

# Effects of *Panax notoginseng* flower extract on the TGF- $\beta$ /Smad signal transduction pathway in heart remodeling of human chymase transgenic mice

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**Abstract.** *Panax notoginseng* is a common Chinese herb extensively used in Chinese medical practice for the treatment of cardiovascular diseases. The present study aimed to evaluate the effects of *Panax notoginseng* flower extract (PNFE) on the TGF- $\beta$ /Smad signal transduction pathway in heart remodeling of human chymase transgenic mice. After treatment with PNFE and soybean trypsin inhibitor (SBTI), the left ventricular mass indexes (LVMI) of transgenic and normal C57 BL/6 mice were analyzed. The mRNA expression of chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7 in myocardium was assessed with RT-PCR, while the protein expression in myocardium was detected by western blotting. The results showed that PNFE and SBTI treatment led to a significant reduction in LVMI in transgenic mice, indicating a beneficial effect on left ventricular remodeling. Mechanistically, PNFE and SBTI treatment attenuated the mRNA expression of chymase, TGF- $\beta$ 1, Smad2 and Smad3, as well as the protein expression in the myocardium tissues of the transgenic mouse model. By contrast, PNFE and SBTI treatment markedly up-regulated the mRNA and protein expression of Smad7. It was concluded that PNFE was able to improve the ventricular

hypertrophy state in human chymase transgenic mice through regulation of the expression of mRNA and protein of TGF- $\beta$ /Smad in ventricular tissues.

## Introduction

Heart remodeling in the form of left ventricular hypertrophy (LVH) is very common during hypertension and is considered as the first step towards myocardial infarction or heart failure (1). Evidence that a large variety of genes and pathways are involved in the alterations of cardiac growth in animal models, such as transgenic mice, and in patients, supports the concept of genetic susceptibility in LVH (2-4). Chymase is one of the angiotensin II (Ang II)-producing enzymes and is a chymotrypsin-like serine protease found in abundance in the secretory granules of mast cells (5). In patients with heart failure, the cardiac mast cell density is markedly increased, and an increased level of chymase may play a role in the development of several cardiovascular diseases (6,7). Several studies have indicated a close association of chymase in the development of heart remodeling (8-12). Chymase inhibitors, such as soybean trypsin inhibitor (SBTI), also a proteinase inhibitor on tryptase, have been found to be highly effective in mitigating tryptase and chymase-induced damage in cardiac tissues (9,13,14).

Transforming growth factor- $\beta$  (TGF- $\beta$ ) has been reported to be crucial in the progression of LV remodeling (15), a pathophysiological process characterized by hypertrophy of cardiomyocytes and an increase in interstitial fibrosis. Cardiac chymase promotes fibrosis by activating the production of TGF- $\beta$  (9,16), and also enhances Ang II production (17). Moreover, chymase also induces cardiac profibrotic response via TGF- $\beta$ 1/Smad signaling activation (18,19). However, the involvement of TGF- $\beta$ 1/Smad signaling activation in heart remodeling has yet to be reported in any chymase gene transgenic study.

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*Panax notoginseng* (Burk) F.H. Chen has long been used in Chinese medicine for the treatment of a wide array of diseases. Modern pharmacological research has found that *Panax notoginseng* exerts a variety of pharmacological effects on the blood, cardio-cerebral vascular system, central nervous system and endocrine system (20). Although the roots of this plant are the most commonly used, other plant parts, such as the rootlets, leaves and flowers, are also used, and their therapeutic activities and safety profiles are found to be similar to that of the roots (21).

*Panax notoginseng* saponins (PNS), the principal ingredients extracted from *Panax notoginseng*, have extensive effects on the cardiovascular system, including, among others, inhibition of platelet aggregation, invigorating blood flow in the coronary arteries (22,23), improving left ventricular diastolic function in hypertensive patients (23), protecting against damage resulting from myocardial ischemia (24), reducing myocardial oxygen consumption and anti-arrhythmic action (25). However, the effect of the *Panax notoginseng* flower extract (PNFE) on heart diseases, such as ventricular hypertrophy, has yet to be evaluated. Based on our unpublished screening data, the ventricular hypertrophy attenuating effect of NGFE is stronger than that of the root extract. In this study, we aimed to investigate the effects of PNS derived from the flower buds of *Panax notoginseng* on ventricular hypertrophy of human chymase transgenic mice, and to elucidate the underlying mechanisms of action by investigating the TGF- $\beta$ /Smads signaling transduction pathway in ventricular tissues.

## Materials and methods

**Preparation and determination of PNFE.** The flowers of *Panax notoginseng* used in the present study were collected in the Wenshan region of Yunnan Province, China, in February 2007, and their botanical identity was authenticated at the Yunnan Wenshan Cotrun Center Co., Ltd. To prepare the saponin-rich PNFE, 1 kg of *Panax notoginseng* flowers was refluxed with 14 liters 70% aqueous ethanol three times, for 2 h each time. The pooled extract was then concentrated to ~0.4 g/ml on a rotary evaporator under negative pressure, and the concentrate was then passed through a filter to remove any large debris. The refined concentrate was then loaded onto a AB-8 macroporous resin (Chemical Factory of Nankai University, Tianjin, China) column using an optimized protocol according to a method used by Li *et al* (26). Briefly, the column was eluted with 70% aqueous ethanol and the elute was then evaporated to yield dry PNFE. The total saponins in the dry PNFE were then quantified to be 87.6% using UV spectrophotometer (UV 8453; Agilent Technologies, USA). The amount of ginsenoside Rb1 and Rb3 was 4.12 and 8.93%, respectively, based on HPLC analysis (1200 series; Agilent Technologies) compared to the ginsenoside Rb1 and Rb3 reference sample solutions (National Institute for the Control of Pharmaceutical and Biological Products, China).

**Animals.** C57 BL/6 mice were obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences [Licence No. SCXK (Beijing) 2005-0013]. In some studies, human chymase gene transgenic mice were produced from C57 BL/6 genetic background mice (8,27). In brief, to

construct the myosin light chain-2 promoter (MLC2)-chymase fusion gene and produce transgenic mice for overexpression of the chymase gene in mouse heart, the MLC2-chymase fusion gene was constructed by splicing the MLC2 promoter sequence with the cloned chymase structural gene, whose promoter sequence had been deleted by nuclease BAL31 and cloned through molecular cloning. Purified fusion gene fragments were microinjected into murine eggs and transgenic mice were produced. PCR amplification, Southern blot analysis and sequencing of the products of PCR amplification were used to identify the positive transgenic mice.

The animals were housed in a 12-h light/dark cycle room and were allowed free access to food and water. Male human chymase gene transgenic mice (7 months of age) were randomly divided into three groups, with 8 animals in each group: i) control group, in which the animals were treated with double distilled water, i.g., once every day; ii) the SBTI group, in which the animals were treated with 20 mg/kg/day SBTI (Sigma), i.g., once every day according to a protocol previously described by Matsumoto *et al* (9); and iii) the PNFE-treated group, in which animals were administered 120 mg/kg/day of PNFE, i.g., once daily. C57 BL/6 mice (7 months of age; n=8) were used as a normal untreated group, in which animals were treated with double distilled water, i.g., once every day. Treatments for all groups lasted for 9 consecutive weeks.

**Left ventricular mass quantification.** At the end of the treatment period, mice were sacrificed by an overdose of pentobarbital sodium. The chests of the mice were cut open, and both the left and right atria and ventricles were dissected and weighed. Left ventricular hypertrophy index (LVHI) was calculated using the following formula: LVHI = ventricle weight/body weight (mg/g). The myocardial tissues were also used for PCR and western blot analysis.

**Reverse transcription (RT)-PCR analysis.** Subsequent to left ventricular mass quantification, the left ventricular tissues were stored in dry ice. Total RNA was extracted from 100 mg ventricular tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The ventricular tissues were pooled in equal amounts in each group to reduce inter-animal variations. RT-PCR for the detection of the mRNA expression of chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7 was performed. Reversed transcription to cDNA was synthesized by analyzing 5 mg of the total RNA sample with SuperScript II reverse transcriptase and oligo(dT) primer (Invitrogen). The reaction was carried out in the presence of first-strand buffer, 1 mmol/l dNTPs and 20 mol/l dithiothreitol. The PCR mixture contained 1 ml of the cDNA reaction mixture, 20 pmol/l primers, PCR buffer, 0.4 mmol/l dNTPs and 2.5 units *Taq* polymerase. The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide, and the samples were then visualized by ultraviolet transillumination. The reaction was performed with a RoboCycler (Stratagene, LaJolla, CA, USA). Sequences of the oligonucleotide primers for PCR are shown in Table I.

**Western blot analysis.** Ventricular tissues (100 mg) were lysed in 0.30 ml lysis buffer consisting of 20 mmol/l Tris/HCl, pH 7.4, 100 mmol/l NaCl, 10 mmol/l sodium pyrophosphate,

Table I. Primers used in the RT-PCR analysis.

Primer pair	Sequence (5'-3')	Fragment size (bp)
$\beta$ -actin	CCTCTATGCCAACACAGTGC	211
Chymase	AGCTCACTGTGCGGGAAGGTCTA	231
TGF- $\beta$ 1	GCACCGGAGAGCCCTGGATAC	226
Smad2	CTCTCCGGCTGAACTGTCTCCTAC	187
Smad3	TCCACGCCTCACAGCCATCCAT	244
Smad7	CCCGGCGGCGAGGACGAGGAG	183

5 mmol/l EDTA, 50 mmol/l NaF, 1 mmol/l sodium vanadate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 10% (w/v) glycerol, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 1  $\mu$ mol/l leupeptin, 0.1  $\mu$ mol/l aprotinin, 1  $\mu$ mol/l phenylmethylsulphonyl fluoride and 1  $\mu$ mol/l pepstatin. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples were loaded onto a 10% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes (Pall, East Hill, NY, USA), analyzed with antibodies according to the manufacturer's instructions and visualized with peroxidase on an enhanced chemiluminescence system (ECL kit; Pierce Biotechnology). The following antibodies were used in the western blot analyses: anti-chymase, anti-TGF- $\beta$ 1, anti-Smad2, anti-Smad3, anti-Smad7 and anti- $\beta$ -actin (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Statistical analysis.** All data are expressed as the means  $\pm$  SD. Comparisons of data among multiple treatment groups were evaluated by one-way analysis of variance (ANOVA) followed by *post-hoc* analysis (Fisher's test).  $p < 0.05$  was found to be statistically significant.

## Results

**PNFE attenuates heart remodeling.** As shown in Table II, the cardiac hypertrophy indexes of human chymase transgenic mice were significantly higher than those of C57 BL/6 mice ( $p < 0.05$ ), whereas PNFE and SBTI treatment resulted in a significant reduction in LVHI, indicating beneficial effects of these two drugs on the cardiac hypertrophic state and an improvement in left ventricular remodeling in this transgenic animal model.

**Expression of ventricular mRNAs encoding chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7.** The RT-PCR analysis showed that expression of mRNAs encoding chymase, TGF- $\beta$ 1, Smad2 and Smad3 mRNA in the transgenic mice was significantly higher than that of the C57 BL/6 mice. Treatment with PNFE effectively lowered the expression of ventricular mRNAs encoding chymase, TGF- $\beta$ 1, Smad2 and Smad3. SBTI, which was used as a positive control in our experiment, also exerted an attenuating effect similar to that of PNFE. On the other hand, Smad7 mRNA was found to be lower in transgenic mice than in C57 BL/6 mice, and treatment with PNFE significantly up-regulated its expression (Fig. 1).

Table II. Left ventricular hypertrophy indexes of all groups.

Treatment group	n	VW (mg)	BW (g)	VW/BW (mg/g)
Normal	8	174.4 $\pm$ 20.73	29.14 $\pm$ 1.57	5.99 $\pm$ 0.54
Control	8	212.5 $\pm$ 35.85 <sup>a</sup>	30.99 $\pm$ 2.33	6.83 $\pm$ 0.83 <sup>a</sup>
SBTI	8	173.5 $\pm$ 17.84 <sup>b</sup>	29.75 $\pm$ 1.84	5.83 $\pm$ 0.41 <sup>b</sup>
PNFE	8	176.1 $\pm$ 18.86 <sup>b</sup>	30.35 $\pm$ 1.97	5.80 $\pm$ 0.76 <sup>b</sup>

All values present the means  $\pm$  SD. <sup>a</sup> $p < 0.05$  compared to the normal group. <sup>b</sup> $p < 0.05$  compared to the control group. PNFE, *Panax notoginseng* flower extract; SBTI, soybean trypsin inhibitor; VW, ventricle weight; BW, body weight.

**Protein expression of chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7.** The protein expression levels of chymase, TGF- $\beta$ 1, Smad2 and Smad3 were markedly up-regulated in the myocardium of human chymase transgenic mice, and the treatment with PNFE and SBTI significantly suppressed their expression. On the contrary, the protein expression of Smad7 was significantly lower than that in the control group. After treatment with PNFE and SBTI, the Smad7 protein levels were significantly elevated when compared to those of the control (Fig. 2).

## Discussion

*Panax notoginseng*, known as San-qi in Chinese, is a well-known and commonly used Chinese medicine. Due to its efficacy in promoting blood circulation, removing blood stasis, relieving swelling and alleviating pain, the root part of *Panax notoginseng* has been widely used to treat hemoptysis and hematomas in Chinese medical practice for centuries. More than 85% of *Panax notoginseng* comes from the Wenshan region in Yunnan Province, China (28). Phytochemical studies on the roots, leaves and flower buds of *Panax notoginseng* over the past several decades have led to the isolation and characterization of more than 50 dammarane-type saponins, which are believed to be the main bioactive components (29). Ginsenoside Rb3, one of the panoxadiols found in many Ginseng species, presents in a higher amount (2.58%) in the flower buds of *Panax notoginseng* than in the root (30). However, since the most commonly used part of *Panax notoginseng* in herbal medicine is the root, the pharmacological effects of other parts, such as the flowers and leaves, have yet to be examined.

It has been reported that *Panax notoginseng* saponins have hemodynamic effects on anesthetized dogs (31). Recently, it was shown that *Panax notoginseng* flower extract possesses anti-cancer activity *in vitro* and *in vivo* (32-34), and protective effect on liver injury (35). The extract has also been shown to inhibit LPS-induced inflammatory response via the inhibition of the NF- $\kappa$ B signaling pathway in macrophages (36). Furthermore, it exhibited a neuroprotective effect on cultured neurons and the underlying action mechanism may involve anti-oxidative activity (37). By using AB-8 macroporous resin as the column packing material, we were able to separate and purify total saponins from *Panax notoginseng* flowers, and panoxadiol

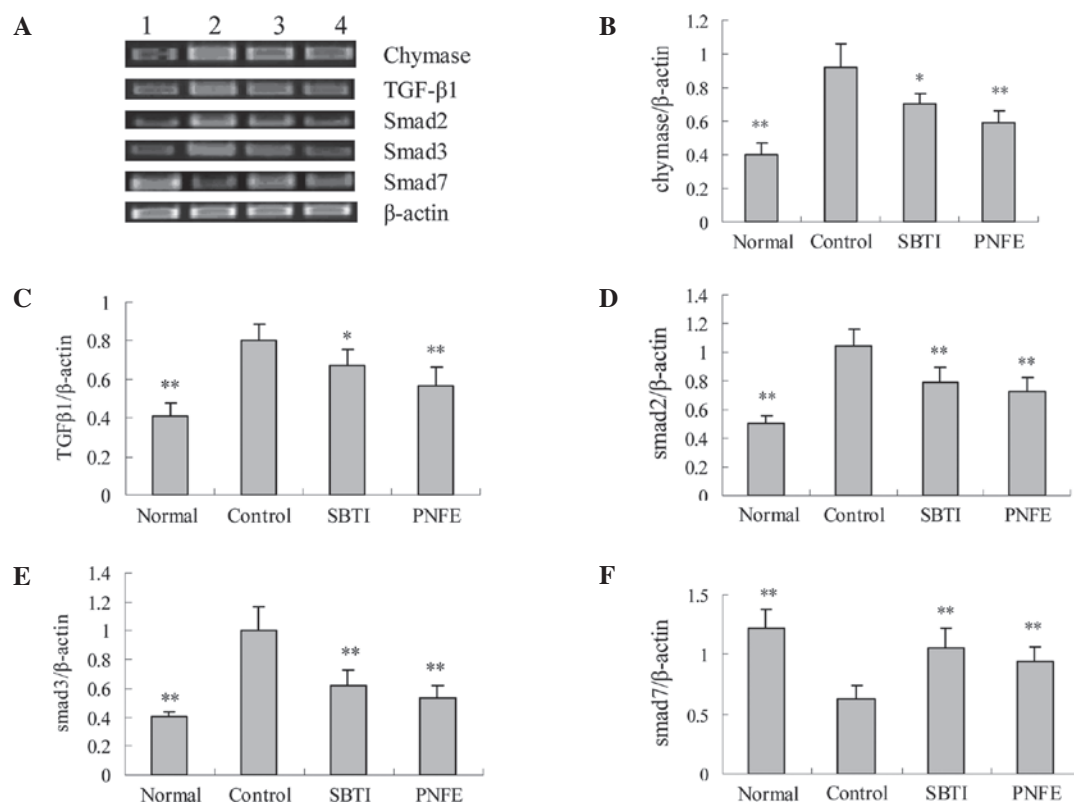


Figure 1. Effects of PNFE on the mRNA expression of chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7 in the myocardium of human chymase gene transgenic mice. (A) Representative examples of RT-PCR results (lane 1, normal; lane 2, control; lane 3, SBTI; lane 4, PNFE). Data are the means  $\pm$  SD (n=5). \*p<0.05; \*\*p<0.01 compared to the control group.

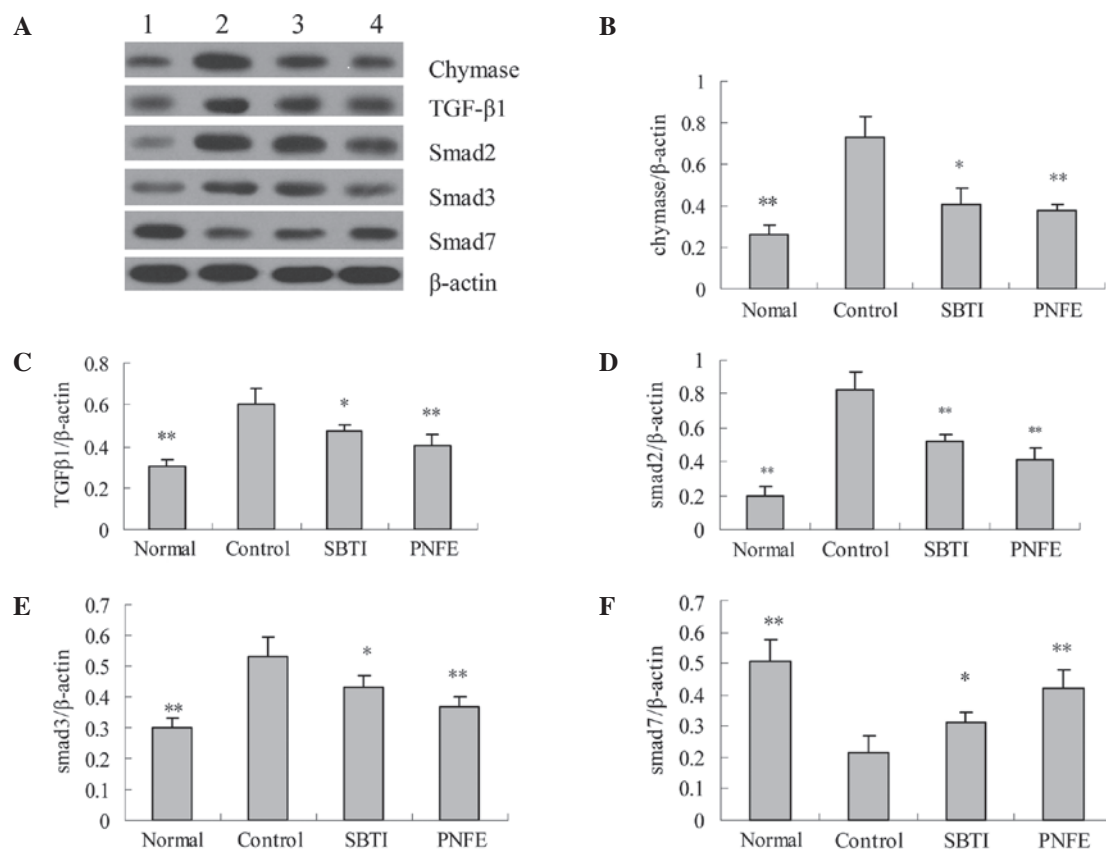


Figure 2. Effects of PNFE on the protein expression of chymase, TGF- $\beta$ 1, Smad2, Smad3 and Smad7 in the myocardium of human chymase gene transgenic mice. (A) Representative Western blotting of protein expression. Bar chart presentation of the protein expression of (B) chymase, (C) TGF- $\beta$ 1, (D) Smad2, (E) Smad3 and (F) Smad7. Data are the means  $\pm$  SD (n=4). \*p<0.05; \*\*p<0.01 compared to the control group.



and notoginsenoside Rb3 were found to be the main saponin components in the extract. In this study, the total saponins in the dry PNFE was quantified to be 87.6%, and the amount of ginsenoside Rb1 and Rb3 was 4.12 and 8.93%, respectively.

Chymase, an alternative pathway for the generation of Ang II, exists in the heart, and has a higher specificity in humans for the conversion of Ang I to Ang II (5). Chymase plays an important role in heart remodeling by increasing Ang II formation, activating matrix metalloproteinase 9 (MMP-9), and regulating collagen I gene expression (8). Human chymase transgenic mice have widely been used as a hypertrophic cardiomyopathy model (38). In the present study, LVMI, chymase mRNA and protein levels in the myocardium of human chymase transgenic mice were significantly increased compared to those of C57 BL/6 mice. Moreover, TGF- $\beta$ 1, Smad2 and Smad3 mRNA and protein levels in the myocardium of human chymase transgenic mice were markedly elevated, while Smad7 expression was reduced. The study also suggested that the expression of the human chymase gene in heart tissues of mice caused increased Ang II production and resulted in the hypertrophy of myocardiocytes, as well as an increase in the left ventricular anterior wall and posterior wall. In addition, it has been reported that chymase activates latent TGF- $\beta$  to form mature TGF- $\beta$ , causing an increased collagen production (39,40). Moreover, it has been shown that chymase inhibition suppresses TGF- $\beta$ 1 transcription levels and prevents cardiac fibrosis in animal models (9,18).

TGF- $\beta$ 1 is the predominant TGF- $\beta$  isoform found in the heart, and through its signaling pathway TGF- $\beta$ 1 activates effector proteins called Smads. The TGF- $\beta$ 1-Smad signaling pathway plays a crucial role in the development of cardiac remodeling (41). Smad2/Smad3 are key mediators of TGF- $\beta$ -mediated activation according to the experimental findings of several *in vitro* and *in vivo* studies (42-45), while Smad6 and Smad7, the inhibitory Smads, are key regulators of TGF- $\beta$  signaling by negative feedback loops (46,47).

Traditional Chinese medicine has long been used to treat many cardiovascular diseases, such as hypertension and coronary heart disease; however, the effect of Chinese herbal medicine on the TGF- $\beta$ 1/Smad signaling activation in hypertrophic remodeling in chymase transgenic animals has not been previously reported. In this study, we demonstrated for the first time that PNFE and SBTI, two proteinase inhibitors of tryptase and chymase (9,14), significantly affected heart remodeling and altered the TGF- $\beta$ /Smad signaling transduction pathway in the myocardium of human chymase gene transgenic mice. PNFE treatment for 9 consecutive weeks was able to attenuate the LVMI, chymase mRNA and protein levels. In addition, PNFE treatment was capable of reducing TGF- $\beta$ 1, Smad2, Smad3 mRNA and protein levels in the myocardium of human chymase transgenic mice. Treatment with PNFE and SBTI markedly accentuated the Smad7 mRNA and protein levels.

Taken together, our experimental results suggest that TGF- $\beta$ 1/Smad signaling activation plays a critical role in the development of cardiac remodeling in human chymase transgenic mice. Treatment with PNFE effectively prevented heart remodeling in human chymase gene mice via modulating the expression of mRNA and protein of TGF- $\beta$ /Smads in the ventricular tissues. The experimental results help confirm a scientific foundation for the use of PNFE for treating cardiac hypertrophy in Chinese medical practice, and pave the way

for further development of this natural product as an effective pharmaceutical agent for the treatment of this common debilitating cardiac condition.

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