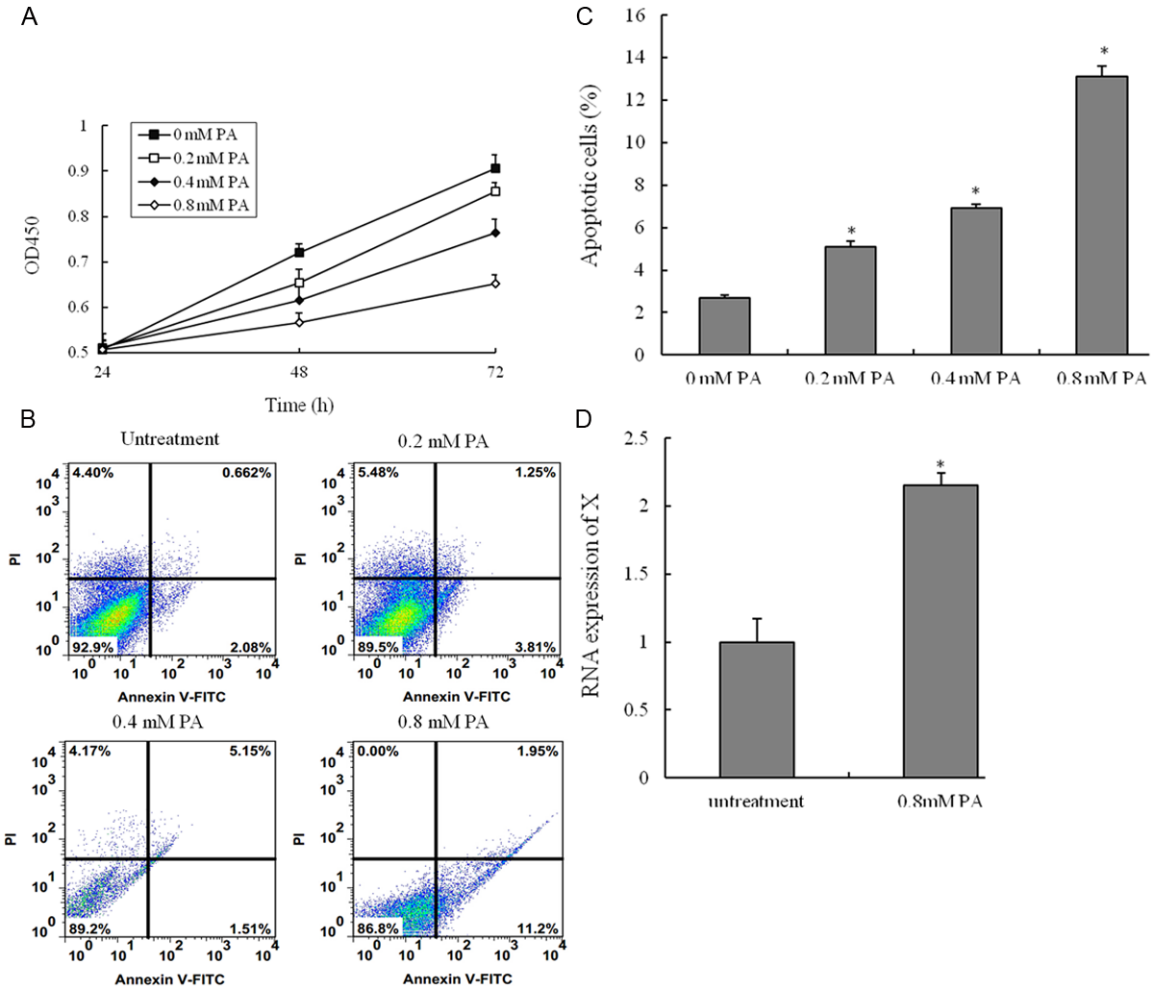

Figure 2. Palmitic acid-induced the apoptosis of vascular smooth muscle cells (VSMCs). VSMCs are incubated with various concentrations of palmitic acid (PA) for 24 h, 48 h or 72 h, and the cell viability was examined by CCK8 assay. (A) Cells are treated with vehicle, 0.2 mM PA, 0.4 mM PA or 0.8 mM PA for 48 h; (B) The percentage of apoptotic cells is also analyzed by flow cytometric analysis of annexin V/PI double staining and (C) the percentage of apoptotic cells (at the right of pictures). (D) Cells are treated with PA (80 mM) for 48 h, RNA expression of HIF 1alpha-antisense RNA 1 (HIF1a-AS1) in VSMCs is measured by quantitative real-time PCR. Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group.

were 2-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. PCR with the following primers: as shown in **Table 2**. U6 as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta ct}$ method.

Western blotting

The VSMCs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to PVDF Transfer Membrane (Millipore). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, caspase3, caspase8 and Bcl-2 (Santa Cruz, USA), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1

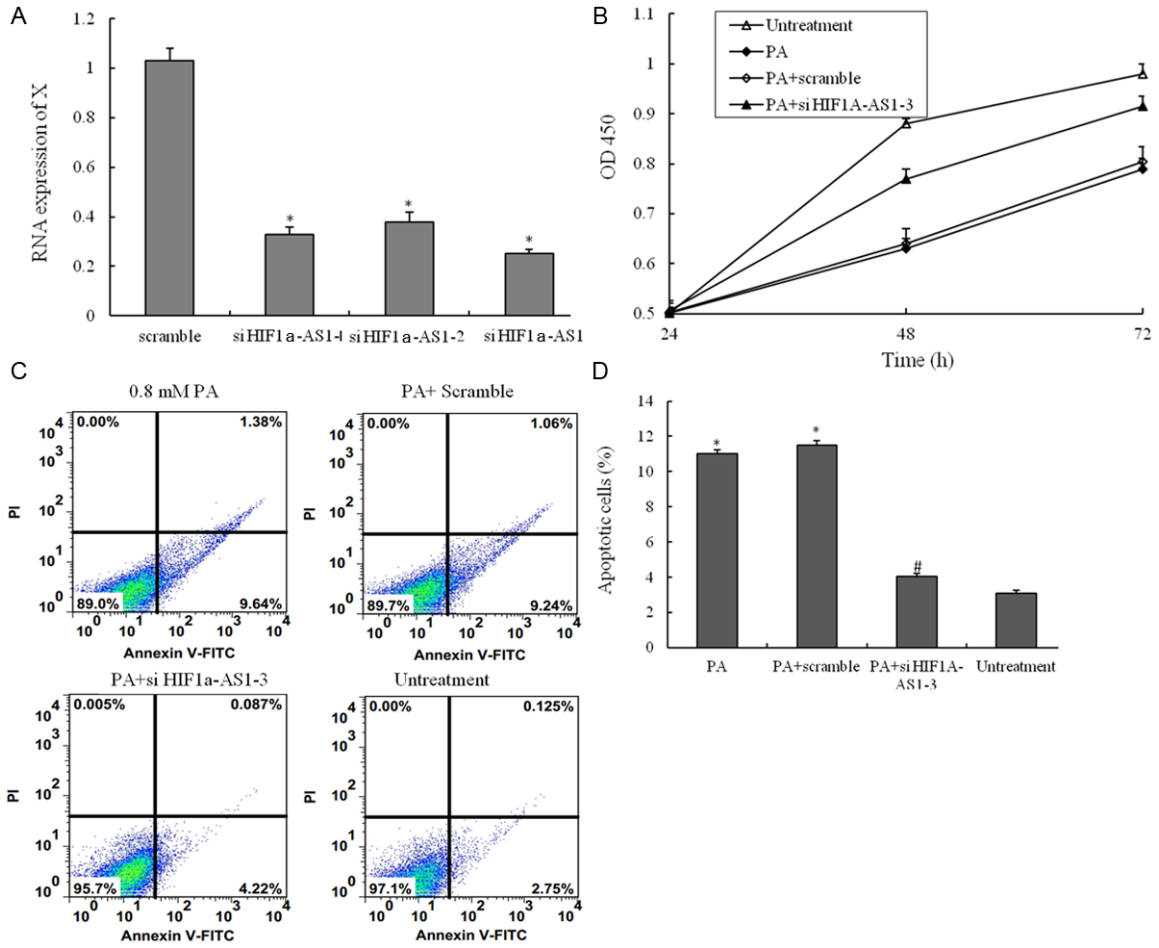

Figure 3. The small interfering RNA for suppressing the function of HIF1a-AS1. (A) Three different small interfering RNA were transfected into VSMCs suppressing the RNA expression of HIF1a-AS1; (B) VSMCs are treated with untreatment, 0.8 mM PA only, 0.8 mM PA plus scramble si-RNA and 0.8 mM PA plus si- HIF1a-AS1-3 for 48 h, and the cell viability was examined by CCK8 assay; (C) VSMCs are treated with untreatment, 0.8 mM PA only, 0.8 mM PA plus scramble si-RNA and 0.8 mM PA plus si- HIF1a-AS1-3 for 48 h, the percentage of apoptotic cells is also analyzed by flow cytometric analysis of annexin V/PI double staining and (D) the percentage of apoptotic cells (at the right of pictures). Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group; $^{\#}P < 0.05$, versus PA group.

hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the monoclonal anti-β-actin antibody (Bioworld Technology, USA).

RNA interference

The small interfering (si) RNA for human PTX3 or scramble siRNA was obtained from Dharmacon (Lafayette, USA). The small interfering with the following primers: siHIF1A-AS1-1, Forward 5'-GAGUCUGUGUGGGACAAGCACUUCA-3' and Reverse 5'-AGUAGAGGAUGUGACUCACUGUCUG-3'; siHIF1A-AS1-2, Forward 5'-GCUAACACUGGUCUGAGCAAGGU-3' and Reverse 5'-UCCUCAAGGAGAGAGGACUAAGC-3', siHIF1A-AS1-3, Forward 5'-GCACAGGAUUCAGUCCACUGUCUU-3' and Reverse 5'-GACACAGGACACUGAAAGCUUGG-3'; scramble, Forward 5'-CACCAGUGGCUAUCACACGUGUGA-3' and Reverse 5'-UCAAGAGGAGUGUAACCCACACGU-3'. The siRNA oligonucleotides (at a final concentration of 100 nM) were transfected into human umbilical vein endothelial cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

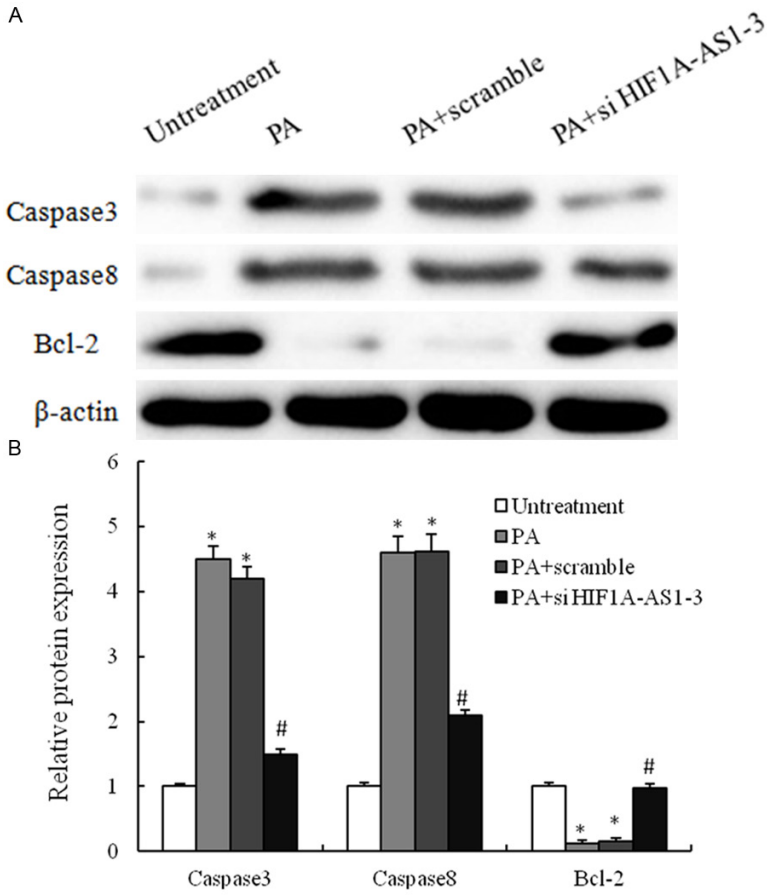

Figure 4. PA-mediated regulation of apoptosis-related proteins in VSMCs. A. VSMCs are treated with untreatment, 0.8 mM PA only, 0.8 mM PA plus scramble si-RNA and 0.8 mM PA plus si-HIF1a-AS1-3 for 48 h, and the expression of caspase3, caspase8 and Bcl2 are analyzed by western blotting; B. These results are confirmed by densitometric analyses. Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group; $^{\#}P < 0.05$, versus PA group.

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

Results

Hierarchical cluster analysis and HIF1a-AS1 expression in vivo

HIF 1alpha-antisense RNA 1 (HIF1a-AS1) plays a key role in the proliferation and apoptosis of vascular smooth muscle cells in vitro, which may contribute to the pathogenesis of thoracic aortic aneurysms. We then investigated the possible mechanisms that Lnc-RNA regulates the thoracoabdominal aorta tumorigenesis. We performed a hierarchical cluster analysis of the differentially expressed Lnc-RNA in the serum of TAA patients that associated with Lnc-RNA expression. After the removal of redundant and unannotated sequences, 10 genes were found to be significantly up-regulated and 15 genes to be significantly down-regulated in the TAA group compared to the normal control group. The results showed that the overexpression of HIF1a-AS1 was associated with TAA, the expression of which was at the highest levels in all 33 Lnc-RNAs in vivo (**Figure 1A**). To further validated the interaction between the TAA and HIF1a-AS1, large sample statistics results showed that compared to the normal control the expression of HIF1a-AS1 was significantly increased (superior to 6 folds) in serum of TAA patients.

PA-induced cell apoptosis and LncRNA HIF1a-AS1 expression in VSMCs

To evaluate the potential cell apoptosis of PA in VSMCs, we analyzed the effect of PA on cell survival in VSMCs. The CCK8 assay was used to measure cell viability. The viabilities of HUVECs treated with PA were significantly lower than those of untreatment group. Treatment of HUVECs with PA induced cell death in a time and dose-dependent manner by using CCK8 assay (**Figure 2A**). We next investigated whether PA induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that the proportion of the apoptotic cells had gained as compared to untreatment group (**Figure 2B**

and **2C**). Moreover, the percentage of the apoptotic cells in a dose-dependent manner. LncRNA HIF1a-AS1 is highly associated with CVD, and HIF1a-AS1 is highly expressed in advanced atherosclerosis tissues. The current study suggested that HIF1a-AS1 was associated with PA-induced dysfunction of VSMCs. The RNA expression of HIF1a-AS1 was significantly higher in VSMCs with PA (0.8 mM) than those of untreatment group (**Figure 2D**). Therefore, our data suggest that up-regulation the expression of HIF1a-AS1 was involved in PA-induced cell death.

Identification of HIF1a-AS1 in the regulation of VSMCs dysfunction

In this work, knock-out of endogenous HIF1a-AS1 with small-interfering RNA (siRNA), the expression of HIF1a-AS1 was down-regulated (**Figure 3A**). To evaluate the potential protective mechanisms of inhibition the function of HIF1a-AS1 in VSMCs, the CCK8 assay was used to measure cell viability. The viabilities of VSMCs inhibited with PA were protected by si- HIF1a-AS1 (**Figure 3B**). Consistent with the CCK8 assay, the Annexin V-PI double-labeling results showed that inhibition the function of HIF1a-AS1 with si-RNA could decrease the proportion of the apoptosis cells inducing by PA treatment (**Figure 3C** and **3D**).

PA-mediated regulation of apoptosis-related proteins

The apoptotic response was further investigated by measuring caspase-3 and caspase-8 activity and apoptosis-related proteins with Western blot techniques. PA administration caused 4.5- and 4.4- fold increases in caspase-3 and caspase activity respectively. However, the combination PA with si-HIF1a-AS1 induced strong and specific suppression of protein expression of caspase-3 and caspase-8 **Figure 4** expression, and expression of which was statistically up-regulated in the PA combination with si-HIF1a-AS1-treated group as compared to PA single treatment group. Therefore, our data suggest that up-regulation the expression of si-HIF1a-AS1 was involved in PA-induced VSMCs death.

Discussion

Research into the pathogenesis of thoracoabdominal aortic aneurysms (TAA) is difficult, because this disease is caused by multiple factors such as hemodynamics, metabolism, inflammation and genetic influences [11]. In addition to activation of proteolysis and inflammation, apoptosis of smooth muscle cells and oxidative stress have been suggested by several clinicopathological studies, and these factors together with many others seem to be intricately interwoven to produce aneurysms [12-14]. Another difficulty is that suitable animal models are not available for the study of aortic aneurysms. In this study, apoptosis of VSMCs were considered to approximately represent the TAA cell injury model. The exposure of PA to VSMCs has been demonstrated to cause a series of dysfunction, and trends to make as the TAA cell injury model. There were two significant findings in this report: (1) patients with TAA were increased HIF1a-AS1 in the serum; (2) we found that PA could induce VSMCs apoptosis in a dose dependent manner and increase the expression of HIF1a-AS1 in VSMCs. VSMCs apoptosis was thought to be involved in TAA [12]. Thus, apoptosis was measured in the present study to better confirm and to analyze the VSMCs injury by PA. We found that the proportion of the apoptotic cells was increased.

The non-coding RNAs that are the predominant transcripts in mammalian genome exceed the number of protein coding genes. It is commonly believed that that alteration of small noncoding RNA expression, especially microRNAs contribute to the pathogenesis of cardiovascular disease [15-18]. More recently, a new class of noncoding RNAs, long non-coding RNAs, which are endogenous RNA transcripts in the genome with no or lower protein coding potential, have been reported to be abundantly transcribed [19]. Related studies indicate that lncRNAs are involved in the alteration of chromatin structure, the control of cellular functions and the regulation of related genes [20-22]. Recent studies are beginning to reveal their importance in tumorigenesis and metastasis in the malignant cell. For example, downregulation of a long noncoding RNA-ncRuPAR contributes to tumor inhibition in colorectal cancer [23], and LncRNA TARID directs demethylation and activation of the tumor suppressor TCF21 via GADD45A [24]. Recently, it has been reported that stroke-induced lncRNAs might associate with CMPs to modulate the post-ischemic epigenetic landscape [25].

In this study, we performed a LncRNA microarray technique using human samples, which can evaluate thousands genes simultaneously, and hierarchical cluster analysis of the differentially expressed Lnc-RNA in the serum of TAA patients to identify the lncRNA HIF1A-AS1 was significantly up-regulated. Our results is consistent with other studies that reported that HIF1A-AS1 is identified through BRG1 knock-down VSMCs, and the expression of HIF1A-AS1 is found to be regulated by BRG1 in VSMCs [4]. These studies will have particular relevance in the future, as the role of lncRNA in cardiovascular disease states becomes increasingly recognized. Moreover, PA dose-dependently decreased the cell viability and increased the apoptosis of VSMCs, and down-regulated the expression of Bcl2 and up-regulated the expression of caspase3 and caspase8. Interestingly, LncRNA HIF1A-AS1 knock-down could suppress PA-induced dysfunction of VSMCs in vitro. Therefore, our data suggest that up-regulation the expression of HIF1a-AS1 was involved in PA-induced cell apoptosis.

In conclusion, our results demonstrate that HIF1A-AS1 was overexpressed in the TAA patients. LncRNA HIF1A-AS1 knock-down could suppress PA-induced apoptosis of VSMCs in vitro, which may contribute to the pathogenesis of thoracoabdominal aorta aneurysm.

Disclosure of conflict of interest

None.

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