EBioMedicine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

A Functional Variant of *SMAD4* Enhances Thoracic Aortic Aneurysm and Dissection Risk through Promoting Smooth Muscle Cells Apoptosis and Proteoglycans Degradation

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ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form 15 June 2017
Accepted 21 June 2017
Available online xxxx

Keywords:
SMAD4
Polymorphism
Thoracic aortic aneurysm and dissection
Proteoglycans degradation
SMCs
Apoptosis

ABSTRACT

Recent studies indicate important roles for *SMAD4* in SMCs proliferation, extracellular matrix maintenance, and blood vessel remodeling. However, the genetic effects of *SMAD4* in the pathogenesis of thoracic aortic aneurysm and dissection (TAAD) are still largely unknown. Here we identified a functional variant of *SMAD4* which might be involved in the pathological progression of TAAD. Five tagging SNPs of *SMAD4* were genotyped in 202 TAAD cases and 400 controls using MALDI-TOF. rs12455792 CT or TT variant genotypes was associated with an significantly elevated TAAD risk (adjusted OR = 1.58, 95%CI = 1.09–2.30) under a dominant genetic model. It was located in the 5'UTR and predicted to influence transcription activity and RNA folding of *SMAD4*. In luciferase reporter assay, rs12455792 T allele markedly decreased luciferase activities. Accordingly, *SMAD4* expression in tissues was lower in patients with CT or TT genotypes, compared with CC. Movat's pentachrome showed that rs12455792 T allele enhanced SMCs loss and fibers accumulation. With angiotensin II induction, rate of Apoptotic SMCs was significantly higher while *SMAD4* silenced. Moreover, rs12455792 T allele also increased Versican degradation via ADAMTS-4. In conclusion, this variant might promote SMCs apoptosis and proteoglycans degradation, and further facilitate the progress of TAAD. Our findings identified rs12455792 as a predictor for progression of vascular media pathological changes related thoracic aortic disorders.

1. Introduction

Aortic aneurysms and dissections are associated with high morbidity and mortality, accounting for over 152,000 deaths in the United States per annum (Benjamin et al., 2017). These are life-threatening diseases due to the predisposition for rupture. About 40% of patients with aortic dissection die immediately and have no enough time to reach a hospital. Aortic medial degeneration (AMD) is considered proceed thoracic aortic aneurysms and dissections (TAAD). AMD is histopathologically characterized by loss of smooth muscle cells (SMCs), and increased proteoglycans degradation.

SMAD4 plays a pivotal role in the pathological progression of vascular disorder (Zhang et al., 2016). *SMAD4*, a member of Smad family, is a central mediator in the canonical TGF-β signaling. It regulates biological processes important for the pathogenesis of TAAD, such as SMCs migration and proliferation, extracellular matrix degradation (Mao et al., 2012). *SMAD4*-deficient SMCs can trigger aortic wall inflammation by producing chemokines and recruiting macrophages (Zhang et al., 2016). Besides, *SMAD4* also play essential roles in cardiogenesis, blood vessel remodeling, maturation and integrity (Jiao et al., 2003; Lan et al., 2007; Qi et al., 2007; Song et al., 2007).

The TGF-β signaling network consists of a complex of ligands, receptors, and transcriptional coregulators that have important effects in vascular remodeling and matrix degradation (Jones et al., 2009). Mutations of members (*TGFBR1/2*, *FBN1*) in this network are causative for disorders hallmarked by aortic aneurysm, e.g. Marfan syndrome (MFS) or Loeys–Dietz syndrome (LDS) (Loeys et al., 2005, 2006). Mutations in a *TGF-β* ligand-*TGF-β2* gene have been recognized as a cause of TAAD with MFS (Boileau et al., 2012). *SMAD4* encodes the only co-smad in TGF-β signaling (Mao et al., 2012). The mutations of *SMAD4* gene are also important in progression of aortopathy. In Heald's retrospective study, the authors described a high prevalence (38%) of aortopathy in patients with juvenile polyposis (JPS) and hereditary hemorrhagic telangiectasia (HHT) is attributed to *SMAD4* mutations (Heald et al., 2015). Gallione and Andrabi also reported that *SMAD4* mutations could cause a combined JPS & HHT syndrome and vascular disorders (for example, aortic root dilation, multiple arteriovenous malformation)

Abbreviations: TAAD, thoracic aortic aneurysm and dissection; SMCs, smooth muscle cells; AMD, aortic medial degeneration; TGF-β, transforming growth factor-β; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SNP, single nucleotide polymorphism; 5′UTR, 5′ untranslated region.

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http://dx.doi.org/10.1016/j.ebiom.2017.06.022

(Andrabi et al., 2011; Gallione et al., 2004). Thus, it is interesting to explore the association between variants of *SMAD4* and the pathological progression of TAAD, which may shed light on the role of *SMAD4* in pathogenesis of TAAD and provide a maker for disease diagnosis.

To investigate the genetic effect of *SMAD4* on TAAD, five tagging SNPs were initially genotyped in 202 subjects and 400 healthy controls. The potential function of the significant SNP in the screening dataset was further analyzed by bioinformatic software. A series of experiments was conducted to investigate the potential molecular mechanism of the significant SNP.

2. Materials and Methods

2.1. Subjects

All the experiments involving human specimens were approved by Institutional Review Board (IRB) at the first affiliated hospital of Soochow University from January 2010 to December 2015. In the case-control study, the response rate for cases was 94% ($n = 223$) and for controls 92% ($n = 437$). Each participant has signed the written informed consent. After selecting, 202 patients with TAAD and 400 healthy controls during physical examination were enrolled in the study. All subjects were Han Chinese from eastern China. Patients with familial TAAD were exclude by the following criteria: (1) He/she had one first-degree relative with a documented TAAD history; (2) He/she had two second-degree relatives with TAAD. Controls were frequency matched by age and gender to cases.

The aortic status of subjects were evaluated by at least one type of imaging examination, such as echocardiography, angiography, CT, MRI. Each subject were interviewed in person and filled a structured questionnaire including demographic information, previous medical histories, diet, tobacco and alcohol use, weight, family history of aortopathy. Freshly frozen aortic tissues from 37 TAAD patients were obtained during the Bentall procedures or other large vessels replacement at Department of Cardiovascular Surgery, the first affiliated hospital of Soochow University. Normal aortic tissues were collected from patients who received aortic valve replacement.

2.2. Blood Sample Collection and Genomic DNA Extraction

Ethylenediamine tetraacetic acid (EDTA) anticoagulated peripheral blood samples were collected from patients before surgery and healthy controls. Genomic DNA was extracted using a RelaxGene Blood DNA System (TIANGEN biotech, Beijing, China) according to the manufacturer's instruction. The laboratory assistant was blinded to the samples about the case-control status.

2.3. SNP Selection and Genotyping

We selected 5 tagger SNPs by analyzing *SMAD4*-related Han Chinese data from 1000 Genome Project resources (http://www.1000genomes.org) using Run Tagger program in Haploview 4.2 (Broad Institute, Cambridge, MA, USA). These SNPs should meet the following criteria: (1) the minor allele frequency (MAF) > 0.05; (2) $r^2 > 0.80$ for each paired SNPs (Fig. 1B). rs12455792 (in the 5′UTR, -650C>T) is located at a transcription factor binding site, with predicted proximal transcriptional regulatory potential (http://rsnp.psych.ac.cn/) (http://snpinfo.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi). rs61751987, rs12456284, rs3819122 and rs2282544 were also predicted as functional SNPs, which located in the enhancer, chromatin interactive region or other transcriptional regulatory region.

SNPs were genotyped using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis on the MassARRAY platform (Sequenom, San Diego, CA, USA). The amplification and single-base extension primers applied in multiple PCR were synthesized by Benegene (Benegene Biotechnology, Shanghai, China) and listed in Supplement Table 1. The product of each sample was dispensed onto a 384-format SpectroCHIP with the MassARRAY Nanodispenser RS 1000. Then the MALDI-TOF MS assay was performed on a MassARRAY Compact Analyzer. Genotype calling was conducted in real time with MassARRAY RT software version 3.0. Data was analyzed with MassARRAY Typer software 4.0.3. Genotyping quality was assessed by Sanger sequencing of ~15% randomly selected samples, yielding a 100% concordance. The success rates of genotyping for these SNPs were >99%.

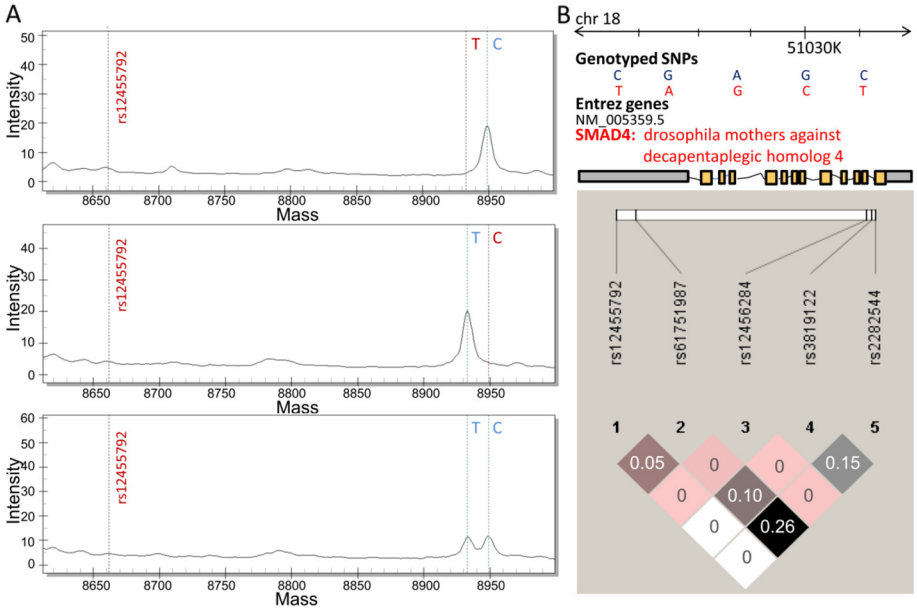

Fig. 1. Representative MALDI-TOF MS spectra of rs12455792 and linkage disequilibrium (LD) analysis of the tagger SNPs in *SMAD4* gene. (A) Genotypes of rs12455792 are determined by plotting peak intensity (y-axis) against mass (Da) (x-axis). (a) MALDI-TOF MS spectrum of a single peak at 8949.0 Da indicates homozygous genotype of CC. (b) MALDI-TOF MS spectrum of a single peak at 8932.5 Da indicates homozygous genotype of TT. (c) MALDI-TOF MS spectrum of 2 peaks at 8932.5 Da and 8949.0 Da represents heterozygous genotype of CT. (B) Pairwise LD among 5 tagger SNPs of *SMAD4*. The r^2 value in each diamond indicates the pairwise correlation between SNPs. The color scale ranges from white to black reflects lower to higher r^2 values.

Please cite this article as: Wang, Y., et al., A Functional Variant of *SMAD4* Enhances Thoracic Aortic Aneurysm and Dissection Risk through Promoting Smooth Muscle Cells Apoptosis and Proteoglycans Degradation, EBioMedicine (2016), http://dx.doi.org/10.1016/j.ebiom.2017.06.022