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Research paper

Functional characteristics of a novel SMAD4 mutation from thoracic aortic aneurysms (TAA)

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ABSTRACT

SMAD4 is as an essential mediator of the transforming growth factor β (TGF-β) signaling pathway, and dysregulated TGF-β signaling is linked with thoracic aortic aneurysms (TAAs). In this study, we functionally characterized the Smad4 S271N mutation (the mutation c. 812G > A in *Smad4* results in the amino acid substitution Ser271Asn) that was isolated from TAA individuals. We first constructed wild-type human Smad4 and Smad4 S271N plasmids. These constructs were then transiently transfected into HEK293T cells, and subsequent real-time PCR and western blotting demonstrated that wild-type Smad4 and Smad4 S271N were successfully expressed in 293T cells. We found that HEK293T cells overexpressing Smad4 S271N showed a strong increase in both cytoplasmic and nuclear Smad4 protein levels in response to TGF-β1. Although TGF-β signaling was the same in wild-type Smad4- and Smad4 S271N-transfected cells following TGF-β1 exposure, interestingly, we observed that transient Smad4 S271N expression in HEK293T cells caused a significant basal activation of TGF-β signaling. These results indicated that Smad4 may not directly induce TAA; rather it may contribute to TAA in combination with other risk factors.

1. Introduction

An aortic aneurysm (AA) is an aortic enlargement or expansion in which the aorta achieves > 1.5 times the normal arterial diameter (Lavall et al., 2012). AA is usually asymptomatic and not inherently dangerous, but an enlarged aorta presents a predisposition for tearing (known as dissection), which has high mortality and morbidity (Lindsay and Dietz, 2011). There are two types of AA: abdominal aortic aneurysm (AAA) and thoracic aortic aneurysm (TAA). Unlike AAA, TAA has little association with cardiovascular risk factors, such as atherosclerosis, but is strongly influenced by hereditary predisposition (Lindsay and Dietz, 2011; Spin, 2011). Understanding of the genetic determinants of TAA has increased dramatically in recent years (Wang et al., 2010; Gillis et al., 2013; Takeda et al., 2015; Ke et al., 2016; Lee et al., 2016; Kuang et al., 2016). An estimated 20%–40% of TAA patients have a familial form. However, until now, data on familial TAA have mainly come from Caucasian populations, and there are few reports of genetic mutations in Chinese TAA patients (Guo et al., 2011; Gillis et al., 2013; Guo et al., 2016).

Smad4 is a critical signal transducer of the transforming growth factor β (TGF-β) superfamily, which regulates a wide range of cellular processes (Euler-Taimor and Heger, 2006; Song et al., 2007). TGFβ includes three isoforms TGFβ1, TGFβ2 and TGFβ3, which combine with latent TGFβ binding proteins at basal levels and are activated by proteolytic cleavage. Activated TGFβ binds to a complex of type I and type II serine/threonine kinase receptors; type II receptors phosphorylate and activate type I receptors, which subsequently show increased ability to phosphorylate the receptor-regulated Smads (R-Smads), Smad2 and Smad3. Activated Smad2/3 form a complex with Smad4 and translocate to the nucleus, where they bind DNA via other transcription factors to regulate target gene expression (Leask, 2007). To date, mutations in genes encoding various components of the TGF-β signaling cascade (*FBN1, TGFBR1, TGFBR2, TGFB2, TGFB3, SMAD2, SMAD3* and *SKI*) have been identified in TAA (Mizuguchi et al., 2004; Gordon and Blobe, 2008; van de Laar et al., 2011). It has been reported that dysregulated TGF-β signaling plays a significant role in the pathogenesis of TAA (Lindsay and Dietz, 2011). Mutations in *Smad4* cause autosomal-dominant diseases, such as juvenile polyposis syndrome (JPS) and Myhre syndrome (Le Goff et al., 2011; D'Inzeo et al., 2012; Teekakirikul et al., 2013). However, the effect of *Smad4* mutations isolated from TAA remains unknown.

In this study, we identified the function of a novel *Smad4* mutation derived from TAA, Smad4 S271N. When Smad4 S271N was transiently transfected in human embryo kidney HEK293T cells, our results

Abbreviations: AA, aortic aneurysm; TAA, thoracic aortic aneurysms; TGF-β, transforming growth factor β; R-Smads, receptor-regulated Smads; FBN1, fibrillin-1; TGFBR1, TGF-β receptor 1; TGFBR2, TGF-β receptor 2; TGFB2, transforming growth factor beta 2; TGFB3, transforming growth factor beta 3

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demonstrated that the mutant Smad4 showed increased nuclear accumulation and also caused a significant activation of TGF-β signaling. These data indicated that Smad4 S271N may have a potential role in TAA pathogenesis.

2. Materials and methods

2.1. Information about Smad4 mutation

The characterization procedure of mutant genes can be referred to a published paper by JunGuo et al. from our team (Guo et al., 2015). Actually, several mutations including *Smad4* mutation were found by targeted exome sequencing in 146 unrelated Chinese Han patients with sporadic thoracic aortic aneurysm and dissections (STAAD), who were evaluated at Beijing Anzhen Hospital and referred for surgery repair, were recruited from the year 2011 to 2012. JunGuo et al. only reported *FBN1* mutation identified in 146 unrelated Chinese patients with STAAD. The detailed characterization procedure of *Smad4* mutation together with other newly found mutations from patients will be described in next paper. Approval for sample collection for characterization procedure was obtained from the Ethical Review Board of Beijing Anzhen Hospital, which has been stated in the published paper by JunGuo et al. The mutation c. 812G > A in *Smad4* (resulting in amino acid substitution Ser271Asn) is a novel mutation, there is no RS number.

2.2. Reagents and constructs

pcDNA3.3 vector, in frame fusion to the C-terminus of flag, was kindly provided by Prof. Han jia huai. pCMV5 CAGA-Luc and Renilla were kindly provided by Prof Chen Ye Guang. Green fluorescent vector pCMV-AC-GFP was obtained from OriGene (Beijing, China). Fast Pfu.DNA polymerase was purchased from TransGen Biotech (China). QuikChange site-directed mutagenesis kit was purchased from Agilent Technologies (CA). Lipofectamin2000 was purchased from Invitrogen (CA). DMEM medium, penicillin, streptomycin and fetal bovine serum (FBS) were all obtained from Sigma-Aldrich, Louis (MO). The human recombinant TGF-β1 isoform was from Rocky Hill (NJ, USA). NE-PER™ Nuclear and Cytoplasmic Extraction Reagents was from Thermo scientific (MA). Antibodies to Smad4, β-actin and Histone1 were all purchased from Santa Cruz biotechnology (CA). Antibodies to flag, DH5α competent cell were all acquired from Kang wei (Beijing). Luciferase assay system was purchased from Promega (WI).

2.3. Construction of wild type plasmid

Smad4 cDNA was subcloned into pcDNA vector which allows in frame fusion to the C-terminus of the tag Flag by PCR using fast Pfu.DNA polymerase and LIC by exonucleaseIII. The target cDNA was transformed into DH5α competent cell according to the manufacturer's protocol. The wild-type Smad4 plasmid was extracted from bacteria solution by manufacturer's protocol and then was selected by sequencing.

2.4. Site-directed mutagenesis, subcloning and transiently transfecting HEK293T cells

Site-directed mutagenesis was used to introduce missense mutations into the human Smad4 cDNA wt inserted in a pcDNA3.3 plasmid. The mutants S271N were constructed by PCR using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's protocol. PCR products were sequenced using an ABI3730 × 1 DNA Analyzer (Applied Biosystems Foster City, CA, USA). The sequencing results were then compared with reference sequence (GenBank, NM_005359) to verify the mutagenesis success.

The resultant constructs and green fluorescent vector pCMV6-AC-GFP were transiently transfected into HEK293T cells (cell bank of Peking Union Medical College Hospital) by lipofectamine method as recommended by the manufacturer. First, HEK293T cells were seeded at a density of 1×10^6 cells per well into six-well culture plates and transfected with 2 μg pCMV6-AC-GFP for 48 hs, transfection efficiency was monitored by determining the GFP autofluorescence in the transfected cells. HEK293T cells were then transfected with 2 μg vector pcDNA3.3 containing FLAG-tagged wild-type or mutant Smad4 cDNAs. HEK293T cells were also transfected with empty vector pcDNA3.3.

2.5. Cell cultures, quantitative real-time PCR and western blotting

HEK293T cells were cultured in DMEM medium supplemented with 10% FBS, and 100 mg/mL penicillin and 100 μg/mL streptomycin, in a humidified incubator with an atmosphere of 5% CO2 at 37 °C (Han et al., 2012). Cells were seeded at a density of 1×10^6 cells per well into six-well culture plates and were transfected with wild-type Smad4 or Smad4 S271N for 48 hs for mRNA and protein analysis. The transfected cells were incubated with TGF-β1 for 1 h or 3 hs for nuclear protein extraction and western blotting.

For quantitative real-time PCR (qRT-PCR), total RNA was extracted from the cells using a TRIzol and reverse-transcribed into cDNA using random primers with a transcriptor first strand cDNA Synthesis Kit. PCR amplifications were quantified using the SYBR Green PCR Master Mix and normalized to β-actin gene expression (Zhu et al., 2013).

For western blotting, culture cells were extracted with lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1 mM β–glycerophosphate, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 4 mg/mL aprotinin, 4 mg/mL leupeptin, 4 mg/mL pepstatin, and 1 mM PMSF (Zhu et al., 2013). Nuclear protein was extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol and then underwent western blotting as described. The protein concentration was determined using the Pierce BCA Protein Assay kit as the manufacturer's describe (Jiang et al., 2014). In total, 40 μg protein lysates were separated by 10% SDS-PAGE before transfer to nitrocellulose membranes (Bio-Rad), The membranes were incubated with primary antibodies against anti-Flag, anti-SMAD4, anti- Histone1 (1:500 diluted in TBS-T), anti-β-actin (1:1000 dilution), then stored at 4 °C overnight, then with IR Dye-conjugated secondary antibodies (1:5000, Rockland Immunochemicals, Gilbertsville, PA) for 1 h. Images were quantified by use of the Odyssey infrared imaging system (LI-COR Biosciences Lincoln, NE) (Ma et al., 2012). The protein contents were normalized to the level of β-actin. All experiments were repeated 3 times.

2.6. Luciferase assays

It is reported that dysregulated TGF-β signaling plays a very important role in the development of TAA. Smad4 is an essential protein in TGF-β signal transduction. To evaluate whether *Smad4* mutation has an effect on TGF-β signaling, we therefore conduct luciferase assays. These assays were carried out as described previously (Labbe´ et al., 1998; Yan et al., 2009; Zhao et al., 2012). HEK293T cells were transiently transfected with CAGA-luciferase (0.5 g), Renilla-luciferase (20 ng), wild-type Smad4 or Smad4 S271N (0.5 g respectively) using lipofectin technique following the manufacturer's instructions. After transfection 24 hs, cells were treated or untreated 24 hs with 10 ng/mL TGF-β1 in serum-free medium and then harvested for luciferase assay. Luciferase activities were measured by the luciferase assay system and normalized using the Renilla control vector. Each result is the mean of three different experiments.

2.7. Statistical analysis

All Data were expressed as the mean ± SEM. For all of the variables, the normality of distribution was checked by the Shapiro-Wilk