ORIGINAL ARTICLE

Rev Bras Cir Cardiovasc 2011;26(3):393-403

Transforming growth factor-ß/Smad signaling function in the aortopathies

Fator transformador de crescimento-ß/Smad como via de sinalização em aortopatias

Shi-Min Yuan[1], Jun Wang[2], Xiao-Nan Hu[3], De-Min Li[4], Hua Jing[5]

DOI: 10.5935/1678-9741.20110014

RBCCV 44205-1295

Abstract

Objectives: **Transforming growth factor (TGF)-ß/Smad signaling pathway in aortic dissection patients and normal subjects has not been previously described. The present study was designed to evaluate the TGF-ß/Smad signaling expressions in the patients with acute type A aortic dissection in comparison with those in the patients with thoracic aortic aneurysm and with coronary artery disease, and (or) the healthy subjects.**

Methods: **Consecutive surgical patients for acute type A aortic dissection (20 patients), aortic aneurysm (nine patients) or coronary artery disease (20 patients) were selected into this study. Blood samples (4 ml) were obtained from the right radial arterial indwelling catheter after systemic heparinization prior to the start of cardiopulmonary bypass in the operating room. Twenty-one young healthy volunteers without underlying health issues who donated forearm venous blood samples (4 ml) were taken as control. The surgical specimens of the aortic tissues were obtained immediately after they were severed during the operations of the replacement of the aorta in the patients with aortic dissection or aortic aneurysm. In patients receiving coronary artery bypass grafting, the tiny aortic tissues were taken when the punch holes of the proximal anastomosis on the anterior wall of the ascending aorta were made. The aortic tissues were for RNA, protein, or supernatant preparations until detection of TGF-ß$_1$ mRNA by quantitative real-time reverse transcription polymerase chain reaction, of TGF-ß$_1$, TGF-ß receptor I, Smad2/3, Smad4 and Smad7 by Western blot, and of TGF-ß$_1$ by enzyme-linked immunosorbent assay, respectively. In particular, the linear correlations of the relative grayscales between different proteins of each group, and those correlations between the quantitative TGF-ß$_1$ by enzyme-linked immunosorbent assay and the time interval from the onset to surgery or the maximal dimensions of the aorta of the aortic dissection group were assessed.**

Results: **Quantitative real-time reverse transcription polymerase chain reaction showed that TGF-ß$_1$ mRNA were upregulated in all surgical groups (1.59 ± 0.33 vs. 1.45 ± 0.34 vs. 1.48 ± 0.48, $P > 0.05$). Western blot revealed that the expressions of TGF-ß$_1$, TGF-ß receptor I, Smad2/3, Smad4 and Smad7 were positive in the aortic tissues of all three investigated groups. Of the quantitative relative grayscales, a significant reverse correlation was noted between TGF-ß$_1$ and Smad2/3 (Y = -0.8552X + 1.6417, r = -0.759, $P < 0.0001$), and a close direct correlation between Smad4 and Smad7 (Y = 0.5905X + 0.2805, r = 0.781, $P < 0.0001$) in the Aortic Dissection Group. In the Aortic Aneurysm Group, Smad4 and Smad7 were also closely correlated (Y = 0.5228X + 0.1642, r = 0.727, $P = 0.026$), and in the Coronary Artery Disease Group, TGF-ß$_1$ and Smad7 were much significantly correlated (Y = 0.5301X + 0.5758, r = 0.917, $P = 0.004$). By enzyme-linked immunosorbent assay, TGF-ß$_1$ level of the aortic tissue was lower in the aortic dissection than in the aortic aneurysm and coronary artery disease groups with no statistical significance (319.52 ± 129.21 pg/mg protein vs. 324.09 ± 49.70 pg/mg protein vs. 304.15 ± 29.39 pg/mg protein, $P > 0.05$). The plasma TGF-ß$_1$ levels were 1158.30 ± 11.54 pg/ml, 1170.27 ± 8.26 pg/ml, 1225.00 ± 174.42 pg/mL and 1160.25**

1. MD, PhD, Department of Cardiothoracic Surgery; Affiliated Hospital of Taishan Medical College, Taian, Shandong Province, People's Republic of China.
2. MD. Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University.
3. Prof. Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University.
4. Prof. Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University.
5. Prof. Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University.

This work was carried out at Department of Cardiothoracic Surgery, Affiliated Hospital of Taishan Medical College, Taian 271000, Shandong Province; and Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University, Nanjing 210002, Jiangsu Province, People's Republic of China

Correspondence:
Shi-Min Yuan, Department of Cardiothoracic Surgery, Affiliated Hospital of Taishan Medical College, Taian 271000, Shandong Province, People's Republic of China.

Article received on February 8th, 2011
Article accepted on May 30th, 2011

± 13.01 pg/mL in the four groups, respectively, showing significant intergroup differences (*P* < 0.05). No significant correlation was found between the aortic or plasma TGF-ß$_1$ levels and the time interval from the onset to surgery or the maximal dimensions of the aorta in the patients of the aortic dissection group.

Conclusions: **Aortic dissection, aortic aneurysm and atheroslerosis might be associated with an enhanced TGF-ß/Smad signaling function, with aortic dissection exhibiting a less prominent upregulation. It might have implications for downstream signal activation presumably translating into matrix degradation in the condition of aortic dissection in comparison to matrix deposition in aortic aneurysm and coronary artery disease.**

Descriptors: **Aorta. Aorta, Thoracic. Smad Proteins. Transforming Growth Factor beta1.**

Resumo

Objetivos: **Fator transformador de crescimento (TGF) -ß/Smad como via de sinalização em casos de dissecção aórtica e indivíduos normais não foi descrito anteriormente. O presente estudo foi elaborado para avaliar as expressões TGF-ß/Smad como via de sinalização nos pacientes com dissecção aguda da aorta, em comparação com que nos pacientes com aneurisma da aorta torácica e com doença arterial coronariana, e (ou) com indivíduos saudáveis.**

Métodos: **Pacientes cirúrgicos consecutivos para o tipo A de dissecção aguda da aorta (20 pacientes), aneurisma da aorta (nove pacientes) ou doença arterial coronária (20 pacientes) foram selecionados para este estudo. Amostras de sangue (4 ml) foram obtidas a partir do cateter arterial radial direito após heparinização sistêmica antes do início da circulação extracorpórea na sala de cirurgia. Vinte e um voluntários jovens e saudáveis, sem problemas de saúde subjacentes que doaram amostras de sangue venoso do antebraço (4 ml) foram tomados como controle. Os espécimes cirúrgicos de tecidos aórtico foram obtidos imediatamente após terem sido cortados durante as operações da substituição da aorta nos pacientes com dissecção aórtica ou aneurisma da aorta. Em pacientes que foram submetidos à cirurgia de revascularização miocárdica, os tecidos da aorta minúsculos foram obtidos quando os orifícios da anastomose proximal na parede anterior da aorta ascendente foram feitos. Os tecidos da aorta foram para a RNA, proteínas ou preparações sobrenadantes até a detecção de TGF-ß$_1$ mRNA pela reação de transcrição reversa quantitativa em tempo real em cadeia da polimerase, de TGF-ß$_1$, receptor I de TGF-ß, Smad2/3, Smad4 e Smad7 por Western Blot, e de TGF-ß$_1$ pelo teste de ELISA, respectivamente. Em particular, as correlações lineares dos tons de cinza relativo entre diferentes proteínas de cada grupo, e aquelas correlações entre os quantitativos TGF-ß$_1$ pelo teste de ELISA e o intervalo de tempo desde o início da cirurgia ou as dimensões máximas da aorta do grupo de dissecção da aorta foram avaliados.**

Resultados: **Reação de transcrição reversa quantitativa em tempo real em cadeia da polimerase mostrou que o mRNA TGF-ß$_1$ foi supra-regulado em todos os grupos cirúrgicos (1,59 ± 0,33 vs 1,45 ± 0,34 vs 1,48 ± 0,48, *P*> 0,05). Western blot revelou que as expressões de TGF-ß$_1$, receptor I de TGF-ß, Smad 2/3, Smad4 e Smad7 foram positivos nos tecidos da aorta de todos os três grupos investigados. Dos tons de cinza quantitativa relativa, uma correlação inversa significativa foi observada entre TGF-ß$_1$ e Smad 2/3 (Y = 1,6417 +-0.8552X, r = -0,759, *P* <0,0001), e uma estreita correlação direta entre Smad4 e Smad7 (Y = 0,5905X + 0,2805, r = 0,781, *P* <0,0001) no Grupo de dissecção da aorta. No Grupo de Aneurisma da Aorta, Smad4 e Smad7 estavam também estreitamente correlacionados (Y = 0,1642 + 0.5228X, r = 0,727, *P* = 0,026), e no Grupo de Coronariopatias, TGF-ß$_1$ e Smad7 estavam muito significativamente correlacionados (Y = 0.5301X + 0,5758, r = 0,917, *P* = 0,004). Por meio do teste de ELISA, o nível de TGF-ß$_1$ do tecido aórtico foi menor na dissecção aórtica do que no aneurisma da aorta e nos grupos de doença arterial coronariana sem significância estatística (319,52 ± 129,21 proteína pg/mg vs 324,09 ± 49,70 pg/mg de proteína vs 304,15 ± 29,39 proteína pg/mg, *P*> 0,05). Os níveis de plasma TGF-ß$_1$ foram 1.158,30 ± 11,54 pg/ml, 1.170,27 ± 8,26 pg/ml, 1225,00 ± 174,42 pg/mL e 1.160,25 ± 13,01 pg/mL nos quatro grupos, respectivamente, mostrando diferenças significativas entre grupos (*P* <0,05). Nenhuma correlação significativa foi encontrada entre a aorta ou níveis plasmáticos de TGF-ß$_1$ e o intervalo de tempo desde o início da cirurgia ou as dimensões máximas da aorta nos pacientes do grupo de dissecção da aorta.**

Conclusões: **A dissecção aórtica, aneurisma da aorta e arterosclerose podem estar associadas a uma via de sinalização TGF-ß/Smad elevada, com dissecção aórtica exibindo uma suprarregulação menos proeminente. Isso poderia ter implicações para a ativação do sinal a jusante, presumivelmente, traduzindo-se em degradação da matriz na condição de dissecção da aorta, em comparação com deposição de matriz no aneurisma da aorta e doença arterial coronariana.**

Descritores: **Aorta. Aorta Torácica. Fatores Transformadores de Crescimento. Proteínas Smad.**

INTRODUCTION

The transforming growth factor (TGF)-ß family, including TGF-ß$_1$, TGF-ß$_2$, and TGF-ß$_3$, is a group of pleiotropic secreted cytokines with a broad spectrum of biologic functions. Of them, TGF-ß$_1$ is a secreted protein with many cellular functions, including cell growth, cell proliferation, cell differentiation and apoptosis. In humans, TGF-ß$_1$ is encoded by the TGF-ß$_1$ gene, either stimulating or inhibiting cell growth depending upon the cellular context [1]. TGF-ß$_1$ can modulate cell differentiation and proliferation in an auto- or paracrine manner [2]. In vascular smooth

muscle cells, TGF-ß may upregulate fibronectin and connective tissue growth factor expressions via activation of Smads, and thus promote the deposit of extracellular matrix [3]. The receptors including TGF-ß receptor (TßR) I and TßRII are glycoproteins of 55 kDa and 70 kDa, respectively, with core polypeptides of 500-570 amino acids [4]. Smads are molecules of 42-60 kDa, with two homology domains at the amino and carboxy terminals termed as terminal Mad-homology domains MH1 and MH2 [5]. Smads can be divided into three classes, receptor-regulated Smads (R-Smads), co-mediator Smads (Co-Smads) and inhibitory Smads (I-Smads). R-Smads are directly phosphorylated and activated by TßRI kinases. Smad2 and Smad3 are involved in TGF-ß signaling transduction and Smad1, Smad5 and Smad8 in bone morphogenic protein signaling transduction [6]. Smad4 was termed as DPC4 (deleted in pancreatic carcinoma locus 4), which was a candidate tumor suppressor gene in chromosome 18q21 frequently subjected to mutation or deletion in pancreatic cancer [7]. Smad2/3 and Smad4 are just the factors of the signaling pathway favoring the deposit of extracellular matrix mediated by TGF-ß [3]. Smad6 and Smad7 inhibit TGF-ß signaling as negative regulators [6].

Elevated TGF-$ß_1$ mRNA was noted in alveolar macrophages of lung tissue from patients with idiopathic pulmonary fibrosis [8], in the hepatic tissue of experimental alcoholic hepatic disease [9], and in the kidney of chronic allograft nephropathy characterized by fibrosis [10]. Many human malignancies including ovarian cancer [11], hepatocellular carcinoma and prostate cancer [12], were associated with overexpressions of TGF-$ß_1$ mRNA and protein, showing close relations to the progress of the disease [11]. Experiments on mammary cancer demonstrated absence of TGF-$ß_1$ reactivities resulted from TßR II or Smad4 genic products [13]. Studies have suggested that colon cancer might be associated with mutations of TßRII, Smad2 or Smad4 resulting in a poor response to TGF-ß stimulus [5].

Aortopathies including aneurysm, dissection, and rupture of the aorta, is a pathological process incorporating vascular damage, repair and remodeling [14,15]. This complex process may incorporate enhanced TGF-ß signaling function and damaged TGF-ß receptors [4]. In either nontransmural infarct rat model [16] or myocardial infarct patients [17], TGF-$ß_1$ mRNA expressions were increased by 2-4 folds 2-10 days after infarction. In the atherosclerotic lesions, TGF-ß was taken as a vascular protecting agent, while TßRs might be adverse factors in angioplasty as it has been observed that TGF-$ß_1$ increased 10 folds and TßRII increased 3 folds within 24 hours following vascular damage, and activin receptor-like kinase 5 increased twice 8 hours after arterial damage [18]. Even though TGF-ß signaling in thoracic aortic aneurysm of different etiologies (Marfan's syndrome, bicuspid aortic valve, or degenerative) has been sufficiently investigated [14,19,20], however, the TGF-ß/Smad signaling pathway in aortic dissection has not been previously described, and moreover the exact mechanisms of TGF-ß/Smad signaling responsible for the development of these aortic disorders still remain uncertain [5]. The present study was designed to evaluate the TGF-$ß_1$ signaling function of aortic dissection in comparison to aortic aneurysm, coronary artery disease, and healthy individuals by way of biomolecular studies.

METHODS

Patients and sampling

From October 2008 to March 2010, consecutive surgical patients for acute type A aortic dissection (20 patients), aortic aneurysm (nine patients) or coronary artery disease (20 patients) who had blood samples and/or surgical specimens of the aortic tissues available were selected randomly into this study, while the Marfan patients were excluded. The surgical patients were comparable in terms of their age and gender. Blood samples (4 ml) were obtained from the right radial arterial indwelling catheter after systemic heparinization prior to the start of cardiopulmonary bypass in the operating room. Twenty-one young healthy volunteers without underlying health issues donated forearm venous blood (4 ml) as control samples. Blood samples were centrifugated at 3000 × g for 5 min, and plasma was collected and stored at -80°C until detection. The surgical specimens of the aortic tissues were obtained immediately after they were severed during the operations of the replacement of the aorta in the patients with aortic dissection or aortic aneurysm. In patients receiving coronary artery bypass grafting, the tiny aortic tissues 0.2~0.4 cm in size were taken when the punch holes of the proximal anastomosis on the anterior wall of the ascending aorta were made. The aortic tissues were stored at -80°C, and were thawed for RNA, protein, or supernatant preparations until detection of TGF-$ß_1$ mRNA by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), of TGF-$ß_1$, TßRI, Smad2/3, Smad4 and Smad7 by Western blot, and of TGF-$ß_1$ by enzyme-linked immunosorbent assay (ELISA), respectively. The patients' demographics were listed in Table 1.

RT-PCR

RNA samples were treated with DNase I to remove genomic DNA contamination before reverse transcription processing. A total of 2-5 μg of RNA from each sample was reverse transcribed into cDNA using the SuperScript™ III first-strand synthesis system (Invitrogen) according to the manufacturer's suggested protocol. Quantitative RT-PCR

Table1. Demographic data of the studying subjects.

Variables	Aortic Dissection	Aortic Aneurysm	Coronary Artery Disease	Healthy Control
Case, n	20	9	21	21 (plasma) 2 (plasma)
Female gender, n	2	3	2	
Age, year	53.78 ± 9.67	46.20 ± 11.16	60.33 ± 4.87	28.17 ± 2.61 (plasma)
Symptom, n	Chest pain (18), chest distress (2)	Chest pain (3), chest distress (2), palpitation (1), laryngeal discomfort (1), abdominal pain (1), asymptomatic (1)	Chest pain (21)	
Hypertension, n	16	6	18	
Diabetes millitus, n	2	0	9	
Renal failure	2	0	0	
Cardiovascular medication, n	12	6	18	
Smoker, n	5	2	12	
Operation, n	Replacement of the aorta (ascending/ arch/descending) with/without aortic valve replacement/ stent graft deployment (20)	Replacement of the ascending aorta (3), Replacement of the ascending aorta and aortic valve (2), aortic arch replacement (1), thoracic and abdominal aorta replacement (1), descending aorta replacement (1), Bentall procedure (1)	Off-pump coronary artery bypass (15), coronary artery bypass grafting (5), beating heart coronary revascularization (1)	
Disease course, month	0.13 ± 1.66	62.82 ± 168.61	41.88 ± 49.74	—
Survival,%	100	85	100	—

reactions were designed and prepared with a KeyGen reaction kit in a final volume of 20 µl containing 1 µl of reverse-transcribed total RNA, 2 µl of primers, and 10 µl of KeyGen Real-time PCR Master Mix (SYBR Green) (KeyGEN Bio, Nanjing, China). PCR reactions were carried out in capillaries in a DA7600 LightCycler instrument (Da An Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, Guangdong, China) and were cycled 40 times. The primers of TGF-ß$_1$ were designed and synthesized by KeyGEN Bio, Nanjing, China as sense 5'-CAAGCAGAGTACACACAGCAT-3' and antisense 5'-TGCTCCACTTTTAACTTGAGCC-3', along with the those of the internal control GAPDH as sense 5'-GGAAGGTGAAGGTCGGAGTCA-3'; and antisense 5'-GTCATTGATGGCAACAATATCCACT-3'. The thermal cycling conditions consisted of a pre-incubation for 5 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C and extension for 30 s at 72°C, and a final extension for 10 min at 72°C. All experiments were done in triplicate to verify the results. The relative expression of TGF-ß$_1$ mRNA to GAPDH mRNA was calculated.

Western blot

Protein extracts (10 mg) of the aortic tissue were denatured in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and separated by 12% SDS-PAGE. Proteins were transferred to a microporous polyvinylidene difluoridemembrane (PVDF) membrane using an electroblotting apparatus and incubated for 1 h at room temperature with 0.5% bovine serum albumin. Membranes were stained with Poinceau S dye, to check for equal loading and homogeneous transfer. The following primary antibodies were utilized: TGF-ß$_1$ (Y369) (Bioworld Technology, Inc., Louis Park, MN, USA), TßRI (E161) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad2/3 (S2) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad4 (L43) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad7 (M09) (Abgent Primary Antibody Company, 10239 Flanders Court, San Diego, CA 92121, USA). Filters were washed and developed using an enhanced chemiluminescence (ECL) system (Amersham Life Science). The optical densities were obtained by scanning densitometry, after normalization for nuclear or

cytoplasmatic housekeeping gene product (ß-actin). The grayscales of the graphs were analyzed using Quantity One software (BIO-RAD Laboratories). Relative grayscales in contrast to those of ß-actin were calculated and analyzed.

ELISA

The expression of TGF-$ß_1$ was determined with commercially available ELISA kit (Human TGF-$ß_1$ ELISA Kit, Cat number: KGEHC107b, KeyGen Biotech Co. Ltd., Nanjing, China) for the detection of the plasma and aortic tissue supernatant by sandwich ELISA according to specialized procedures described in the instructions for users of the product.

Statistics

Data were expressed as mean ± standard deviation. Intergroup comparisons of quantitative variables were made by using one-way ANOVA model, and meanwhile by rank sum test as well. A two-tailed *P* value less than 0.05 was considered significant. The linear correlations of the relative grayscales between different proteins of each group, and those correlations between the quantitative TGF-$ß_1$ by ELISA and the time interval from the onset to surgery or the maximal dimensions of the aorta of the Aortic Dissection Group were assessed. $|r| < 0.3$ was taken as a non-significant correlation, while $0.3 \leq |r| < 0.5$, $0.5 \leq |r| < 0.8$, and $|r| \geq 0.8$ were taken as a slight, middle, and striking correlation, respectively.

Ethics

This study was approved by the institutional ethical committee, and was conducted following the guidelines of the Declaration of Helsinki. Informed consent was obtained from each patient before commencing treatment.

RESULTS

Quantitative RT-PCR

The melting curves showed the changing rate of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis displayed a single peak at the melting temperature (Tm) of 87°C for the samples, and of 84°C for the control, respectively (Fig. 1). The expressions of TGF-$ß_1$ mRNA were positive in all three groups. The results of TGF-$ß_1$ mRNA were calculated quantitatively by 2-ΔΔCT method, however, they did not show any intergroup differences (1.59 ± 0.33 vs. 1.45 ± 0.34 vs. 1.48 ± 0.48, $P > 0.05$ by rank sum test).

Western blot

Western blot assay revealed TGF-$ß_1$, TßRI, Smad2/3, Smad4 and Smad7 were positive in all three groups (Fig. 2). Smad4 was weakly present in the aortic tissues of the coronary patients. In spite of scanty of significant intergroup differences, quantitative results of relative grayscales of the five investigated proteins showed TGF-$ß_1$ was less pronounced in the aortic dissection than in the aortic aneurysm or coronary artery disease group, and a more pronounced TGF-$ß_1$ was present in the latter group than others. The expressions of Smad2/3 was somehow higher in the aortic dissection than in the aortic aneurysm and coronary patients, and Smad4 were the highest in the Aortic DissectionGroup. TßRI and Smad7 expressions were similar in all three groups (Fig. 3, Table 2).

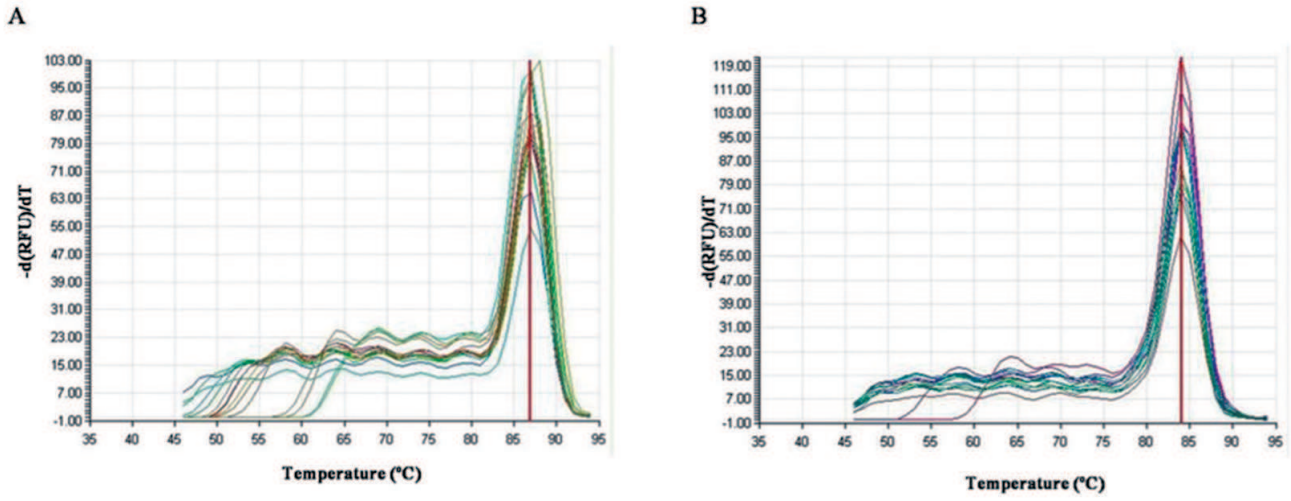

Fig. 1. The melting curves of (A) the samples, and (B) the control. Note the melting temperatures were 87°C and 84°C, respectively

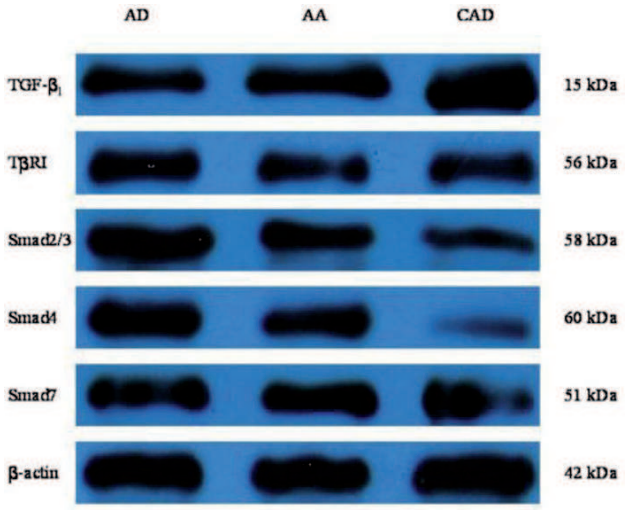

Fig. 2. Western blot of TGF-ß$_1$, TßRI, Smad2/3, Smad4 and Smad7. AD: aortic dissection; AA: aortic aneurysm; CAD: coronary artery disease

Of the quantitative relative grayscales, a significant reverse correlation was noted between TGF-ß$_1$ and Smad2/3 (Y = -0.8552X + 1.6417, r = -0.759, *P* < 0.0001), and a close direct correlation between Smad4 and Smad7 (Y = 0.5905X + 0.2805, r = 0.781, *P* < 0.0001) in the aortic dissection group. In the aortic aneurysm group, Smad4 and Smad7 were also closely correlated (Y = 0.5228X + 0.1642, r = 0.727, *P* = 0.026), and in the coronary artery disease group, TGF-ß$_1$ and Smad7 were much significantly correlated (Y = 0.5301X + 0.5758, r = 0.917, *P* = 0.004) (Fig. 4).

ELISA

The expressions of TGF-ß$_1$ in the aortic tissue were 319.52 ± 129.21 pg/mg protein, 324.09 ± 49.70 pg/mg protein, and 304.15 ± 29.39 pg/mg protein in the three groups, respectively. Despite no significant differences, a less pronounced elevation could be seen in the aortic dissection in comparison to either aortic aneurysm or coronary artery disease group (Fig. 5).

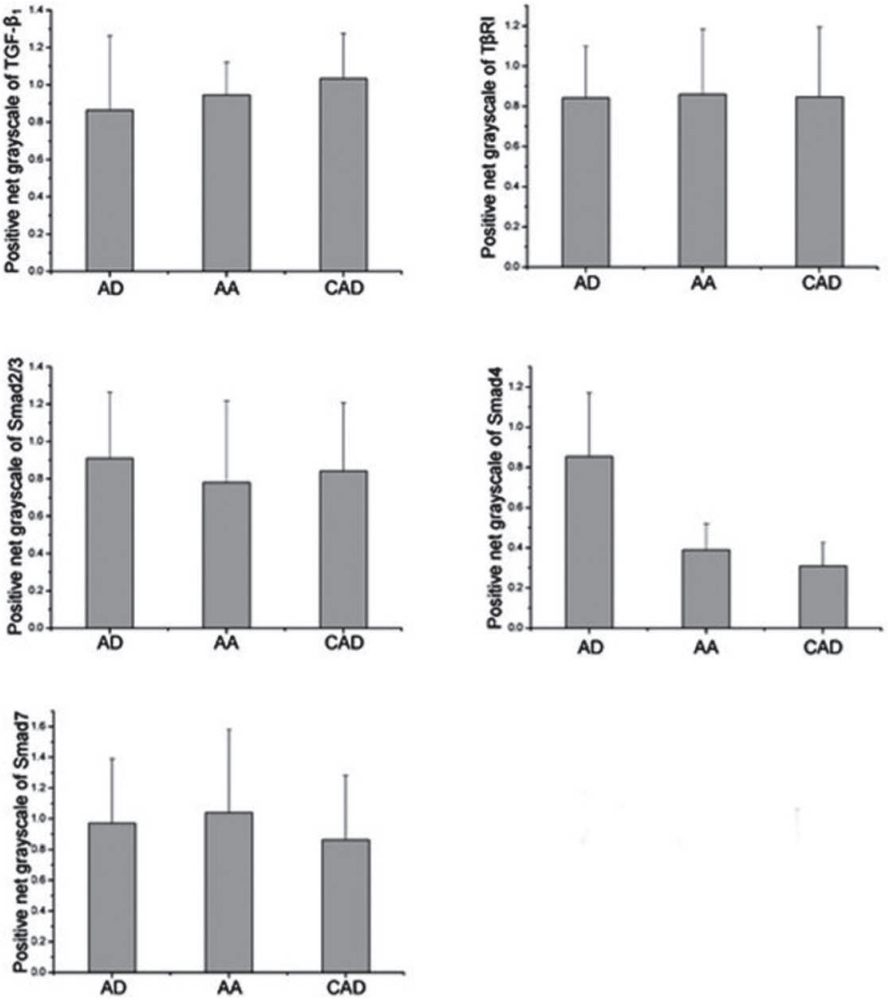

Fig. 3. Relative positive net grayscales of TGF-ß$_1$, TßRI, Smad2/3, Smad4 and Smad7. Note the expression of TGF-ß$_1$ was lower in aortic dissection patients

Table 2. Non-significance of relative net grayscales of Western blot assay by ANOVA

Variable	Mean ± standard deviation			*P* value		
	AD	AA	CAD	AD vs. AA	AD vs. CAD	AA vs. CAD
TGF-ß$_1$	0.95 ± 0.64	1.10 ± 0.55	1.07 ± 0.54	0.530	0.660	0.905
TßRI	0.75 ± 0.51	1.02 ± 0.54	1.06 ± 0.62	0.229	0.204	0.879
Smad2/3	0.81 ± 0.46	0.57 ± 0.37	0.73 ± 0.30	0.153	0.629	0.464
Smad4	0.82 ± 0.68	0.59 ± 0.27	0.68 ± 0.41	0.311	0.591	0.731
Smad7	0.99 ± 0.65	1.01 ± 0.57	0.97 ± 0.53	0.967	0.932	0.915

AA: aortic aneurysm; AD: aortic dissection; CAD: coronary artery disease; TßRI: receptor I of transforming growth factor ß; TGF-ß$_1$: transforming growth factor ß$_1$

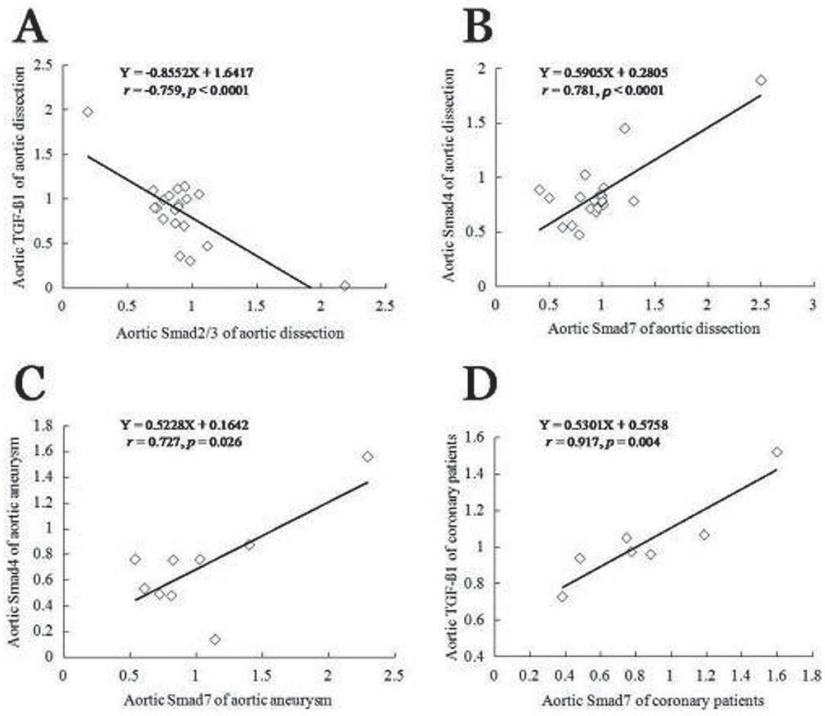

Fig. 4. Significant correlations of the relative positive net grayscales between (A) TGF-ß$_1$ and Smad2/3 of the aortic dissection; (B) Smad4 and Smad7 of the aortic dissection; (C) Smad4 and Smad7 of the aortic aneurysm; and (D) TGF-ß$_1$ and Smad7 of the coronary artery disease group

Plasma TGF-ß$_1$ values were 1158.30 ± 11.54 pg/ml, 1170.27 ± 8.26 pg/ml, 1225.00 ± 174.42 pg/mL and 1160.25 ± 13.01 pg/mL in the four groups, respectively. A similar but less pronounced increasing trend was found to that in the supernatant of the aortic tissues in the aortic dissection and the aortic aneurysm groups (Fig. 6). However, the plasma TGF-ß$_1$ level was remarkably enhanced in the coronary patients, and significant intergroup differences were present by rank sum test ($P < 0.025$).

The time interval from the onset to surgery was 4.76 ± 7.85 days (range: 8 hours to 1 month) in patients with aortic dissection. This time interval did not correlate with aortic or plasma TGF-ß$_1$ values (aorta: Y=23.757X + 827.68, $r^2 = 0.0411$, r = -0.203, $P = 0.420$; plasma: Y=0.3148X + 1156.70, $r^2 = 0.0324$, r = 0.180, $P = 0.670$), neither did the maximal dimension of the thoracic aorta with aortic or plasma TGF-ß$_1$ (aorta: Y= 145.52X + 1807.67, $r^2 = 0.0400$, r = -0.200, $P = 0.493$; plasma: Y=1.9537X + 1145.03, $r^2 = 0.0649$, r = 0.255, $P = 0.626$) (Fig. 7).

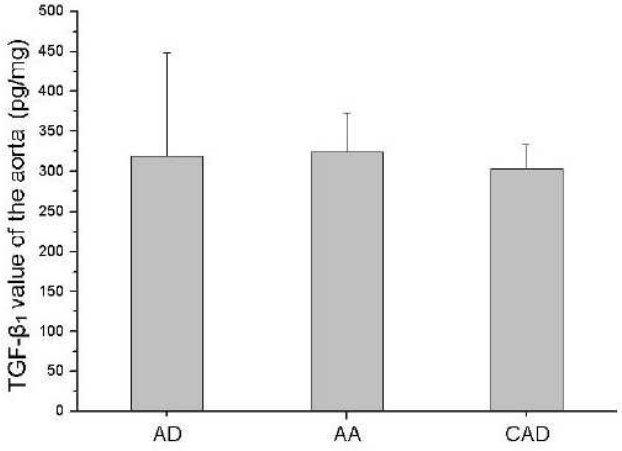

Fig. 5. TGF-ß$_1$ value of the aorta by ELISA. AD: aortic dissection; AA: aortic aneurysm; CAD: coronary artery disease. AD: aortic dissection; AA: aortic aneurysm; CAD: coronary artery disease. P > 0.05 by rank sum test

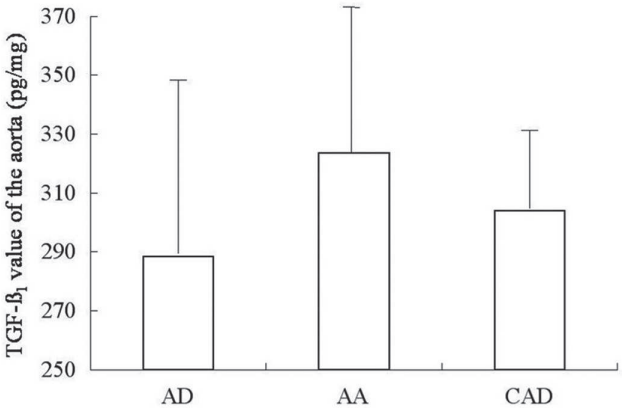

Fig. 6. Plasma TGF-ß$_1$ level by ELISA. AD: aortic dissection; AA: aortic aneurysm; CAD: coronary artery disease. P < 0.025 by rank sum test

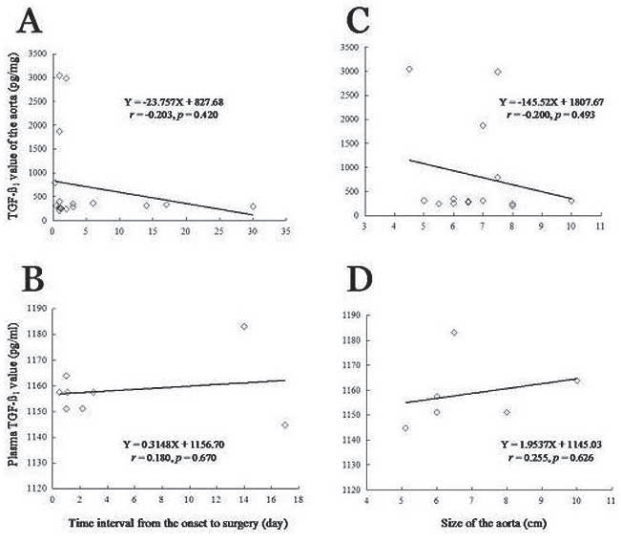

Fig. 7. Correlation between the plasma or aortic TGF-ß$_1$ level and the time interval from the onset to surgery or the maximal dimension of the aorta in aortic dissection patients: (A) aortic TGF-ß$_1$ and (B) plasma TGF-ß$_1$ corresponding to the time interval from the onset to surgery; and (C) aortic TGF-ß$_1$ and (D) plasma TGF-ß$_1$ corresponding to the maximal dimensions of the aorta in the patients with aortic dissection.

DISCUSSION

Studies on TGF-ß signaling revealed that Smad4 was unlikely to be involved in matrix contraction induced by TGF-ß, whereas Smad2/3 was distributed in the cytoplasm but relatively lower in the nucleus [21]. On the contrary, Smad7 overexpression may inhibit the TGF-ß-induced fibronectin and connective tissue growth factor expressions [3]. Nevertheless, the intensity and duration of TGF-ß signals and Smad2/3 nuclear translocation may largely depend on the regulation by Smad7 on the one hand [21], and Smad7 overexpression may prevent injury-induced α-smooth muscle actin expression as well [22]. Besides, Smad7 overexpression may remarkably reduce the ß-galactose-labelled cells in the neointima, decrease the loss of the lumen, reduce the collagen content of the vascular adventitia, and delay the process of vascular fibrosis following balloon angioplasty [23].

In aortic dissection, Smad4 may promote, while Smad7 may abolish, this signaling pathway, leading to matrix degradation by attenuating laminin expression and increasing expression of matrix metalloproteinases, making the balance between deposition and degradation a shift to the latter. Similar to what has been described previously, upregulations of TGF-ß$_1$ and Smad2, Smad3 and Smad7 may be responsible for cardiac hypertrophy induced by abdominal aortic constriction in the rat models [24]. In addition, Smad4 was upregulated as well, despite few other studies have directly investigated this issue, but an attenuated expression of Smad4 in a murine model of thoracic aortic aneurysm with enhanced other ligands of the signaling pathway has been reported [25]. In the vascular smooth muscle cells, in the condition of angiotensin II stimulation, a rapid Smad2 phosphorylation, nuclear translocation of phosphorylated-Smad2 and Smad4 might occur [26]. In contrast, Smad4 functional loss may result in increased laminin expression and decreased expression of matrix metalloproteinases, which, with increased levels of laminin α 1, cause excessive basement membrane deposition [27].

Madri et al. [28] found in the balloon-injured rat carotid artery model the neointima of the arteries showed intense staining of TGF-ß$_1$ at 10 weeks after vascular injury. Majesky et al. [29] also observed an increased TGF-ß$_1$ in neointimal

smooth muscle cells with antecedent transcripts for TGF-$ß_1$ 6 hours after balloon injury. serum TGF-$ß_1$ between the patients with abdominal aortic aneurysm and the subjects without an aneurysm did not display any significant difference (32.6 ± 9.9 ng/mL vs. 33.2 ± 8.3 ng/mL, $P = 0.098$) [30]. However, TGF-$ß_1$ might be released from the platelets into the serum when blood coagulates, and this would largely influence the serum detection [31]. Therefore, one should always bear in mind such influence factors when confronting TGF-$ß_1$ results detected by ELISA especially when the patients are at risks of coagulopathies.

TGF-$ß_1$ mRNA can be upregulated in cancer and disorders involving fibrotic process, and it is especially more expressed in malignant than in benign lesions. In comparison with non-atherosclerotic disease, atherosclerotic aortic smooth muscle cells showed much more TGF-$ß_1$ mRNA expressions. In this study, TGF-$ß_1$ mRNA was expressed in all the aortic tissues of the patients of each group, with a slight higher level in the aortic dissection than in the aortic aneurysm and coronary artery disease group, but lack of significant differences. The results indicated that TGF-β1 may participate the development of the aortopathies, with no difference in the extent at the genetic level while displaying its major biological function. But the potential disparities of the functioning ways in various aortopathies could not be excluded. Anyway, interruption of TGF-ß/Smad signaling pathway at the genetic level might represent an alternative of reversing the pathological process of these lesions [32].

Substraction of the background gray levels may facilitate correct measurement of the grayscale at each pixel across the image in immunostaing [33] and Western blot analyses [34], and maximize the signal strength and minimize the non-specific bands [35]. We therefore adopted positive net grayscale in evaluating the positiveness of quantitative Western blot results, from which we noted the close correlations between Smad4 and Smad7 of the aortic dissection and aortic aneurysm patients, which may indicate an intense abolishing effect of Smad7 in the signaling transduction. However, such a relation was scanty in coronary artery disease patients, indicating a less inhibitory effect of Smad7 associated with atherosclerotic changes. The negative regressions between TGF-$ß_1$ and Smad2/3 in aortic dissection highlighted a probable impetus of matrix degradation. Background noise is often associated with the problematic samples such as plasma, serum or cell culture. It may influence on all values, but influence more on the lower and non-expressed genes at a large extent [36]. Our Western blot disclosed an enhanced TGF-ß/Smad transduction in the aortopathies, including aortic dissection, aortic aneurysm and atherosclerosis. Furthermore, TGF-$ß_1$ was less pronounced in the aortic dissection than in the aortic aneurysm or coronary artery disease group, and a more pronounced TGF-$ß_1$ was present in the latter group than others. The expressions of Smad2/3 was somehow higher in the aortic dissection than in the aortic aneurysm and coronary patients, and Smad4 were the highest in the Aortic Dissection Group, but was weakly present in the aortic tissues of the coronary patients. TßRI and Smad7 expressions were similar in all three groups. Linear correlations revealed a somehow damaged TGF-$ß_1$ in the aortic dissection. We postulated that TGF-ß/Smad signaling transduction varied in various aortopathies: R-Smad was slightly upregulated, Co-Smad was remarkably upregulated and I-Smad was moderately upregulated in the aortic dissection; and R-Smad and Co-Smad moderately attenuated and I-Smad enhanced in the aortic aneurysm, while Co-Smad was remarkably attenuated in the coronary patients.

In this study, the ELISA showed a distinguished increase of TGF-$ß_1$ in the aortic tissue in the Aortic Aneurysm Group, and a distinguished increase of TGF-$ß_1$ in the plasma in the coronary artery disease group, indicating TGF-β_1 might be expressed in the aortic tissues prior to its release into the circulation. As such, TGF-$ß_1$ upregulation may play a role in inhibiting the progression of aortic dilation as described in the literature [37].

There were four limitations confronted in this study that should be mentioned: small sample, small aortic tissues from the coronary patients, the lack of normal aortic tissues from heart transplant donors, and the different sources of healthy controls for blood and aorta sampling. Further studys on larger patient population and sufficient sampling sources can be helpful for obtaining more precise information.

CONCLUSION

In conclusion, TGF-ß/Smad signaling transduction varied in the functioning way in different aortopathies. In patients with aortic dissection, the signaling was enhanced, in comparison to aortic aneurysm and coronary artery disease, characterized by a less pronounced TGF-$ß_1$ expression, but a somehow pronounced I-Smad and Co-Smad upregulation, suggesting a prominent matrix degradation in aortic dissection, but a prominent matrix deposition in the aortic aneurysm and coronary artery disease.

REFERENCES

1. Leof EB. TGF beta receptors and cell proliferation. http://www.researchgrantdatabase.com/g/5R01GM054200-03/TGF-BETA-RECEPTORS-AND-CELL-PROLIFERATION/. [accessed on January 21, 2010].

2. Hamet P, Hadrava V, Kruppa U, Tremblay J. Transforming growth factor ß$_1$ expression and effect in aortic smooth muscle cells from spontaneously hypertensive rats. Hypertension. 1991;17(6 Pt 2):896-901.

3. Wang W, Huang XR, Canlas E, Oka K, Truong LD, Deng C, et al. Essential role of Smad3 in angiotensin II-induced vascular fibrosis. Circ Res. 2006;98(8):1032-9.

4. Massagué J. TGF-beta signal transduction. Annu Rev Biochem. 1998;67:753-91.

5. Chai Y, Ito Y, Han J. TGF-ß signaling and its functional significance in regulating the fate of cranial neural crest cells. Crit Rev Oral Biol Med. 2003;14(2):78-88.

6. Makkar P, Metpally RP, Sangadala S, Reddy BV. Modeling and analysis of MH1 domain of Smads and their interaction with promoter DNA sequence motif. J Mol Graph Model. 2009;27(7):803-12.

7. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 1996;271(5247):350-3.

8. Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor ß$_1$ is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A. 1991;88(15):6642-6.

9. Kamimura S, Tsukamoto H. Cytokine gene expression by Kupffer cells in experimental alcoholic liver disease. Hepatology. 1995;22(4 Pt 1):1304-9.

10. Dahiya R, Lee C, Haughney PC, Chui R, Ho R, Deng G. Differential gene expression of transforming growth factors α and ß, epidermal growth factor, keratinocyte growth factor, and their receptors in fetal and adult human prostatic tissues and cancer cell lines. Urology. 1996;48(6):963-70.

11. Dunfield LD, Dwyer EJ, Nachtigal MW. TGF ß-induced Smad signaling remains intact in primary human ovarian cancer cells. Endocrinology. 2002;143(4):1174-81.

12. Elliott RL, Blobe GC. Role of transforming growth factor ß in human cancer. J Clin Oncol. 2005;23(9):2078-93.

13. Pouliot F, Labrie C. Expression profile of agonistic smads in human breast cancer cells: absence of regulation by estrogens. Int J Cancer. 1999;81(1):98-103.

14. Jones JA, Spinale FG, Ikonomidis JS. Transforming growth factor-ß signaling in thoracic aortic aneurysm development: a paradox in pathogenesis. J Vasc Res. 2009;46(2):119-37.

15. Lesauskaite V, Tanganelli P, Sassi C, Neri E, Diciolla F, Ivanoviene L, et al. Smooth muscle cells of the media in the dilatative pathology of ascending thoracic aorta: morphology, immunoreactivity for osteopontin, matrix metalloproteinases, and their inhibitors. Hum Pathol. 2001;32(9):1003-11.

16. Dietz HC. TGF-ß in the pathogenesis and prevention of disease: a matter of aneurysmic proportions. J Clin Invest. 2010;120(2):403-7. doi: 10.1172/JCI42014.

17. Youn TJ, Kim HS, Oh BH. Ventricular remodeling and transforming growth factor-ß$_1$ mRNA expression after nontransmural myocardial infarction in rats: effects of angiotensin converting enzyme inhibition and angiotensin II type 1 receptor blockade. Basic Res Cardiol. 1999;94(4):246-53.

18. Thompson NL, Bazoberry F, Speir EH, Casscells W, Ferrans VJ, Flanders KC, et al. Transforming growth factor ß-1 in acute myocardial infarction in rats. Growth Factors. 1988;1(1):91-9.

19. Gomez D, Al Haj Zen A, Borges LF, Philippe M, Gutierrez PS, Jondeau G, et al. Syndromic and non-syndromic aneurysms of the human ascending aorta share activation of the Smad2 pathway. J Pathol. 2009;218(1):131-42.

20. Ward MR, Agrotis A, Kanellakis P, Dilley R, Jennings G, Bobik A. Inhibition of protein tyrosine kinases attenuates increases in expression of transforming growth factor-ß isoforms and their receptors following arterial injury. Arterioscler Thromb Vasc Biol. 1997;17(11):2461-70.

21. Dawes LJ, Sleeman MA, Anderson IK, Reddan JR, Wormstone IM. TGFß /Smad4-dependent and -independent regulation of human lens epithelial cells. Invest Ophthalmol Vis Sci. 2009;50(11):5318-27.

22. Saika S, Ikeda K, Yamanaka O, Sato M, Muragaki Y, Ohnishi Y, et al. Transient adenoviral gene transfer of Smad7 prevents injury-induced epithelial-mesenchymal transition of lens epithelium in mice. Lab Invest. 2004;84(10):1259-70.

23. Mallawaarachchi CM, Weissberg PL, Siow RC. Smad7 gene transfer attenuates adventitial cell migration and vascular remodeling after balloon injury. Arterioscler Thromb Vasc Biol. 2005;25(7):1383-7.

24. Huang J, Wei C, Peng J, Zheng Z, Peng X. The effects of signal protein Smads on rat cardiocyte hypertrophy. J US-Chin Med Sci. 2005;2(1):29-44.

25. Jones JA, Barbour JR, Stroud RE, Bouges S, Stephens SL, Spinale FG, et al. Altered transforming growth factor-ß signaling in a murine model of thoracic aortic aneurysm. J Vasc Res. 2008;45(6):457-68.

26. Rodríguez-Vita J, Sánchez-López E, Esteban V, Rupérez M, Egido J, Ruiz-Ortega M. Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming

growth factor-ß-independent mechanism. Circulation. 2005;111(19):2509-17.

27. Costello I, Biondi CA, Taylor JM, Bikoff EK, Robertson EJ. Smad4-dependent pathways control basement membrane deposition and endodermal cell migration at early stages of mouse development. BMC Dev Biol. 2009;9:54.

28. Madri JA, Reidy MA, Kocher O, Bell L. Endothelial cell behavior after denudation injury is modulated by transforming growth factor-$ß_1$ and fibronectin. Lab Invest. 1989;60(6):755-65.

29. Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA. Production of transforming growth factor $ß_1$ during repair of arterial injury. J Clin Invest. 1991;88(3):904-10.

30. Golledge J, Clancy P, Jones GT, Cooper M, Palmer LJ, van Rij AM, Norman PE. Possible association between genetic polymorphisms in transforming growth factor ß receptors, serum transforming growth factor $ß_1$ concentration and abdominal aortic aneurysm. Br J Surg. 2009;96(6):628-32.

31. Grainger DJ, Mosedale DE, Metcalfe JC. TGF-ß in blood: a complex problem. Cytokine Growth Factor Rev. 2000;11(1-2):133-45.

32. Yuan S, Jing H. Cardiac surgery and hypertension: a dangerous association that must be well known. Rev Bras Cir Cardiovasc. 2011;26(2):273-81.

33. Boushell LW, Kaku M, Mochida Y, Bagnell R, Yamauchi M. Immunohistochemical localization of matrixmetalloproteinase-2 in human coronal dentin. Arch Oral Biol. 2008;53(2):109-16.

34. Zhou JD, Luo CQ, Xie HQ, Nie XM, Zhao YZ, Wang SH, et al. Increased expression of heat shock protein 70 and heat shock factor 1 in chronic dermal ulcer tissues treated with laser-aided therapy. Chin Med J (Engl) 2008;121(14):1269-73.

35. Quaglino A, Salierno M, Pellegrotti J, Rubinstein N, Kordon EC. Mechanical strain induces involution-associated events in mammary epithelial cells. BMC Cell Biol. 2009;10:55.

36. Kroll TC, Wölfl S. Ranking: a closer look on globalisation methods for normalisation of gene expression arrays. Nucleic Acids Res. 2002;30(11):e50.

37. Dai J, Losy F, Guinault AM, Pages C, Anegon I, Desgranges P, et al. Overexpression of transforming growth factor-$ß_1$ stabilizes already-formed aortic aneurysms: a first approach to induction of functional healing by endovascular gene therapy. Circulation. 2005;112(7):1008-15.