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Original Article

S1004A4 mediated TGFβ/Smad signal pathway contributes to the development of thoracic aortic dissection (TAD)

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Abstract: Thoracic aortic dissection (TAD) is a catastrophic acute disease with a high postoperative mortality, including extensive degenerative, genetic, structural and acquired disease states, and may be complicatedly by potentially life-threatening thoracic aortic ruptures. The calcium-binding protein S100A4 was frequently over-expressed in several tumors and cancer cells, and it was associated with invasion and metastasis of various cancer cells. In the present study, the effect of S100A4 on human aortic vascular smoothmuscle cells (VSMCs) and the development of TAD were investigated. The expression level of S100A4 in TAD aortic tissue was increased significantly compared with normal tissue, and the TGFβ/Samd signal pathway was activated by S100A4. The protein levels of MMP-2, MMP-9 and VCAM-1 were increased and SM-MHC and SM-α-actin were decreased in tissues of TAD patients. In vitro cell experiments showed that up-regulate the expression of S100A4 inhibits the proliferation and induces the apoptosis of VSMCs. In VSMCs, the TGFβ/Samd signal pathway was activated in S100A4 group and the protein expression of MMP-2, MMP-9, VCAM-1, SM-MHC and SM-α-actin showed a trend similar to that of TAD group. In conclusion, this study first investigated the effect of S100A4 on the development of TAD and revealed that S100A4 might promote TAD development by regulated the TGFβ/Samd signal pathway and some important molecular regulatory proteins.

Keywords: S100A4, thoracic aortic dissection, TGFβ/Smad, apoptosis

Introduction

Thoracic aortic dissection (TAD) as a serious cardiovascular disease characterized with a spontaneous tear through the intima and into the media of theaortic wall [1, 2]. Arterial blood entering the tear leading to the media split along the lengthof the vessel [3]. The outer aortic wall of TAD patient is weakened due to tearing and becomes tends to dilatation andrupture, which is usually fatal. It was reported that the mortality rate of untreated TAD cases during first week following occurrence of dissection reached 70% [4-6]. At present, despite the diagnostic and treatment techniques has made great progress, it is still associated with high morbidity and mortality and the exact mechanism underlying TAD remains unclear [7-10].

Normal aortic mainly consist of concentricarrangements of vascular smoothmuscle cells (VSMCs) and extracellular matrix (ECM) in elastic fibers, and the interaction of SMCs and ECM plays vital role for the structural and functional integrity of the aortic wall [11, 12]. A common pathogenic feature TAD is characterized by gene mutation cause aortic VSMCs dysfunction [13, 14]. TGFβ may induce fibronectin andconnective tissue growth factor expressions by activation of Smads in VSMCs, and thus promotesthe deposit of extracellular matrix [15]. Samd2 and Smad3 are involved in TGFβ signaling transduction by interaction with TGF-β receptors typeI and II (TβRI and TβRII, respectively). The nuclear phosphorylated Smad2 (pSmad2) level in aortic smooth muscle cells was increased significantly of patients with diseased aortic tissue, suggesting increased TGFβ cellular signaling [16, 17].

S100A4 is an 11 kDa calcium-binding protein that enhances metastasis of several types of

cancer cells [18]. In the extracellular, S100A4 interacts with the receptor for advanced glycation end the products (RAGE) [19]. In the cell, S100A4 interacts and regulates the function of several intracellular cytokines and signaling proteins involved in cell motility, invasiveness, growth, and angiogenesis such as muscle myosin II, nuclear factor kappa B (NF-κB), p53, TGFβ and matrix metalloproteinase 9 (MMP-9) [20-23]. Induced the expression of S100A4 is involved in human cancer cell migration and proliferation by interacting with MMP-2/9 and TGFβ/Samd signal pathway and transgenic mice overexpressing S100A4 develop occlusive pulmonary vascular changes [24-26]. In the present study, we found that S100A4 was up-regulated in the tissue of patients with TAD on both mRNA and protein levels. In addition, the overexpression of S100A4 promoted the TGFβ/Samd signal pathway induces MMP-2/9 and VCAM-1 (vascular cell adhesion molecule 1) up-regulation and significantly inhibits the expression of smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α-actin (SM-α-actin) in human aortic VSMCs.

Materials and methods

Patients and sample collection

Eleven tissue samples were obtained from patients with TAD who underwent surgical treatment at Renmin hospital of Wuhan University between October 2015 and May 2016 were enrolled in this study. TAD diagnosis was confirmed by noninvasive imaging such as helical computed tomography, magnetic resonance imaging, echocardiography and angiography [27].

Inclusion and exclusion criteria of TAD: Patients with aortic diameters ≥55 mm, as well as included patients who were previously treated with stents that developed typeI or III endoleaks. Patients with a proximal aorticneck containing thrombi or calcifications >50% of theneck diameter, an external iliac artery diameter <7 mmor a creatinine clearance <30 mL/min were excluded from the study. In addition, the study also excluded patients with Marfan syndrome, Loeys-Dietzaneurysm, aortic coarctation, or other aortic diseases. All patients had nopreoperative diagnosis of known coronary artery diseases, peripheral artery disease, diabetes, arthritis, and/or membranous nephropathy.

Nine cases of normal thoracic aorta tissue were obtained from donors who died from traffic accidents or brain death; all donors had nocardiovascular disease, and the pathology of theaortic tissue was not diseased in all cases. Data on demographic factors, weight and body mass index,smoking habits, alcohol consumption, and family history of cardiovascular disease in first-degree relatives were recorded.

All protocols involving human specimens were approved by Medical Ethical Committee of the Renmin Hospital of Wuhan University, and all patients enrolled provided informed consent.

Periaortic fat and intraluminal thrombus were trimmed away, and the samples were washed with saline to remove blood. The protein expression difference of S100A4, TGFβ1, TGFβ2, Samd2, Samd4, MMP-2/3, MMP-9, VCAM-1, SM-MHC and SM-α-actin in TAD tissue and control tissue were evaluated by western blot.

In vitro cell culture

Human aortic VSMCs were obtained from Gaining Biological (China) and cultured in 5% CO_2 and 95% humidified air atmosphere at 37°C in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin. Cells were split every 3 days to ensure logarithmic growth.

Construction of S100A4 overexpression vector and cell transfection

The S100A4 genes was amplified by primers (S100A4F: 5'-AGCTACTGACCAGGGAGCTG-3'; S100A4R: 5'-TGCAGGACAGGAAGACACAG-3') and was inserted into the corresponding site of the pGPU6/GFP/Neo vector site to construct theoverexpressionvectorpGPU6/GFP/Neo-S100-A4-Homo. Cells were seeded in cell plates and cultured until the cells were grown 70-80% confluence and then transfected with 800 ng/well overexpression plasmids using Lipofectamine 2000 (Invitrogen, USA) as specified by the manufacturer.

Quantitative real-time PCR

Total RNA was extracted from samples with Trizol reagent (TaKaRa, China), and detected by an ultraviolet spectrophotometer and agarose electrophoresis. For each sample, 1 μg RNA was reverse transcribed to obtain first-strand

Table 1. Baseline clinical characteristics of study subjects

Variable	TAD (n=11)	Control (n=9)	*P* value
Age (years)	52.09±9.98	47.3±8.27	0.051
Gender, male, n (%)	8 (72.7)	7 (77.8)	0.856
Hypertension, n (%)	7 (63.6)	4 (33.3)	<0.001**
Systolic arterial pressure (mmHg)	145.55±30.43	119.44±6.82	<0.001**
Diastolic arterial pressure (mmHg)	83.55±20.57	79.22±8.51	0.084
Mean arterial pressure (mmHg)	114.55±23.64	100.22±8.39	0.045*
Smoking, n (%)	6 (54.5)	4 (44.4)	0.135

Dates were expressed as either mean ± S.E.M or n (%). *P<0.05 or **P<0.05.

cDNA using the PrimeScript®RT reagent Kit with gDNA Eraser (TaKaRa) according to manufacturer's instructions. The primes used were: S100A4 forward primer 5'-CCAGATCCTGACTGCTGTC-3'; S100A4 reverse primer 5'-GACTCACTCAGGCACTACCC-3'; β-actin forward primer 5'-CAACTGGGACGACATGGAGAAT-3'; β-actin reverse primer 5'-CCAGAGGCGTACAGGATAGCA-3'. Reaction (20 μL total volume) contained 10 μL of 2 × SYBRPremix Ex TaqTM (TaKaRa), 0.50 μmol/L each primer and 0.2±0.02 μg of cDNA template. The following three-step qRT-PCR reaction was performed: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 20 s and elongation at 72°C for 20 s. The transcriptional levels of genes were calculated using ΔΔCt. The threshold cycle (Ct) was determined for each reaction, by using ΔΔCt method can make Ct values for eachgene of interest were normalized to the endogenous control gene (*β-actin*). For each group, three samples were measured and three technical replicates of each measurement were obtained.

Western blot analysis

Protein expression levels were analyzed by Western blot analysis and conducted using standard methods with modification [28]. For total protein extraction, cells washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (Beyotime, China) containing protease inhibitor at 4°C. For in vivo study, the tissue samples were homogenizes in RIPA lysis buffer containing protease inhibitor at 4°C. Both cell lysate and tissue lysate were centrifuged at 12000 × g for 15 min and supernatants were collected. The protein concentration was determined by BCA kit (Bioswamp, China). Equal amounts of protein (30 μg) were separated by 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto PVDF membrane (Millipore, USA) at 200 mA for 2 h. The membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (20 mmol/L Tris, 500 mmol/L NaCl, and 0.05% Tween 20). Subsequently, the membrane was incubated with primary antibodies against S100A4, TGFβ1, TGFβ2, Samd2, pSamd2, Samd4, pSamd4, MMP-2/3, MMP-9, VCAM-1, SM-MHC and SM-α-actin overnight at 4°C. Anti-β-actin antibody was selected as internal reference. Then, the membranes were washed with Tris-buffered saline and incubated in biotinylated goat anti-rabbit IgG secondary antibody for 2 h at room temperature. Immunoreactivity was visualized by colorimetric reaction using ECL substrate buffer (Millipore, Massachusetts, USA). Membranes were scanned with Gel Doz EZ imager (Bio-rad, USA).

MTT assay

Cell proliferation was evaluated with MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) colorimetric method. The human aortic VSMCs were seeded in 96-well plate and transfected with overexpression vectors or empty vector. MTT (20 μL) was added into each well after transfection for 24 h, 48 h and 72 h, respectively, and incubated for 2-4 h at 37°C. After the purple precipitate was visible, the medium was removed and 150 μL DMSO was added to each well. After shaking at low speed for 10 min, the absorbance was recorded at 570 nm. For each detect, the total procedure was repeated 3 times.

Cell apoptosis assay

Apoptosis analysis of human aortic VSMCs was performed with the AnnexinV-FITC/PI (propidium iodide) flow cytometry kit (BD) according to the manufacturer's instructions. Cells were transfected with overexpression vector or empty vector of S100A4 using Lipofectamine 2000. After cultured for 48 h, cells were washed with ice-cold PBS three times and resuspend 200 μL of binding buffer at a concentration of 1 × 10^6 cells/mL. 10 μL Annexin V-FITC and 10 μL PI were added and cells were incubated for 30 min at 4°C in the dark. Finally, 300 μL binding

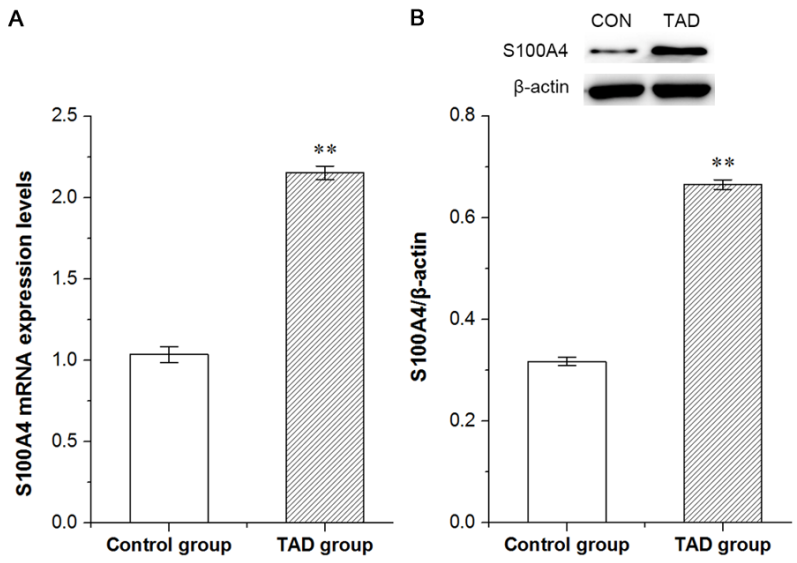

Figure 1. S100A4 was increased in the aortic tissues of TAD patients. A. The mRNA expression level of S100A4 in TAD and normal tissues. B. The protein levels of S100A4 in TAD and normal tissues. All values are expressed as the mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; TAD: TAD group.

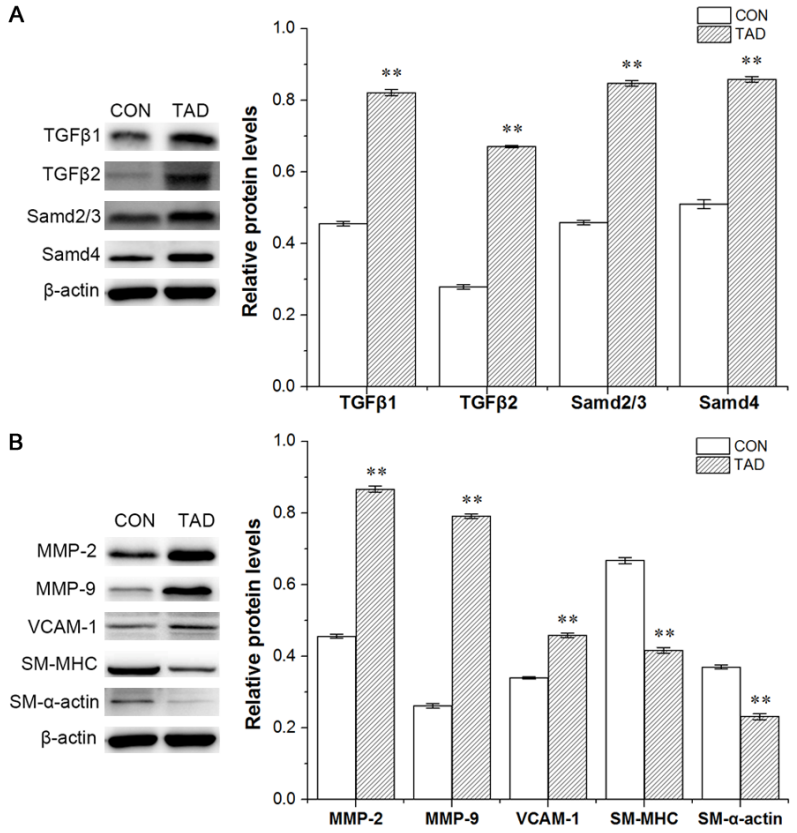

Figure 2. Abnormal aortic tissue composition. A. TGFβ/Smad signal pathway was detected using western blot. B. The levels of MMP-2, MMP-9, VCAM-1, SM-MHC and SM-α-actin from TAD and normal tissue were evaluated by western blot. All values are expressed as the mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; TAD: TAD group.

buffer was added and analyzed by flow cytometry (Beckman Coulter, Cytomics FC 500, CA) within 1 h.

Statistical analysis

The statistical differences of the experimental data were evaluated by Dunnett's one-way analysis of variance (ANOVA) using SPSS 19.0 software package. Independent sample t-test was used for mean comparison between 2 groups. Differences were considered as statistically significant at $p<0.05$ and very significant at $p<0.01$. All results were expressed as mean ± S.E.M.

Results

Baseline clinical characteristics

The clinical features of the subjects were shown in **Table 1**. A total of 11 TAD patients (age, 31-62 years) and 9 healthy persons (age, 34-60 years) were studied. The baseline subjectcharacteristics listed for the 2 groups were accuratelymatched for age and gender. In all groups, previous cardiovascular complications were absent. Comparing these 2 groups, we found that hypertension, systolic arterial pressure and mean arterial pressure in TAD patients were significantly higher than that of normal control group (P<0.01 or P<0.05).

Expression levels of S100A4 were increased in TAD tissues

RT-qPCR and western blot were used to evaluate the

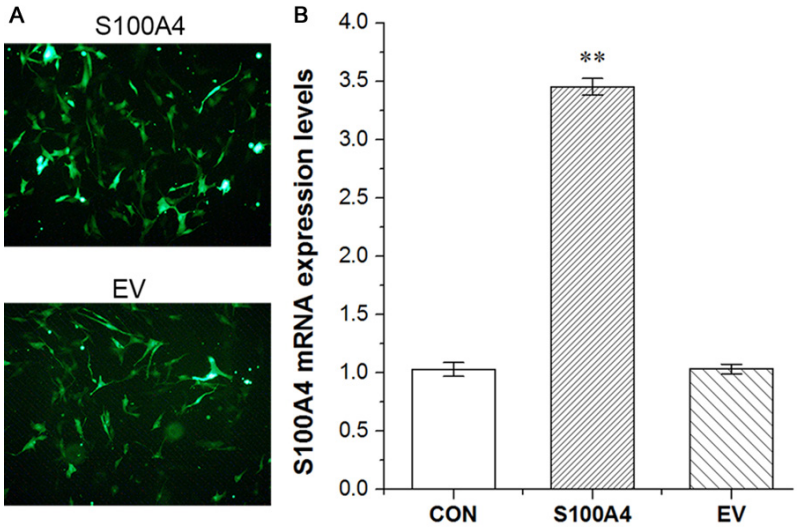

Figure 3. Expression levels of S100A4 in human aortic VSMCs. A. The overexpression vector of S100A4 and empty vector were insert in VSMCs and detected by fluorescence microscope. B. The mRNA level of S100A4 in VSMCs after transfect with S100A4. All values are expressed as the mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; S100A4: transfect S100A4 overexpression vector group; EV: transfect empty vector group.

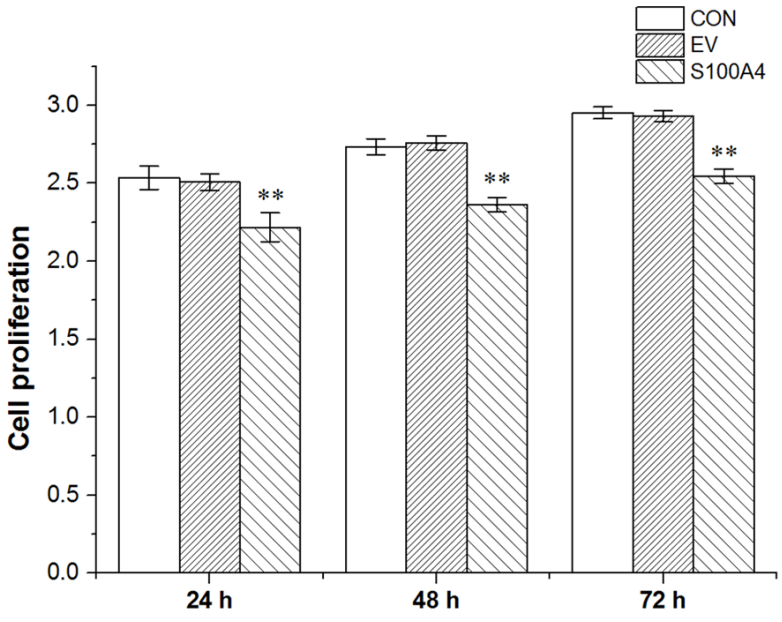

Figure 4. S100A4 inhibited human aortic VSMCs proliferation, as measured by MTT. The proliferation of VSMCs with upregulate S100A4 was lower than control group. Data are shown by mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; S100A4: transfect S100A4 overexpression vector group; EV: transfect empty vector group.

S100A4 expression levels in TAD patients and normal tissues. The results was shown in **Figure 1**, in the tissue of patients with TAD, the mRNA and protein levels of S100A4 were increased significantly compared with normal tissue.

TGFβ/Smad signal pathway and TAD related protein levels

In the present study, the TGFβ/Smad signal pathway related protein levels (TGFβ1, TGFβ2, Samd2/3 and Samd4) were detected by western blot and the results were shown in **Figure 2A**. In the TAD group, the protein levels of TGFβ1, TGFβ2, Samd2/3 and Samd4 were increased significantly compared with control group.

In order to reveal the mechanism of TAD, the protein levels of MMP-2, MMP-9, VCAM-1, SM-MHC and SM-α-actin were also detected using western blot. Compare with normal tissue, we noted an increase of MMP-2, MMP-9 and VCAM-1 and a reduction of SM-MHC and SM-α-actin in the aortic tissue of TAD patients (**Figure 2B**).

Overexpression of S100A4 in human aortic VSMCs

The S100A4 overexpression vector and empty vector were successfully transfected into VSMCs and the S100A4 mRNA and protein levels in the S100A4 group were higher than that of the control group (**Figure 3**).

The viability of VSMCs was suppressed by overexpression of S100A4

The proliferation of VSMCs was measured by MTT. Three groups were performed: CON (control group), EV (transfection of empty vector) and S100A4 group (transfection of expression vector of S100A4). The results showed that the proliferation of cells in S100A4 group was lower than that of the control group (**Figure 4**).

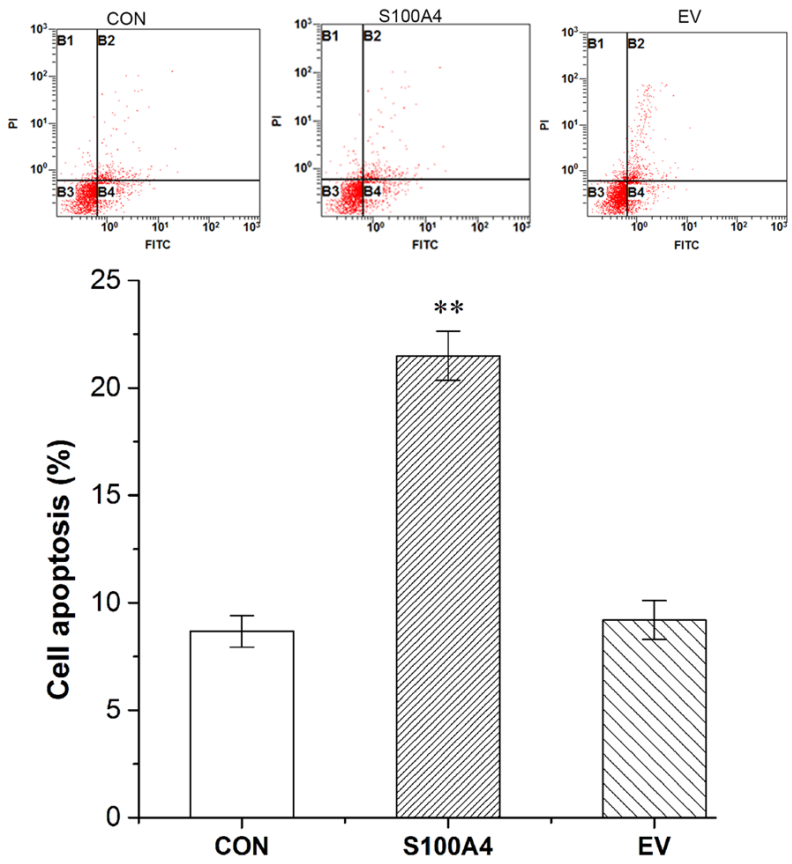

Figure 5. The upregulation of S100A4 induced apoptosis in human aortic VSMCs. The percentage of apoptotic VSMCs in S100A4 group was significantly higher than that of control group. Data are shown by mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; S100A4: transfect S100A4 overexpression vector group; EV: transfect empty vector group.

Overexpression of S100A4 in VSMCs induced cellapoptosis

The apoptosis of human aortic VSMCs was measured by AnnexinV-FITC/PI flow cytometry. As shown in **Figure 5**, the percentage of apoptotic cells in S100A4 group was significantly higher than that of the control group which indicated that S100A4 promotes the apoptosis of human aortic VSMCs.

S100A4 effects on VSMCs are TGFβ/Smad signal pathway dependent

The signaling processes triggering cellular effects of S100A4 are not well understood, but at least in human aortic VSMCs include TGFβ/Smad pathway. Therefore, the effect of S100A4 on the expression of TGFβ1, TGFβ1, Smad2/3 and Samd4 and their phosphorylation state was detected by western blot. The results revealed increased TGFβ1, TGFβ1, Smad2/3 and Samd4 phosphorylation in VSMCs with up-regulate S100A4 (**Figure 6**).

S100A4 regulate TAD-related protein expression levels

In the present study, the protein levels of MMP-2, MMP-9, VCAM-1, SM-MHC and SM-α-actin in human aortic VSMCs were detected by western blot which involved TAD incidence. The results were shown in **Figure 7**, in the S100A4 group the protein levels of MMP-2, MMP-9 and VCAM-1 were higher than that of the control group, and the protein levels of SM-MHC and SM-α-actin were lower than that of the control group.

Discussion

S100A4 belongs to the S100 family of EF-hand calcium-binding proteins which characteristic as several functions including cell motility, angiogenesis, promoting metastasis and neuronal [29-32]. Previous studies indicated that S100A4 can controls the invasive potential of human cancer cells through regulates the expression of MMP-2 or MMP-9 and as a critical mediator of invasion interaction with TGFβ signaling pathway in endometrial cancer [33, 34]. It was also reported that S100A4 can increased the secretion of MMPs from endothelial cells and fibroblasts, and the expression of S100A4 was increased in smooth muscle cells of atherosclerotic lesions and pulmonary vascular diseases [35, 36]. S100A4 is endogenously expressed in VSMC and promotes VSMC dedifferentiation [37]. In the present study we indicated that the expression of S100A4 was increased significantly in the tissue of TAD patients and the MMP-2, MMP-9 and VCAM-1 were increased significantly, the SM-MHC and SM-α-actin were decreased significantly. Furthermore, Samd2/3, Samd4 and the TGFβ1 and TGFβ2 were increased in TAD aortic tissue. We speculate that enhance

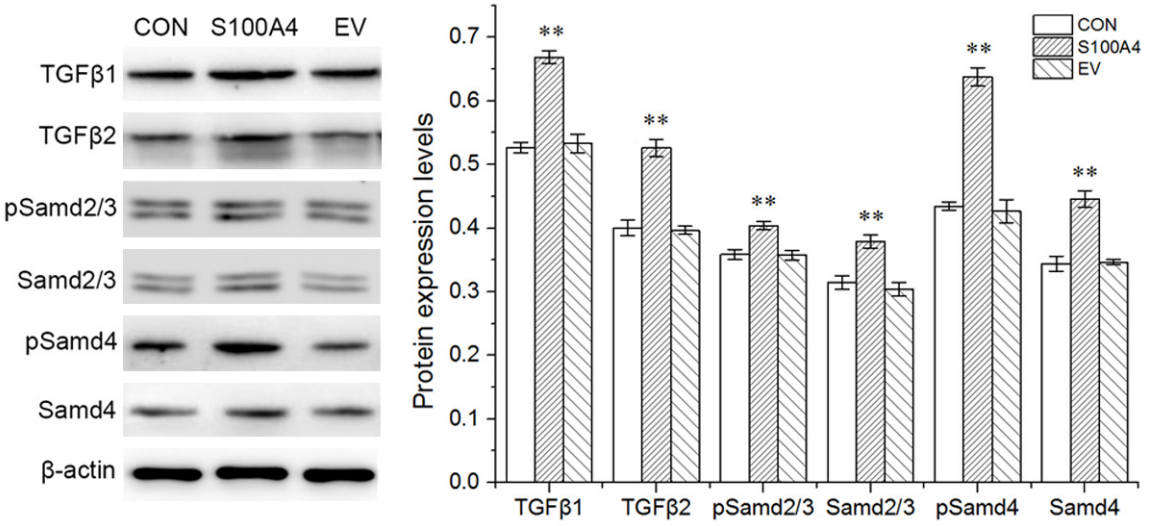

Figure 6.TGFβ/Smad signal pathway was activated by S100A4. The TGFβ1, TGFβ2, Samd2/3, pSamd2/3, Samd7 and pSamd7 were detected by western blot. Bands were quantified using Quantity One 5.0 and the fold changes in each protein to β-actin ratio are shown. Data are shown by mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; S100A4: transfect S100A4 overexpression vector group; EV: transfect empty vector group.

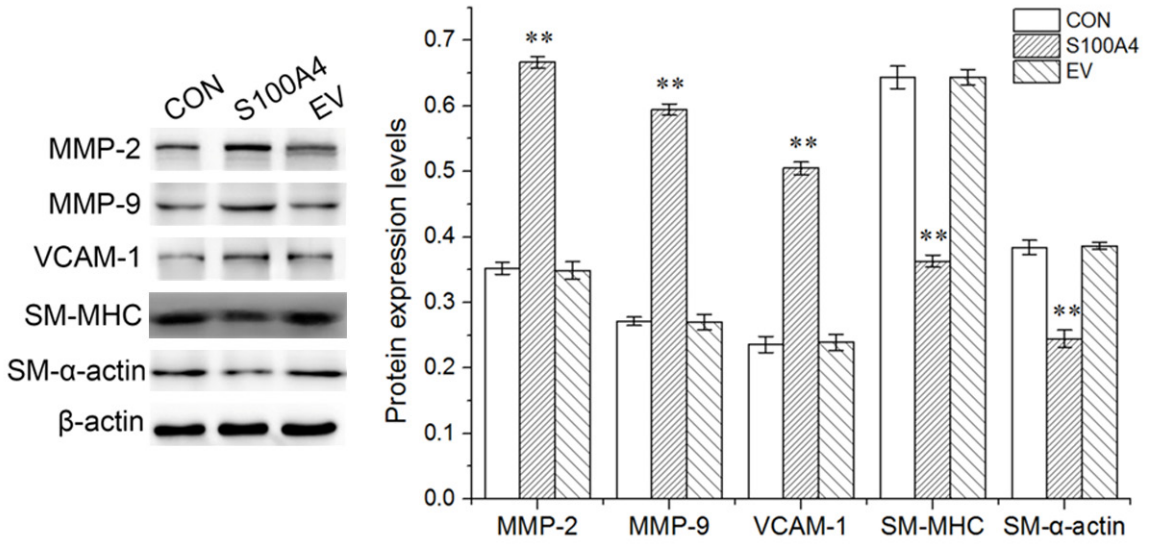

Figure 7. Protein analysis of VSMCs with overexpressed S100A4. The protein levels of MMP-2, MMP-9, VCAM-1, SM-MHC and SM-α-actin were detected by western blot. Bands were quantified using Quantity One 5.0 and the fold changes in each protein to β-actin ratio are shown. Data are shown by mean ± S.E.M (n=3). **P<0.01 as compared with control group. CON: control group; S100A4: transfect S100A4 overexpression vector group; EV: transfect empty vector group.

the expression of S100A4 might promote the development of TAD by stimulate the release of MMP-2/9 to activate the TGFβ/Samd signaling pathway.

In order to verify our suggestion, we found that up-expressed the S100A4 in human aortic VSMCs induced cell apoptosis and inhibited cell proliferation through upregulate the expression of MMP-2 and MMP-9 and activated the TGFβ/Samd signaling pathway. Studies on TGFβ/Samd signaling pathway revealed that Samd4 and Samd2/3 involved in cell matrix contraction induced by TGFβ [38]. In aortic dissection, Samd2 and Samd4 may lead to matrix degradation by attenuating laminin expression

and increasing expression of matrix metalloproteinases and making the balance between deposition and degradation shift to the latter by promote TGFβ/Samd signaling pathway. MMP-2 and MMP-9 belongs to the family of zinc dependent enzymes which play an important role in extracellular matrix degradation and involved in pathogenesis of some diseases tissue remodeling [39, 40]. It was reported that MMP-2, MMP-9 and membrane typeI MMP to be able to cleave and active the latent TGFβ [41]. In the present research we found that the protein levels of MMP-2 and MMP-9 were increased in human aortic VSMCs which insect overexpression S100A4 vector, thus activate TGFβ/Samd signaling pathway. Similar to previously described, in abdominal aorticconstriction rat models, cardiac hypertrophy induced the TGFβ1 and Samd2/3 expression levels increased significantly [42].

VCAM-1 as a specific adhesion molecule was secreted by endothelial cells which play an important role in defining the types of leukocytes recruited and mediating mononuclear adhesion [43]. Hofmann et al. reported that in S100A12 transgenic mice, the protein levels of VCAM-1 was increased and the protein levels of SM-MHC and SM-α-actin was decreased which demonstrated that S100A12 modulate aortic wall remodeling and induce pathogenic in aortic aneurysms [44]. In the present study, VCAM-1 was upregulated and SM-MHC and SM-α-actin were downregulated in TAD tissue. Furthermore, cultured human aortic VSMCs transfected with overexpression S100A4 vector had induces the expression of VCAM-1 and inhibits the expression of SM-MHC and SM-α-actin, suggesting that upregulate S100A4 induced the pathological remodeling of the aorta with disarray of elastic fibers. In vitro, overexpression of S100A4 inhibits the proliferation and induces the apoptosis of human aortic VSMCs.

In conclusion, the present study firstly demonstrated that S100A4 level is significantly increased in the tissue with TAD, and its overexpression in human aortic VSMCs can induced the expression of MMP-2 and MMP-9 which activated the TGFβ/Samd signaling pathway and finally leading to apoptosis and inhibiting cell proliferation. We inferred that S100A4 might promote TAD development by release some important molecular regulatory proteins to activate the TGFβ/Samd signal pathway.

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Disclosure of conflict of interest

None.

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