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Case Report

Expression of TGF-β/SMAD Signaling Pathway of Cardiac Fibrosis in Patients with Left Ventricular Failure

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Abstract

Left ventricular failure, a common type of cardiovascular diseases complicated with cardiac fibrosis, is related to different changes in various gene expression. In order to explore the expression of genes related to cardiac fibrosis in patients with left ventricular failure. Case Report: The present study is to determine the expression of genes related to cardiac fibrosis in the blood samples from ten patients with left ventricular failure and ten healthy subjects using RT-PCR and western blot. Using ACTB (β-actin) as a reference, five genes related to cardiac fibrosis were identified. Compared with healthy subjects, the level of five genes, including SMAD2, SMAD3, ACTA2 (α-SMA), COL3A1 and COL1A1 were found to be significantly elevated in the blood samples from patients with left ventricular failure ($P < 0.01$). Conclusion: This study suggests a potential relationship between the increased expression of five genes of TGF-β/SMAD signaling pathway and cardiac fibrosis in left ventricular failure.

Keywords: Left ventricular failure; Cardiac fibrosis; Blood; TGF-β/SMAD signaling pathway

Introduction

According to statistics, cardiovascular diseases are still the disease with the highest mortality rate in the world [1]. Heart failure, as a cardiovascular disease, is caused by insufficient blood supply due to abnormal cardiac function [2]. Left ventricular failure, defined as heart failure caused by left ventricular compensatory insufficiency, is the most common condition of heart failure [3]. The pathogenesis of left ventricular failure is slow, gradual, and irreversible, and it is accompanied by pulmonary circulatory congestion and cardiac fibrosis [4]. Cardiac fibrosis, also known as myocardial calcification, is the main reason for the irreversible development of left ventricular failure [5]. Cardiac fibrosis, is caused by damage of myocardial tissues. Fibers and collagen replace the necrotic myocardial tissues, resulting in excessive accumulation of extracellular matrix, and eventually

leading to impaired cardiac function and cardiac fibrosis [6]. In terms of pharmacological mechanism, the mechanism of cardiac fibrosis is closely related to the regulation of various genes in cardiomyocytes [7]. The occurrence of cardiac fibrosis can lead to hardening of the heart, heart conduction problem, reduced oxygen diffusion, damages in the electromechanical coupling of the heart, and arrhythmia [8]. Previous studies have proved that cardiac fibrosis is tightly linked with the TGF-β/SMAD signaling pathway [9]. TGF-β promoted fibrosis occurrence by activating SMAD2 and SMAD3 regulators, while SMAD7 inhibited TGF-β-mediated fibrosis [10]. Although a few compounds could improve cardiac fibrosis in animals [11,12], there was still no effective treatments in clinical. In this study, we detected the expression levels of genes related to cardiac fibrosis in blood samples from patients with left ventricular failure by real-time fluorescent quantitative PCR (RT-PCR) and western blot methods, for finding potential biomarkers of cardiac fibrosis and breaking through cardiac fibrosis in the future.

Materials and Methods

Clinical Samples: Blood samples from 10 patients with left ventricular failure were provided by Guangdong Provincial People's Hospital. And blood samples from 10 healthy subjects without any known cardiac or other health impairment were provided by The University Hospital of Macau University of Science and Technology (MUST). Details of all subjects are presented in Table 1.

RNA extraction: Total RNA purification was extracted from the blood samples of subjects using the QIAam RNA Blood Mini Kit (Cat. 52304, Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Total RNA was spectrophotometrically quantitated (NanoDrop One/OneC, Thermo, USA). 1 μg total RNA of each sample were reverse-transcribed using the 5 × EVO M-MLVRT Master Mix reagent (Cat. AG11706, Accurate Biology, China). Subsequently, cDNA was kept at -20 ℃.

Real-time quantitative PCR: Thermal cycling and SYBR Green fluorescence detection were done in a real-time PCR thermal cycler (ViiA 7 Real-Time PCR System, Thermo, USA). The 10 μL PCR reactions prepared in 384-well plates (Applied Biosystems) included mixtures of 4 μL cDNA and 6 μL primers, which included 5 μL SYBR Green and 1 μL primers workstation of forward and reverse primer each 10 μmol/μL. According to the manufacturer's instructions, the primers used in the experiment were listed as follows (Table Ⅱ.). 10 μL total RNA was mixed with 90 μL RNase free water and the primers were added into SYBR preparing for PCR condition. PCR conditions included hold stage (50 ℃ for 2 minutes and 95 ℃ for 10 minutes), PCR stage (95 ℃ for 15 seconds and 60 ℃ for 1 minute with 45 cycles) and melt curve stage (95 ℃ for 15 seconds, 60 ℃ for 1 minute and 95 ℃ for 15 seconds). Furthermore, the experiments were repeated three times. Threshold Cycle (Ct) represents the PCR cycle, which determines the fluorescence of different genes in samples. Relative quantitation was performed by normalizing Ct value of each sample genes with Ct value of the ACTB (β-actin) gene (ΔCt). ΔCt corresponds to the difference between the Ct value of the gene of target genes and the Ct of the ACTB gene.

Western blot: Protein was extracted from the blood samples from subjects. The protein concentration was determined by the Detergent Compatible Bradford protein assay kit (Beyotime, Nantong, China). Protein samples were subjected to 8% SDS-PAGE and blotted to nitrocellulose filter membranes. Primary antibodies included mouse anti-β-actin (1:1000, Cell Signaling), rabbit α-SMA (1:100, Santa), mouse anti-COL3A1 (1:500, Huabio), goat anti-COL1A2 (1:500, Huabio), rabbit anti-SMAD2 (1:1000, Cell Signaling) and rabbit anti-SMAD3 (1:1000, Cell Signaling). Secondary antibodies included HRP-conjugated goat anti-mouse IgG (1:10000, ICL Lab, USA), HRP-conjugated goat anti-rabbit (1:10000, ICL Lab, USA) and HRP-conjugated rabbit anti-goat (1:5000, ICL Lab, USA). Protein bands were visualized by GE Amersham Imager 600 RGB (EG, USA) with ECL western blotting detection reagent (Millipore, USA).

Statistical analysis: Data were expressed as mean±standard error of the mean (mean±SEM). All data analysis was performed using GraphPad Prism 8.0 software (GraphPad-Prism Software Inc., San Diego, CA, USA). Differences between mean values of multiple groups were analyzed by one-way analysis of variance with Tukey's test for post hoc comparisons. $P<0.01$ was considered statistically significant.

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Clinically, patients with left ventricular failure are usually determined by examining indicators related to cardiac function. The statistical results of classical indicators related to cardiac function in patients with left ventricular failure were shown in Table Ⅲ, including AO (29.0±2.59 mm), LA (49.7±4.92 mm), RVOT anteroposterior diameter (27.8±2.37 mm), LVDd (53.4±5.10 mm), LVDs (40.0±5.61 mm), AAO (34.6±2.43 mm), RAD2 (57.4±5.33 mm), RVD2 (56.4±3.28 mm), MPA (24.0±1.19 mm), IVS (11.5±0.47 mm), LVPW (11.1±0.47 mm), MP E peak (1.1±0.14 m/s), MP A peak (0.8±0.13 m/s), AV (1.9±0.37 m/s), AV peak pressure difference (37.7±14.40 mmHg), AV mean pressure difference (22.3±9.87 mmHg), PV (0.9±0.09 m/s), TV E peak (0.5±0.04 m/s), LVEF (53.7±6.76 %), CW S' (6.0±0.60 cm/s), CW E' (5.0±0.45 cm/s), CW A' (6.7±0.97 cm/s). CW E/E' (23.1±3.08), RV FAC (29.0±2.59 %), PVPW (29.0±2.59 mm), RVCM (29.0±2.59 mm) and TARVWTV (29.0±2.59 cm/s). Among them, LVEF, serving as the most important clinical indicator, was about 55 % to 65 % in healthy subjects. LVEF level of patients with left ventricular failure was 53.7±6.76 %, suggesting cardiac

insufficiency. Other common indicators include LA, LVDd, LVDs, LVPW and E/E' were 19 to 33 mm, 35 to 50 mm, 20 to 37 mm, 7 to 11 mm and 8 to 15 in healthy subjects. The levels of LA, LVDd, LVDs, LVPW and E/E' were 49.7±4.92 mm, 53.4±5.10 mm, 40.0±5.61 mm, 11.1±0.47 mm and 23.1±3.08, which were classified as left ventricular failure.

Types of subjects (n)	Sex ratio (male/female)	Age (years old)
Patient (n=10)	07-Mar	59.3±16.5
Health (n=10)	06-Apr	32.0±10.0

Table 1: Clinical characteristics of all subjects.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
COL1A1	TTTGGATGGTGCCAAGGGAG	CACCATCATTTCCACGAGCA
COL3A1	CGCCCTCCTAATGGTCAAGG	TTCTGAGGACCAGTAGGGCA
SMAD2	CCATCGGAAGAGGAAGGAACA	TCCCAGCAGTCTCTTCACAAC
SMAD3	CCGGGGGTTGGACTTTCCT	CAGAAGTTTGGGTTTCCGCA
ACTA2	GGACCTTTGGCTTGGCTTGT	TTGAAGCGGAAGTCTGGGAA
ACTB	CTTCGCGGGCGACGAT	CCACATAGGAATCCTTCTGACC

Table 2: Primer sequences of genes.

Indicators	Mean±SEM
AO (mm)	29.0±2.59
LA (mm)	49.7±4.92
RVOT anteroposterior diameter (mm)	27.8±2.37
LVDd (mm)	53.4±5.10
LVDs (mm)	40.0±5.61
AAO (mm)	34.6±2.43
RAD2 (mm)	57.4±5.33
RVD2 (mm)	56.4±3.28
MPA (mm)	24.0±1.19
IVS (mm)	11.5±0.47
LVPW (mm)	11.1±0.47
MP E peak (m/s)	1.1±0.14
MP A peak (m/s)	0.8±0.13
AV (m/s)	1.9±0.37
AV peak pressure difference (mmHg)	37.7±14.40
AV mean pressure difference (mmHg)	22.3±9.87
PV (m/s)	0.9±0.09
TV E peak (m/s)	0.5±0.04

LVEF (%)	53.7±6.76
CW S' (cm/s)	6.0±0.60
CW E' (cm/s)	5.0±0.45
CW A' (cm/s)	6.7±0.97
CW E/E'	23.1±3.08
RV FAC (%)	41.5±0.87
PVPW (mm)	4.0±0.37
RVCM (mm)	21.0±1.83
TARVWTV (cm/s)	11.8±1.03

AO: Aorta; LA: Left Atrium; RVOT: Right Ventricular Outflow Tract; LVDd: Left ventricularend end-diastolic Dimension; LVDs: Left ventricularend end-systolic Dimension; AAO: Aorta ascendens; RAD2: Right Atrium D2; RVD2: Right Ventricle D2; MPA: Main Pulmonary Artery; IVS: Interventricular Septum; LVPW: Left Ventricular Posterior Wall; MP: Mitral Valve; AV: Aortic Valve; PV: Pulmonic Valve; TV: Tricuspid Valve; LVEF: Left Ventricular Ejection Fraction; CW: Chest Wall; RV: Right Ventricle; FAC: Fractional Area Change; PVPW: Pulmonic Valve Posterior Wall; PVCM: Pulmonic Valve; TARVWTV: Tricuspid Annulus Right Ventrcular Wall Tissue Velocity; All data in the Table Ⅲ. were expressed as Mean±SEM.

Table 3: Analysis of patients' cardiac ultrasound data.

Total RNA samples were extracted from blood samples in patients with left ventricular failure and healthy subjects (shown in Table Ⅰ). The samples were reverse-transcribed into cDNA, amplified, and quantitated by SYBR Green detection. And the messenger RNA expression of five genes relevant to cardiac fibrosis were detected using RT-PCR, as shown in Table Ⅱ. RT-PCR results showed the Ct values of different genes after three times. Relative messenger RNA expression was calculated using ACTB (also named β-actin) gene expression as a reference gene. As RT-PCR results shown in Figure 1, the messenger RNA expressions of five genes relevant to cardiac fibrosis including SMAD2, SMAD3, ACTA2 (α-SMA), COL3A1 and COL1A1 were significantly increased in patients with left ventricular failure compared with healthy subjects ($P<0.01$), which were in consistence with western blot results. Western blot results showed that the expression of five proteins including α-SMA, SMAD2, SMAD3, COL3A1 and COL1A2 (its gene named COL1A1 gene) were increased in patients with left ventricular failure compared with healthy subjects (shown in Figure 2).

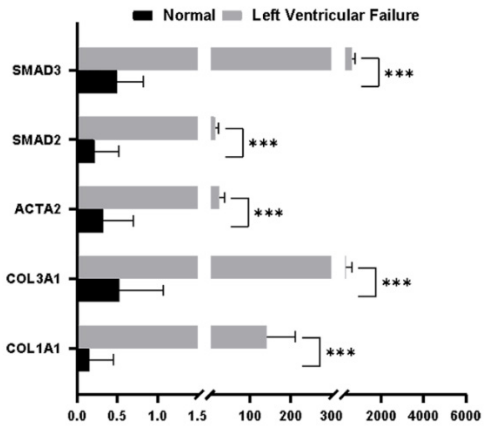

Figure 1: Expression of cardiac fibrosis-related genes in blood samples of patients with left ventricular failure and healthy subjects using RT-PCR. The expression of genes including SMAD2, SMAD3, ACTA2 (α-SMA), COL3A1 and COL1A1 were increased in patients with left ventricular failure compared with healthy subjects. *$P<0.01$, vs. healthy subjects.

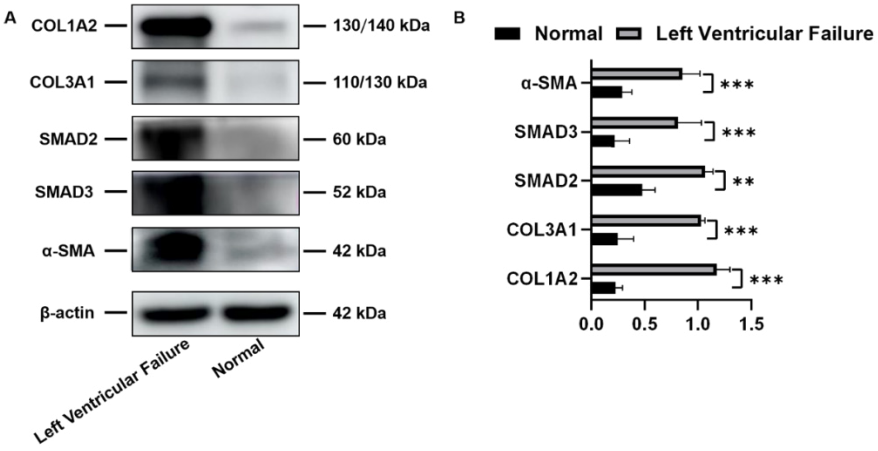

Figure 2: Expression of cardiac fibrosis-related proteins in blood samples of patients with left ventricular failure and healthy subjects using western blot with β-actin as a reference protein. The expression of proteins including α-SMA, SMAD2, SMAD3, COL3A1 and COL1A2 were increased in patients with left ventricular failure compared with healthy subjects. *P<0.01, vs. healthy subjects.

ACTA2 (α-SMA), COL3A1and COL1A1 genes are related to the deposition of collagen and fibers, and cardiac fibrosis. SMAD2 and SMAD3 play an important part in the TGF-β/SMAD signaling pathway, which is the canonical signaling of cardiac fibrosis. The TGF-β/SMAD signaling pathway is the current focus of cardiac fibrosis, and the genes on this signaling pathway could be served as potential targets for the treatment of cardiac fibrosis in the future.

Discussion

Left ventricular failure, a type of heart failure, is clinically diagnosed by determining various indicators related to cardiac function. Common cardiac function indicators mainly include AO, LA, RVOT anteroposterior diameter, LVDd, LVDs, AAO, RAD2, RVD2, MPA, IVS, LVPW, MP E peak, MP A peak, AV, AV peak pressure difference, AV mean pressure difference, PV, TV E peak, LVEF, CW S', CW E', CW A', CW E/E', RV FAC, PVPW, RVCM and TARVWTV [13]. In this study, patients diagnosed with left ventricular failure were evaluated and the level of LVEF served as a typical indicator (as shown in Table 3).

Left ventricular failure occurs in the heart tissue and is closely associated with cardiac fibrosis. The pathogenesis of cardiac fibrosis is complex, which is related to signaling pathways involving various genes and proteins. As a classic signaling pathway associated with trans-differentiation of myofibroblasts, the TGF-β/SMAD signaling pathway could promote the secretion of collagen and fibers [7,9]. And TGF-β promotes cardiac fibrosis by activating the phosphorylation of the transcription factor SMAD2/3 [10,14]. SMAD2/3 activation promotes the conversion of cardiomyocytes to myofibroblasts and upregulates the expression of genes related to cardiac fibrosis such as COL3A1, COL1A1, α-SMA, etc [15]. In this study, the RT-PCR was used to study the expression levels of five genes relevant to cardiac fibrosis in blood samples from patients with left ventricular failure and healthy subjects. Due to ethical issues, the heart tissue samples were restricted and we used the blood samples instead of heart tissues in this study.

Our data showed that the expression of five genes which are associated with cardiac fibrosis were significantly up-regulated in patients with left ventricular failure. The cardiac fibrosis-related genes identified in this study include SMAD2, SMAD3, ACTA2 (α-SMA), COL3A1 and COL1A1. In patients with left ventricular failure, the TGF-β/SMAD signaling pathway was activated with an up-regulated expression of SMAD2 and SMAD3. Results indicated that upregulated expression of ACTA2 (α-SMA) and collagen (including COL3A1 and COL1A1) in patients with left ventricular failure triggered myofibroblasts transdifferentiation and collagen secretion [16].

Conclusions

In this study, we found for the first time that the transcription levels of five genes of TGF-β/SMAD signaling pathway related to cardiac fibrosis in the blood samples of patients with left ventricular failure were significantly elevated compared with healthy subjects. Specific roles of these genes in the process of cardiac fibrosis will become a major focus rendering further investigation. This study may play a key role in breaking through the problem of the reversal of cardiac fibrosis in future.

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Authors' contributions

ZYL prepared manuscript. XL, SYC, JZ, YJW and LML provided samples. ZYL and YZZ reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate: The ethics were approved by the Medical Research Ethics Committee of Guangdong Provincial People's Hospital (KY-A-2021-414-02).

Patient consent for publication: Informed consent was obtained from all subjects involved in this study.

Competing interests: The authors declare no conflict of interest.

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