BRG1 expression is increased in thoracic aortic aneurysms and regulates proliferation and apoptosis of vascular smooth muscle cells through the long non-coding RNA HIF1A-AS1 in vitro

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Received 13 January 2014; received in revised form 14 April 2014; accepted 16 April 2014

Abstract

OBJECTIVES: Brahma-related gene 1 (BRG1) and long non-coding RNAs (lncRNAs) play important roles in cellular processes. However, little is known regarding their roles in thoracic aortic aneurysms. We investigated BRG1 expression in thoracic aortic aneurysms and the roles of BRG1 and the lncRNA HIF 1 alpha-antisense RNA 1 in regulating the proliferation and apoptosis of aortic smooth muscle cells in vitro.

METHODS: BRG1 mRNA and protein expression in human aortic media specimens were examined by quantitative real-time polymerase chain reaction, immunohistochemical staining and western blot. BRG1 expression was up-regulated by lentiviral vectors. Vascular smooth muscle cell proliferation and apoptosis were studied using Cell Counting Kit-8 and terminal deoxynucleotidyl transferase dUTP nick-end labelling assays. We performed western blots to detect Caspase3 and Bcl2 protein expression. LncRNAs regulated by BRG1 were identified through microarray in BRG1 gain- and loss-of-function vascular smooth muscle cells. Finally, the expression of HIF 1 alpha-antisense RNA 1 was reduced by siRNA and cell proliferation and apoptosis was studied using Cell Counting Kit-8 assays, caspase-3 activity assays and western blot.

RESULTS: BRG1 expression in the aortic media was significantly higher in thoracic aortic aneurysms than in normal controls. Overexpression of BRG1 in human aortic smooth muscle cells promoted apoptosis and reduced proliferation. The expression of HIF 1 alpha-antisense RNA 1 was significantly down- and up-regulated in BRG1 knock-down and overexpressing vascular smooth muscle cells, respectively. We further demonstrated that suppression of HIF 1 alpha-antisense RNA 1 by siRNA in vascular smooth muscle cells reduced apoptosis and promoted proliferation.

CONCLUSIONS: BRG1 is overexpressed in the aortic media of thoracic aortic aneurysms and the interaction between BRG1 and HIF 1 alpha-antisense RNA 1 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.

Keywords: Brahma-related gene 1 • Long non-coding RNA • Thoracic aortic aneurysm • Vascular smooth muscle cells • Apoptosis • Proliferation

INTRODUCTION

Thoracic aortic aneurysms (TAAs) are localized dilatations of the supra-diaphragmatic aorta that result from weakening and expansion of the arterial wall. When the ascending aorta is involved, patients may die of aortic rupture, haemopericardium, pericardial tamponade or acute aortic insufficiency. Based on the size of the ascending aorta and patient’s risk factors, tight control of arterial blood pressure or even ascending aortic replacement may be necessary. However, patients surviving surgery are at risk of significant postoperative morbidity due to myocardial infarction, renal failure, stroke, neurological deficits and/or paraplegia [1]. The formation and expansion of TAAs seem to result from many factors, which include an interplay between genetic predisposition and cellular imbalances and some haemodynamic factors. Although there has been progress in understanding the physiopathology of TAA, the molecular mechanism of pathogenesis is not completely clear.

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. Increased apoptosis of VSMCs observed in the aortic wall of patients with TAAs is considered to be an important cause for TAA [2].

Brahma-related gene 1 (BRG1) is the central catalytic subunit of numerous chromatin-modifying enzymatic complexes, which can use the energy derived from ATP hydrolysis to change the chromatin architecture of target promoters. In various tissues and physiological conditions, BRG1 is involved in the activation and repression of gene expression through chromatin remodelling. Previous studies have shown that BRG1 plays an important role in vascular cells [3]. However, it is unclear how the proliferation and apoptosis of smooth muscle cells are regulated by BRG1.

Non-coding RNAs (ncRNAs) include short/small ncRNAs and long ncRNAs based on a cut-off length. NcRNAs under 200 nucleotides are usually called short/small ncRNAs, including microRNAs, and those greater than 200 bases are called long non-coding RNAs (lncRNAs). With recent genome-wide transcriptome studies, it has been revealed that approximately 10–20 times more genomic sequences are transcribed to lncRNA than to protein-coding RNA. MicroRNA expression appears to be regulated by BRG1-containing chromatin remodelling complexes. SWI/SNF-mediated chromatin remodelling also regulates the phenotype of smooth muscle by affecting expression of protein-coding genes and microRNAs [4]. Similarly, as regulatory factors of chromatin remodelling [5], lncRNAs influence gene expression by interacting with a key subunit of the chromatin remodelling complex [6]. lncRNAs also play important roles in cell proliferation and apoptosis [7]. However, the mechanisms through which BRG1 interacts with lncRNAs and their regulatory roles in the proliferation and apoptosis of VSMCs are far from clear.

In the present study, we assayed BRG1 expression in the aortic media of TAA tissues by quantitative real-time polymerase chain reaction (qRT-PCR), immunohistochemistry and western blotting. We also investigated the role of BRG1 and the lncRNA HIF 1 alpha-antisense RNA 1 (HIF1A-AS1) in vitro in regulating the proliferation and apoptosis of aortic VSMCs.

**MATERIALS AND METHODS**

**Tissue collection**

Aortic media specimens were obtained from 20 TAA patients who underwent ascending aorta replacement procedures at Changhai Hospital in 2011. Control aortic media specimens were obtained from 10 donors without vascular diseases. The specimens were fixed in formalin for immunohistochemical staining or frozen fresh for western blot analysis and qRT-PCR. This study was conducted in accordance with the Declaration of Helsinki and was

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**Figure 1:** Expression of BRG1 in aortic media measured by qRT-PCR (A), western blot (B and C) and immunohistochemical staining (D and E). (B) Representative image of 20 TAAs and 10 control samples. (F) Integrated optical density results of immunohistochemical staining for BRG1. BRG1 mRNA and protein were increased in TAA. *Significant from the control group (P < 0.05). CTL: control; BRG1: Brahma-related gene 1; qRT-PCR: quantitative real-time polymerase chain reaction; TAAs: thoracic aortic aneurysms; IOD: integrated optical density.
approved by the Medical Ethics Committee of Changhai Hospital. All patients gave written informed consent.

Cell culture

A human aortic VSMC line was obtained from Cascade Biologics. The cells were cultured according to the supplier’s instructions and were grown in Medium 231 with smooth muscle growth supplements (Cascade Biologics). Cells from Passages 3–8 were used for experiments.

Transfections in vascular smooth muscle cells

A BRG1-expressing lentivirus vector (Lenti-BRG1) and a negative control vector (Lenti-GFP) were purchased from FreeBio Technology, Ltd (Shanghai, China). The BRG1-specific small interfering RNA lentivirus vector (sh BRG1) and HIF1A-AS1-specific small interfering RNAs (sh HIF1A-AS1) were purchased from GenePharma (Shanghai, China). Transfections of sh HIF1A-AS1 were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions when the cells reached 40–50% confluence.

![Figure 2: The transfection efficiency of lentivirus vectors in VSMCs was assessed under confocal microscope (A and B). BRG1 mRNA and protein expression were examined by qRT-PCR (C and D) and western blot (E and F) after transfection with BRG1 overexpressing (Lenti-BRG1) and silencing (sh BRG1) lentivirus vectors. Lenti-GFP and Scramble served as control, respectively. *P < 0.05 versus control. VSMCs: vascular smooth muscle cells; BRG1: Brahma-related gene 1; qRT-PCR: quantitative real-time polymerase chain reaction; GFP: green fluorescent protein.](image-url)
Terminal deoxynucleotidyl transferase dUTP nick-end labelling staining

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) reaction was performed with a One-Step TUNEL Apoptosis Assay Kit (Beyotime, Haimen, China), according to the manufacturer's instructions, to detect the apoptosis of VSMCs. TUNEL-positive nuclei (green fluorescence) were counted in 10 random fields (×200) and then averaged.

Cell proliferation assay

Cell proliferation was assessed by using a Cell Counting Kit-8 (Beyotime, Haimen, China). Cells were plated into 96-well cell culture plates at a density of 2000 cells/well in 100 µl DMEM. Twenty-four hours later, the cells were transfected with Lentivirus vectors (Lenti-BRG1 or sh BRG1) at 7 days (0.31 ± 0.01 vs 0.41 ± 0.01, P < 0.05) and 10 days (0.60 ± 0.07 vs 0.78 ± 0.09, P < 0.05) (Fig.3C). The differences were statistically significant (both P < 0.05).

Caspase-3 activity assay

The caspase-3 activity was determined by using a caspase-3 activity kit (Beyotime) [9]. Caspase 3 activity was assayed according to the instruction manual. All experiments were carried out in triplicate.

Statistical analysis

Continuous data were expressed as mean ± standard deviations. Data were evaluated with normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). The Student's t-test was applied for analysis of differences between the groups. In case of inhomogeneity of variances or deviation from the normal distribution, the Mann-Whitney U-test was used. All data in the study were evaluated by using SPSS 19.0 software. A P-value of <0.05 was considered statistically significant.

RESULTS

Increased expression of BRG1 in the aortic media of thoracic aortic aneurysm patients

First, we investigated the expression of BRG1 in TAA (n = 20) and control (n = 10) aortic media specimens. QRT-PCR and western blot showed significantly higher levels of BRG1 mRNA (5.54 ± 1.79 vs 1.24 ± 0.42, P < 0.05) and protein (1.05 ± 0.21 vs 0.45 ± 0.13, P < 0.05) in TAA (Fig. 1A–C). Next, immunohistochemistry assays showed that BRG1 was expressed at higher levels in VSMCs in TAA than in the control group (1460 ± 312 vs 253 ± 37, P < 0.05) (Fig.1D–F).

Overexpression of BRG1 promotes vascular smooth muscle cell apoptosis and suppressed vascular smooth muscle cell proliferation

To study the function of BRG1 in apoptosis and proliferation of VSMCs, we transfected in vitro cultured normal VSMCs with a BRG1-expressing lentivirus vector (Lenti-BRG1). Transfection efficiency of lentivirus vectors reached up to 90% (Fig. 2A and B). In VSMCs that were transfected with Lenti-BRG1, BRG1 mRNA and protein expression was 10.2- and 2.5-fold of the control Lenti-GFP group (Fig. 2C, E and F). Cell apoptosis rate was significantly higher in the Lenti-BRG1 group than that in the Lenti-GFP group (0.28 ± 0.01 vs 0.048 ± 0.002, P = 0.001) (Fig. 3A and B). Western blot showed BRG1 overexpression resulted in up-regulated expression of the apoptosis promoting gene Caspase 3 (2.6-fold of control, P < 0.05) and down-regulated expression of the antiapoptosis gene Bcl2 (0.3-fold of control, P < 0.05) (Fig. 3C), which was consistent with the cell apoptosis assay. Using a CCK-8 cell proliferation assay, we found that cell proliferation was decreased in VSMCs that were transfected with Lenti-BRG1 at 7 days (0.31 ± 0.01 vs 0.41 ± 0.01, P < 0.05) and 10 days (0.60 ± 0.07 vs 0.78 ± 0.09, P < 0.05) (Fig. 3D).

HIF1A-AS1 expression is positively regulated by BRG1

BRG1 mRNA and protein expression were successfully up- and down-regulated in VSMCs by using Lenti-BRG1 or sh BRG1 (Fig. 2C–F). The expression of 95 apoptosis-related IncRNAs were examined in VSMCs with overexpression or silencing of BRG1. The changes in HIF1A-AS1 expression were the most significant (Fig. 4A). We further transfected VSMCs with Lenti-BRG1 and sh BRG1 and found that expression levels of HIF1A-AS1 were up-regulated by 3.26-fold compared with control by BRG1 overexpression, and down-regulated to 0.37-fold by BRG1 silencing (Fig. 4B). The differences were statistically significant (both P < 0.05).
HIF1A-AS1 silencing suppresses vascular smooth muscle cell apoptosis and promotes vascular smooth muscle cell proliferation

In VSMCs transfected with HIF1A-AS1-specific silencing RNA (shHIF1A-AS1), HIF1A-AS1 expression was knocked down to 0.29-fold of control (Scramble) (Fig. 5A). Lower protein levels of Caspase 3 (0.7-fold of control, P < 0.05) and higher levels of Bcl2 were observed (1.3-fold of control, P < 0.05) (Fig. 5B). Caspase 3 activity was also decreased (836.0 ± 98.5 vs 1371.3 ± 102.6, P < 0.05) (Fig. 5C). Increased cell proliferation was observed through the CCK-8 cell proliferation assay. The differences between the two groups at 2 days (0.31 ± 0.01 vs 0.22 ± 0.01), 3 days (0.51 ± 0.07 vs 0.30 ± 0.02) and 4 days (0.60 ± 0.04 vs 0.45 ± 0.02) were statistically significant (P < 0.05, respectively) (Fig. 5D).

DISCUSSION

The present study reports two novel findings: (i) patients with TAA have increased BRG1 in the media of the aortic wall and (ii) BRG1 promotes apoptosis and inhibits the proliferation of VSMCs through the mediator HIF1A-AS1 in vitro.

Previous studies suggest that BRG1, a key chromatin remodeling protein, plays an important role in regulating cardiac growth, differentiation and gene expression. In embryos, BRG1 promotes myocyte proliferation by maintaining BMP10 and suppressing p57kip2 expression [10]. BRG1 expression is up-regulated in hypertrophic cardiomyopathy [10]. In addition, BRG1 expression is also up-regulated and involved in tumour progression in prostate cancer [11]. So far, little is known about the expression pattern of BRG1 in TAA. Our data demonstrate that BRG1 expression is significantly increased in the aortic media of TAA patients compared with the normal control group. This implies that BRG1 may play a role in the development and progression of TAA.

Multiple studies have documented that matrix metalloproteinase-2 and -9 (MMP2/9) are increased in the aortic media of TAA patients [12, 13], which results in a change in the composition of the extracellular matrix. This change may be a key cause for apoptosis of VSMCs [14]. Previous studies also suggest that BRG1 is also involved in the regulation of expression of MMP9 and MMP2 [15, 16]. Related studies show that BRG1 overexpression can significantly increase the apoptotic rate of mesenchymal stem cells [17]. Similarly, a study by Zhang et al. [18] showed that BRG1 is up-regulated in VSMCs of patients with primary atherosclerosis and is involved in the pathological process of atherosclerosis, presumably by inducing cell apoptosis. In our in vitro assay, our results suggest that BRG1
Figure 4: LncRNA HIF1A-AS1 showed the most significant changes in VSMCs transfected with Lenti-BRG1 or sh BRG1 (A). HIF1A-AS1 expression changes were further assayed by qRT-PCR (B). *P < 0.05 versus control. LncRNA: long non-coding RNA; VSMCs: vascular smooth muscle cells; BRG1: Brahma-related gene 1; qRT-PCR: quantitative real-time polymerase chain reaction; HIF1A-AS1: HIF 1 alpha-antisense RNA 1.

Figure 5: Silencing efficiency of sh HIF1A-AS1 was verified by qRT-PCR (A). Decreased Caspase 3 and increased Bcl2 protein expression were detected through western blot in VSMCs transfected with sh HIF1A-AS1 (B). Decreased caspase 3 activity was further confirmed (C). Higher optical density at 450 nm (OD450) in CCK-8 proliferation assay was observed in sh HIF1A-AS1 group at 2, 3 and 4 days, respectively. *P < 0.05 versus Scramble (D). qRT-PCR: quantitative real-time polymerase chain reaction; VSMCs: vascular smooth muscle cells; sh HIF1A-AS1: HIF 1 alpha-antisense RNA 1-specific small interfering RNAs.
overexpression can promote cell apoptosis and reduce cell proliferation in VSMCs. However, BRG1 may influence the pathogenesis of TAA through many routes.

For the moment, at least three processes are known to control the assembly and regulation of chromatin: DNA methylation, histone modifications and ATP-dependent chromatin remodelling [19]. Related studies indicate that IncRNAs are involved in the alteration of chromatin structure, the control of cellular functions and the regulation of related genes [20–22]. However, there have been few articles discussing the manner of the interaction between IncRNA and ATP-dependent chromatin remodelling complexes. Accumulating evidence indicates that IncRNAs play critical roles in the regulation of cellular processes such as cell growth and apoptosis [23]. In the present study, the IncRNA HIF1A-AS1 was identified through microarray in BRG1 knock-down VSMCs, and the expression of HIF1A-AS1 was found to be regulated by BRG1 in VSMCs. Our finding is consistent with other studies that reported that IncRNAs are regulated by epigenetic processes in neuronal and glial cell [24]. These studies will have particular relevance in the future, as the role of IncRNA in cardiovascular disease states becomes increasingly recognized.

The long intergenic noncoding RNA erythroid prosurvival (LincRNA-EPS), is up-regulated in the terminal differentiation of murine erythroid cells and inhibits apoptosis [25]. Similarly, a study of melanoma cell lines showed that by down-regulating the sprouty homolog 4 intronic transcript 1, IncRNAs inhibit cell proliferation and apoptosis [7]. However, loc285194 inhibits tumour cell growth and, on the contrary, suppression of loc285194 by RNAi promotes tumour cell growth. The function of IncRNAs may have tissue/cell-type specificity. HIF1A-AS1 acts as an inhibitor or promoter of cell proliferation and apoptosis depending on its binding partners and cell types. In our study, suppression of the IncRNA HIF1A-AS1 by siRNA in VSMCs reduced cell apoptosis and promoted cell proliferation. Therefore, we conclude that the IncRNA HIF1A-AS1 plays an important role in the pathophysiology of VSMCs.

However, there are several limitations in our study that should be mentioned. First of all, although it is tempting to suggest that BRG1 overexpression may predispose to TAA, our clinical observation is limited by the nature of cross-sectional design in the study. Our data do not distinguish whether overexpression of BRG1 occurred before or after the TAA. Second, it was an observational study with normal human aortic VSMCs as the study was focusing on the relationship between BRG1, HIF1A-AS1 and the pathophysiology of VSMCs. Nevertheless, the demonstration of the overexpression of BRG1 in TAA and the interaction between BRG1 and the IncRNA HIF1A-AS1 in VSMCs might provide clues to the molecular mechanisms involved in TAA, which should be pursued by using isolated VSMCs from TAA patients and appropriate animal models in future studies.

In conclusion, our results demonstrate that the SWI/SNF complex protein BRG1 is overexpressed in the aortic media of TAA patients. In addition, BRG1 overexpression in a human aortic VSMC line can promote apoptosis and inhibit cell proliferation. The interaction between BRG1 and the IncRNA HIF1A-AS1 plays a key role in VSMCs. Although more studies are clearly needed to identify a causal relationship between BRG1 and the IncRNA HIF1A-AS1, a delicate balance of proliferation and apoptosis in VSMCs in the aortic wall is clearly essential for a normal aorta, and the IncRNA HIF1A-AS1 associated with BRG1 may play a vital role in maintaining this balance. On the other hand, appropriate animal models are warranted to evaluate the causal relationship in which the effects of BRG1 and IncRNA HIF1A-AS1 knock-down or overexpression on the development of TAA can be properly explored. Such research may also provide novel therapeutic targets in that tailored inhibitory strategies aimed specifically at BRG1 and IncRNA HIF1A-AS1 activity may decrease or halt TAA formation while minimizing the untoward side effects associated with global inhibition.

**Funding**

This work was supported by National Natural Science Foundation of China (81300233) and the Shanghai Municipal Natural Science Foundation (12JC140702 and 12JC1408102).

**Conflict of interest:** none declared.

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