Biomarkers, 2012; 17(5): 407–415

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.677860

informa
healthcare

RESEARCH ARTICLE

LTBP-2 acts as a novel marker in human heart failure – a preliminary study

Yuanyuan Bai, Peng Zhang, Xiaoling Zhang, Jie Huang, Shengshou Hu, and Yingjie Wei

State Key Laboratory of Cardiovascular Disease, National Center for Cardiovascular Disease and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, P. R. China

Abstract

Background: We have observed increased expression of latent TGF-β binding protein (LTBP)-2 mRNA in human failing hearts. This study was aimed to further confirm LTBP-2 act as a novel marker in human acute heart failure.

Methods and results: We demonstrated that median level of LTBP-2 in myocardial samples from heart failure patients was significantly elevated, and TGF-β1 significantly promoted LTBP-2 expression in neonatal rat cardiomyocytes. To investigate the potential of LTBP-2 as a biomarker to diagnose heart failure with reduced ejection fraction (HFREF), another cohort of 133 consecutive patients with dyspnea were enrolled. In receiver operating characteristic (ROC) curve analyses to detect HFREF, LTBP-2 achieved an area under curve (AUC) of 0.67 (95% confidence intervals (CI) 0.58–0.75), comparable to the diagnostic ability of NT-proBNP 0.68 (95% CI 0.59–0.77).

Conclusion: The serum LTBP-2 levels might act as a promising biomarker in HFREF.

Keywords: Latent TGF-β binding protein 2 (LTBP-2), heart failure, heart failure with reduced ejection fraction, signaling pathway, biomarker

Introduction

The latent TGF-β binding protein (LTBP) family consists of four members, namely LTBP-1, -2, -3, and -4 (Michel et al. 1998). LTBPs were initially identified as part of the large latent TGF-β complex (Miyazono et al. 1998). They have generally been considered to be involved in the assembly, secretion, and targeting of TGF-β to sites at which it is stored and/or activated, suggesting these proteins may play critical roles in controlling and directing the activity of TGF-β (Oklü et al. 2000). LTBPs may also exert effects independently of those associated with TGF-β, for example as a component of the extracellular matrix (ECM) glycoproteins of various cell types and tissues (Oklü et al. 2000; Taipale et al. 1996). Human LTBP-2 is expressed mostly in the lung and to a lesser extent in the liver, skeletal muscle placenta and heart (Moren et al. 1994).

Regarding a functional role of LTBP-2 in the cardiovascular system, it was reported that LTBP-2 synthesis increased following angioplasty-induced injury to the arteries in a porcine model of coronary angioplasty, suggesting some role for LTBP-2 in tissue repair processes (Sinha et al. 2002). Recent advances in genomics have led to the discovery of novel pathways in the pathophysiology of heart failure. Using gene microarray technology, we (Wei et al. 2008) and Gabrielsen group (Gabrielsen et al. 2007) observed a significant increase of LTBP-2 mRNA in failing hearts compared with non-failing control hearts. In a mice model of heart failure, LTBP-2 mRNA was also shown to be upregulated in failing myocardium compared with non-failing control myocardium (Bilchick et al. 2006). This study was designed to further validate the increase of cardiac LTBP-2 at protein level in

Address for Correspondence: Shengshou Hu, State Key Laboratory of Cardiovascular Disease, National Center for Cardiovascular Disease and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, P. R. China. Tel: +8610-88398494. Fax: +8610-88396050; Yingjie Wei, State Key Laboratory of Cardiovascular Disease, National Center for Cardiovascular Disease and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, P. R. China. Tel: +8610-88398494. Fax: +8610-88396050. E-mail: weiyingjie@fuwaihospital.org

(Received 09 February 2012; revised 12 March 2012; accepted 17 March 2012)

human failing hearts and clarify the underlying signaling pathways responsible for elevated LTBP-2 in cultured rat cardiomyocytes. We also tested the hypothesis that human serum LTBP-2 levels are increased in patients with heart failure with reduced ejection fraction (HFREF) and analyzed potential value of serum LTBP-2 as a biomarker to confirm and rule out the presence of HFREF in patients with dyspnea.

Methods

Patients and sample collection

For histological analyses and enzyme-linked immunosorbent assay (ELISA) of cardiac LTBP-2, human left ventricular (LV) myocardial samples were obtained from 34 patients diagnosed with end-stage heart failure (HF) due to dilated cardiomyopathy (DCM, n = 19), ischemic cardiomyopathy (ICM, n = 8), and arrythmogenic right ventricular cardiomyopathy (ARVC, n = 7) during cardiac surgery at the time of heart transplantation and eight control subjects without heart failure who had died from accidents and had no history of heart disease. The clinical and hemodynamic features of the 34 HF patients were referred to our previous study (Wei et al. 2011). The clinical diagnosis of ARVC was made with echocardiography and nuclear magnetic resonance imaging (MRI). Diagnosis of ICM was based on coronary angiography and MRI, and diagnosis of DCM was based on electrocardiogram and echocardiography. All the patients before heart transplantation had a relatively standard therapeutic regimen, including diuretics, digoxin or intravenous inotropes, and angiotensin-converting-enzyme inhibitors, and none of them had other organ failures or detected diseases. The transplantation was performed according to the standard procedure developed at Fuwai hospital.

For determination of serum LTBP-2 and NT-proBNP, blood samples were acquired from a cohort of 133 enrolled consecutive patients with dyspnea referred to out-patient clinic or admitted into our institution (Fuwai Hospital, Beijing, China) between September 2007 and December 2008. On enrollment, data including detailed history, biochemistry measurements, electrocardiogram and echocardiography were collected. Left ventricular ejection fraction (LVEF) was used to divide the patients into two groups according to the severity of the ventricular impairment based on assessment by echocardiography (Remme and Swedberg 2002). Patients were diagnosed as HFREF if the LVEF was ≤40% (n = 67), or heart failure with preserved ejection fraction (HFPEF) if the LVEF was >40% (n = 66). Another cohort of subjects (n = 87) who had routine health check-up over the same period were enrolled as healthy controls without clinical risk factors and echocardiographic abnormalities. The mean age of healthy controls was 40.5 years and most of the subjects were males (87%). Blood samples (5 mL) were collected from all the enrolled patients and control subjects. Serum was prepared by clotting the blood at 4°C for 2 h followed by centrifugation at 3000 rpm for 15 min, and subsequently subdivided and stored at –70°C until test.

All patients and control subjects gave written informed consent for this investigation, which was approved by the Institutional Ethical Review Board of Fuwai Hospital. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

Histopathology, immunohistochemistry, and immunocytochemistry

For histopathological analysis, samples were fixed in 10% neutral buffered formalin. Dehydration was accomplished through alcohol and xylene gradients, followed by embedding in paraffin. Serial 5-μm sections were prepared and stained with hematoxylin and eosin (HE) to assess morphologic features of the failing and non-failing hearts.

For immunohistochemical analysis, sections were fixed for 10 min in 4% paraformaldehyde. For immunocytochemical analysis, cardiomyocytes were grown on coverslips, washed in phosphate buffered saline (PBS), and fixed for 15 min in 4% paraformaldehyde. Samples were permeabilized with 0.2% Triton X-100 for 5 min, blocked in 3% bovine serum albumin (BSA), and then incubated with rabbit anti-human LTBP-2 antibody (1 : 200, Cat.#17708-1-AP, ProteinTech Group Inc., IL, USA) for 1 h at room temperature and washed in PBS buffer for 10 min, followed by incubation with IgG-peroxidase conjugated secondary antibody (Sigma, St. Louis, MO, USA) for 1 h at room temperature, washed in PBS buffer for 10 min, and incubated with 0.5mg/mL diaminobenzidine tetrahydrochloride 2-hydrate plus 0.05% H_2O_2 for 5 min. Negative control samples were incubated with secondary antibody alone. All the slides were stained with hematoxylin, dehydrated, mounted, and captured by Olympus BX61 (Tokyo, Japan) microscope.

Enzyme-linked immunosorbent assay

The commercial human LTBP-2 ELISA Kit (Cat. # ESBL2312, Ever Systems Biology Laboratory Inc., Sacramento, CA, USA) and human NT-proBNP ELISA kit (Cat. # ESBL2196, Ever Systems Biology Laboratory Inc., Sacramento, CA, USA) were used in this study to determine cardiac or serum LTBP-2 and NT-proBNP levels according to the manufacturer's protocols. The minimum detectable dose of human LTBP-2 and NT-pro BNP was typically less than 20 pg/mL and 15 pg/mL, respectively. The sensitivity of this assay, or lower limit of detection was defined as the lowest detectable concentration that could be differentiated from zero. The detection range of human LTBP-2 and NT-pro BNP was 40–10,000 pg/mL and 40–8400 pg/mL, respectively. Intra- and inter-assay coefficient of variation was below 10% and 15%. The resultant reaction was read at a wavelength of 450 nm on 96-well Microplate Rader (Bio Rad, Mode 680, Tokyo, Japan).

Culture and treatment of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured as described previously (Jeyaseelan et al. 1997). After the cells started beating 48 h post-culture, they were cultured in serum-free medium for 24 h and then treated without or with human transforming growth factor-β1 (TGF-β1) (R&D Systems, Minneapolis, MN, USA) at doses of 2, 5, and 10 ng/mL for 24 h or at a dose of 5 ng/mL for different time points of 3, 6, 12, and 24 h. In experiments involving inhibitors, the cells were pretreated with different specific inhibitors for 2 h prior to the addition of TGF-β1 at 5 ng/mL and the incubations continued for 24 h, at which time point the cells were harvested for real-time quantitative RT-PCR and western blot analysis.

The specific inhibitors were used in this study. SB431542 (TGF-β type Ireceptor inhibitor), SB203580 (p38 MAP kinase inhibitor), PD-98059 (ERK1/2 inhibitor), and LY294002 (PI3 kinase inhibitor) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). SP600125 (JNK1/2 inhibitor) was purchased from Calbiochem (MerckBiosciences, Darmstadt, Germany). The final concentrations of inhibitors were 10 μM in SB431542, 10 μM in SB203580, 20 μM PD-98059, 10 μM in LY294002, and 10 μM in SP600125.

The procedure using rats for primary NRVMs culture was approved by the Animal Laboratory Use and Care Committee at Fuwai Hospital. Studies also conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996).

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA from cultured NRVMs was isolated and the corresponding cDNA was reversely transcribed (RT) from 500 ng of purified total RNA. Primers specific for LTBP-2 (forward, ATG CTC TTG TGA GCC AGG CTA TGA -3′; reverse, 5′- AGT GCC ATC TTC GTT CAC CCA GTA -3′) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (forward, 5′-GGC ACA GTC AAG GCT GAG AAT G-3′; reverse, 5′-ATG GTG GTG AAG ACG CCA GTA-3′) were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). Optimized primer/probe mixture solution of LTBP2 and GAPDH were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Real-time quantitative polymerase chain reaction (PCR) was performed as described previously (Cui et al. 2012).

Western blot analysis

The relative abundance of LTBP-2 was examined in cultured NRVMs using standard immunoblotting procedures as described previously (Seeland et al. 2007). Western blot was performed using specific primary antibodies for rat LTBP-2 (Santa Cruz Biotechnology, CA, USA), Smad3, P-Smad3, ERK1/2, P-ERK1/2, p38, P-p38, JNK1/2, P-JNK1/2, Akt, P-Akt (Cell Signaling Technology, MA, USA), and β-tubulin (Abmart, Shanghai, China).

Statistical analysis

Continuous and categorical variables are presented as the mean ± standard deviation and percentages, respectively. Non-normally distributed data are presented as median (25th ~ 75th percentile). Group comparisons of continuous and categorical variables were made using Pearson's χ^2 tests and *t* tests. Group comparisons of non-normally distributed data were made using Mann–Whitney's *U* test. Receiver operating characteristic (ROC) curves were plotted to assess the diagnostic accuracy of LTBP-2 and NT-proBNP and their combination. A cut-off value reflecting the best combination of sensitivity and specificity was ascertained, and the corresponding positive and negative predictive values [positive predictive value (PPV) and negative predictive value (NPV)] were calculated. Linear regression analysis was performed to examine the correlation between two variables. All *p* values reported are two-sided and were regarded as statistically significant if $p < 0.05$. Data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Elevation of cardiac LTBP-2 protein in human failing hearts

In the light microscope examination by HE staining, compared to normal hearts, varying degree of interstitial fibrosis in failing hearts due to ICM and DCM, and fibrofatty or fatty replacement in failing hearts due to ARVC were observed (Figure 1A). In the immunohistochemical analyses using specific antibody against LTBP-2, strongly immunoreactive LTBP-2 was observed in the failing hearts due to ICM, DCM, and ARVC in contrast to normal control hearts with few and weak immunoreactive LTBP-2 (Figure 1B). The diffuse staining pattern of LTBP-2 protein in failing hearts might reflect the fact that they are secreted.

ELISA further confirmed that the median level of cardiac LTBP-2 were significantly increased in heart failure patients ($n = 34$) compared with normal control subjects ($n = 8$) (974 pg/mg vs. 193 pg/mg, $p < 0.001$) (Figure 1C).

Promotive effects of TGF-β1 on LTBP-2 expression and its underlying signaling pathway in NRVMs

We observed that LTBP-2 mRNA expression increased after 24 h treatment with TGF-β1 in a dose-dependent manner in cultured NRVMs (Figure 2A), and TGF-β1 at the optimal dose (5 ng/mL) promoted LTBP-2 expression at both the mRNA (Figure 2B) and protein levels (Figure 2C) in a time-dependent manner. By immunocytochemical analysis, we further observed strong immunoreactivity of LTBP-2 in cultured NRVMs after TGF-β1 treatment for 12 h at a dose of 5 ng/mL, in a striking contrast to the control cells with weak

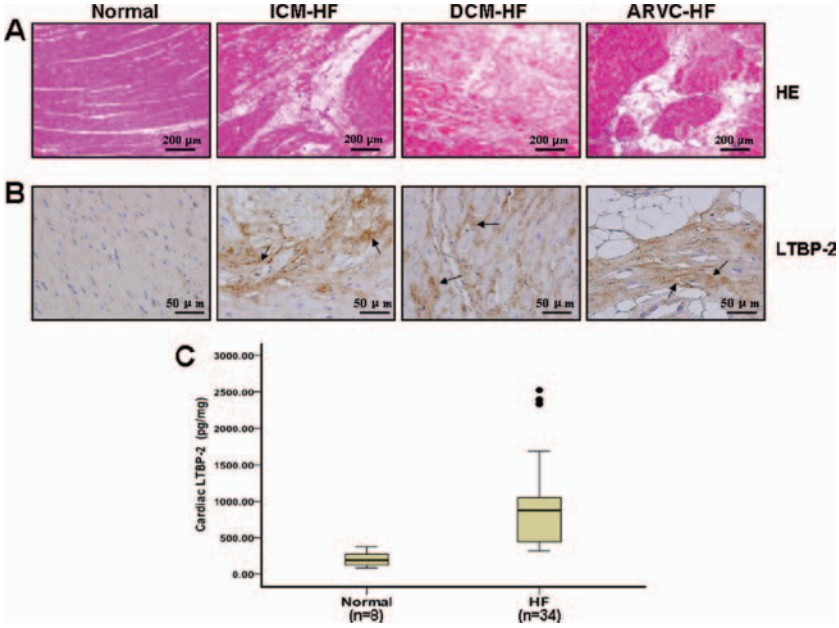

Figure 1. Detection of cardiac latent TGF-β binding protein (LTBP)-2 in the human failing heart myocytes. (A) Hematoxylin and eosin (HE) staining shows the normal appearance of myocardial fibers with central nuclei in non-failing control hearts (normal). Extracellular matrix remodeling and fibrosis are observed in the failing hearts due to ischemic cardiomyopathy (ICM-HF), dilated cardiomyopathy (DCM-HF), and arrhythmogenic right ventricular cardiomyopathy (ARVC-HF). (B) Immunohistochemical analysis shows few and weak immunoreactivity of LTBP-2 in non-failing control hearts (normal), but more and strong immunoreactivity of LTBP-2 can be seen in the failing hearts due to the three kinds of cardiomyopathy. Arrows indicated the positive staining. Scale bar = 200 μm for HE staining. Scale bar = 50 μm for immunochemistry. (C) Enzyme-linked immunosorbent assay shows the median level of cardiac LTBP-2 were significantly elevated in heart failure patients (HF, n = 34) compared with the normal control subjects (normal, n = 8) (974 pg/mg vs. 193 pg/mg, $p < 0.001$). The bottom and top I bars indicate the 5th and 95th percentile levels; the lower and upper boundaries of the boxes, the 25th and 75th percentile levels; and the horizontal lines within the box, the median levels.

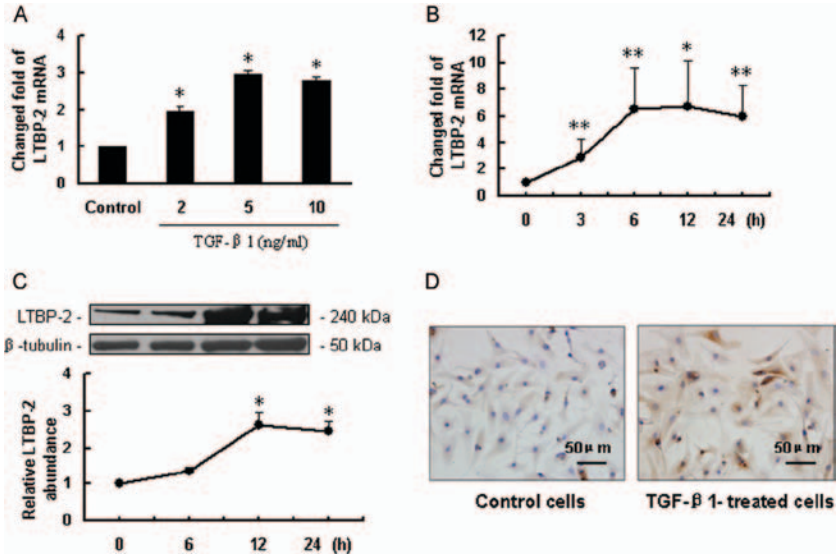

Figure 2. Promotive effect of TGF-β1 on latent TGF-β binding protein (LTBP)-2 expression in cultured neonatal rat ventricular myocytes (NRVMs). (A) LTBP-2 mRNA expression increased in a dose-dependent manner after TGF-β1 treatment for 24 h (*$p < 0.05$ compared with control group). (B and C) TGF-β1 at the optimal dose (5 ng/mL) promoted LTBP-2 expression at both the mRNA and protein levels in a time-dependent manner (*$p < 0.05$, $p < 0.01$ compared with the 0 h time point). (D) Strong immunoreactivity of LTBP-2 was observed after TGF-β1 treatment for 12 h at a dose of 5 ng/mL, in contrast to the control cells with weak immunoreactivity of LTBP-2. Scale bar = 50 μm. Data are presented as mean ± standard deviation (n = 3).

immunoreactivity of LTBP-2 (Figure 2D). Further signaling pathway study demonstrated that TGF-β1 induced expression of LTBP-2 in NRVMs via ERK pathway and PI3K pathway (Figure 3).

Serum LTBP-2 levels and its correlation with LVEF in patients and healthy controls

The baseline characteristics of 133 enrolled patients with dyspnea are presented in Table 1. Box plots show

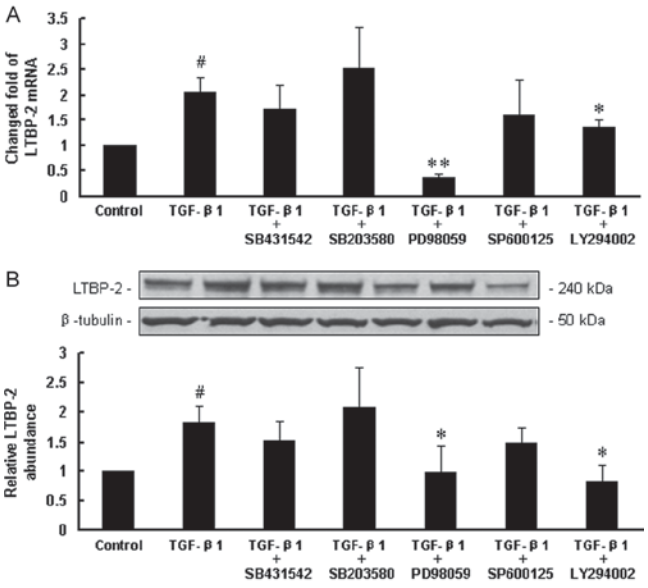

Figure 3. TGF-β1-induced latent TGF-β binding protein (LTBP)-2 expression can be attenuated by specific inhibitors of PI3K and ERK1/2 in cultured neonatal rat ventricular myocytes (NRVMs). The cultured NRVMs were pretreated with different specific inhibitors for 2 h prior to the addition of TGF-β1 at a dose of 5 ng/mL and the incubations continued for 24 h, at which time point the cells were harvested for total RNA and protein isolation. (A) Real-time quantitative reverse transcription polymerase chain reaction shows that upregulation of LTBP-2 mRNA induced by TGF-β1 is attenuated by PD-98059, inhibitor of ERK1/2 and LY-294002, inhibitor of PI3K. (B) Western blot and densitometric analyses show that upregulation of LTBP-2 protein induced by TGF-β1 is attenuated by PD-98059, inhibitor of ERK1/2 and LY-294002, inhibitor of PI3K. The internal standard β-tubulin was used to normalize for equal protein loading. Band intensities of the LTBP-2 and β-tubulin signals were assessed by densitometry. Data are presented as mean ± standard deviation ($n = 3$). #$p < 0.05$ compared with control group; *$p < 0.05$, $p < 0.01$ compared with TGF-β1-treated group.

the median levels of serum LTBP-2 (Figure 4A) and serum NT-pro BNP (Figure 4B) measured in normal control subjects (Normal, n = 87) and patients with a final diagnosis as HFREF (n = 67) and HFPEF (n = 66). The median LTBP-2 level in the normal group was 365.8 pg/mL, significantly lower than either those with HFREF and HFPEF (857.6 pg/mL and 587.1 pg/mL, $p <$ 0.001 in both cases). Similarly, the median NT-pro BNP level in the normal group was 109.1 pg/mL, significantly lower than those with HFREF (229.3 pg/mL, $p < 0.001$), and lower than those with HFPEF (126.5 pg/mL), but with no significant difference ($p = 0.08$). Patients with HFREF had significantly higher LTBP-2 and NT-proBNP levels than those with HFPEF (857.6 pg/mL vs. 587.1 pg/mL, 229.3 pg/mL vs. 126.5 pg/mL, all $p < 0.001$).

Linear correlation analyses in the enrolled 133 patients showed serum LTBP-2 was not correlated with NT-proBNP levels (r = 0.04, p = 0.26) (Figure 5A), but both serum LTBP-2 and NT-proBNP levels were negatively correlated with left ventricular ejection fraction. ($r = -0.31$, $p = 0.001$ and $r = -0.32$, $p < 0.001$, respectively) (Figure 5B and 5C).

Table 1. Baseline characteristics of 133 enrolled patients with dyspnoea.

	HFREF (LVEF ≤ 40%)	HFPEF (LVEF > 40%)	p Value
Case number	67	66	
Age (years)	51 ± 12.6	56.3 ± 11.5	0.012*
Male (%)	57	61	0.18
BMI (kg/m^2)	23.4 ± 3.3	25.6 ± 3.2	<0.001**
BSA (m^2)	1.79 ± 0.19	1.84 ± 0.16	0.13
Current smoker (%)	49.3	56.1	0.43
Drinking (%)	41.8	40	0.78
Hypertension (%)	25.4	48.5	0.006*
DM (%)	25.3	18.1	0.315
MI (%)	26.8	78.8	<0.001**
CAD (%)	31.3	78.8	<0.001**
Angina (%)	20.9	30.3	0.2
Primary valve disease (%)	13.4	16.7	0.6

BMI, body mass index; BSA, body surface area; CAD, coronary arterial disease; DM, diabetes mellitus; HFPEF, heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; LVEF, left ventricular ejection factor; MI, myocardial infarction.
*$p < 0.05$; **$p < 0.01$.

The ability of LTBP-2 to reflect human HFREF

ROC analyses showed similar diagnostic performance for LTBP-2 and NT-proBNP (AUC = 0.67 and 0.68, respectively) in separating HFREF from HFPEF (Figure 5D). A summary of the analytical parameters and predictive values is listed in Table 2. The cut off values for the diagnosis of HFREF were 601 pg/mL for LTBP-2 and 152 pg/mL for NT-proBNP. LTBP-2 showed a little higher positive predictive values and negative predictive values than NT-proBNP (58.5% vs. 54% and 62.7% vs. 57%, respectively). The sensitivity of both LTBP-2 and NT-proBNP are equal (71.6% and 71.6%, respectively). LTBP-2 showed lower specificity than NT-proBNP (51.5% vs. 62.10%).

Using a logistic regression model, the diagnostic capability of LTBP-2 and NT-proBNP were combined, generating a ROC curve with an AUC of 0.73 (95% CI 0.58–0.76) (Figure 5D), in which the specificity (83.3%), PPV (78%), and NPV (66.3%) were increased, but the sensitivity (58.2%) is decreased. The AUC of combined LTBP-2 and NT-proBNP showed a little better performance but was not significantly different from the AUC of LTBP-2 (0.73 v.s. 0.67, p = 0.11), and NT-pro BNP (0.73 vs. 0.68, p = 0.31) alone.

Discussion

Studies conducted in human failing hearts have identified a number of differentially expressed genes that may be importantant in the pathophysiology of heart failure but previously received little attention. Using gene microarray technology, we (Wei et al. 2008) and Gabrielsen group (Gabrielsen et al. 2007) observed a significant increase of LTBP-2 mRNA in failing hearts compared with non-failing control hearts. In a mice model of heart failure, LTBP-2 mRNA was also shown to be upregulated in

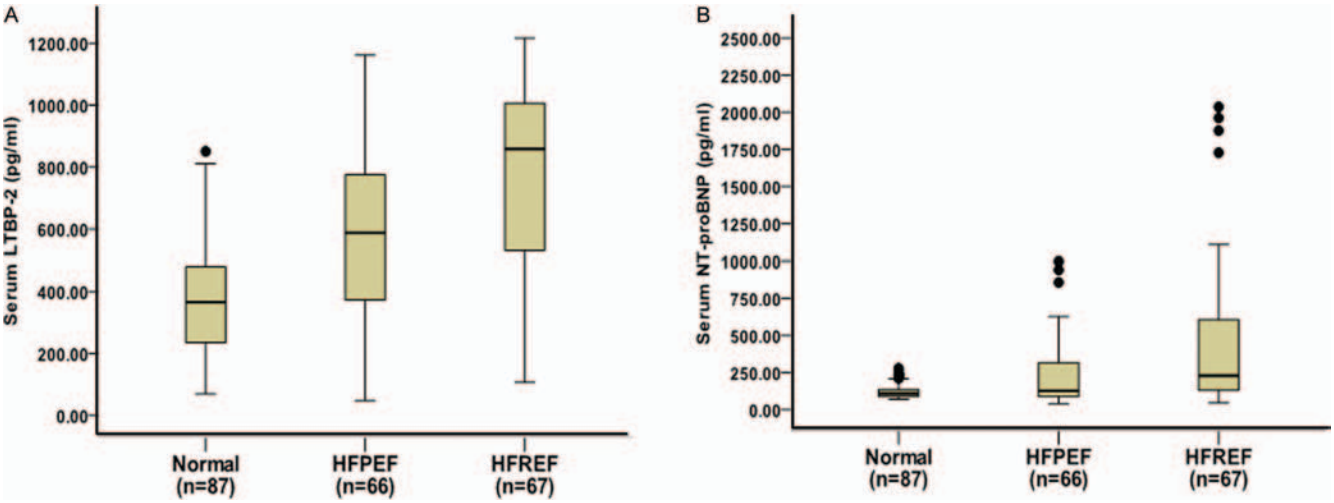

Figure 4. Median levels of serum latent TGF-β binding protein-2 (A) and NT-pro BNP (B) in patients with a final diagnosis as heart failure with reduced ejection fraction (HFREF) are significantly elevated compared with patients with a final diagnosis as heart failure with preserved ejection fraction (HFPEF) (both $p < 0.01$) and normal control subjects (both $p < 0.01$). The bottom and top I bars indicate the 5th and 95th percentile levels; the lower and upper boundaries of the boxes, the 25th and 75th percentile levels; and the horizontal lines within the box, the median levels.

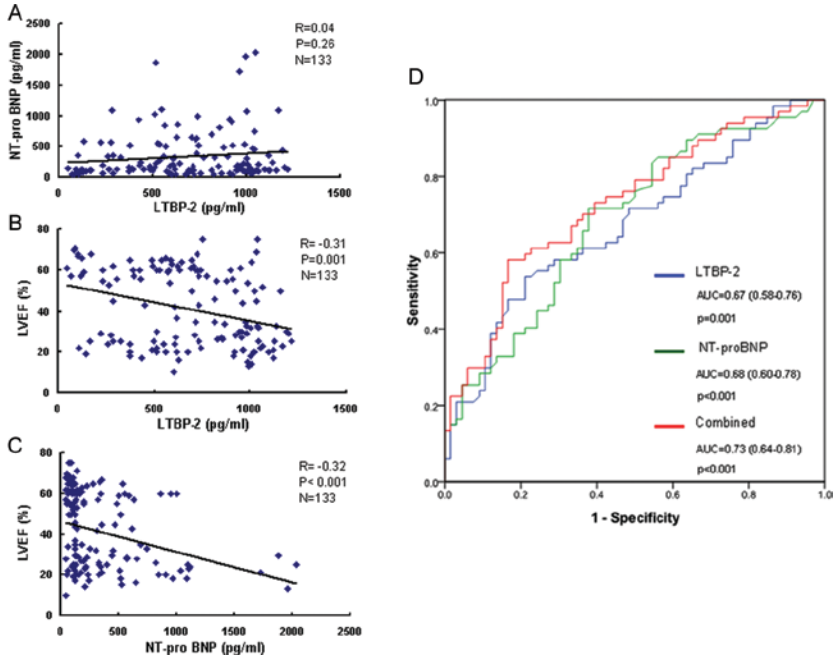

Figure 5. The correlation of serum LTBP-2 with LVEF and the ability of LTBP-2 to reflect HFREF. Scatter plots demonstrates no correlation between serum latent TGF-βbinding protein (LTBP)-2 and NT-proBNP (A), significant correlation between left ventricular ejection fraction (LVEF) and serum levels of LTBP-2 (B), NT-proBNP (C), and receiver-operator characteristic (ROC) curves for detection of heart failure with reduced ejection fraction by LTBP-2 (blue line), NT-proBNP (green line), and combined both (red line) (D).

failing myocardium (Bilchick et al. 2006). In this study, elevated cardiac LTBP-2 was further confirmed at protein level by ELISA and immunohistochemistry analyses in 34 patients with heart failure (Figure 1). Little is known about the pathophysiological and clinical implications of LTBP-2 in heart failure, which attract our interest.

First of all, we are very interested in the relationship between LTBP-2 and TGF-β1. The domain structure of LTBP-2 clearly places the molecule in the LTBP family (Oklü et al. 2000). Like other LTBPs, LTBP-2 was initially recognized as part of the large latent TGF-β1 complex (Moren et al. 1994), which may facilitate the secretion and activation of TGF-β1 (Oklü et al. 2000). However, later evidence indicates that LTBP-2 does not form covalent complexes with latent TGF-β1 in contrast to other LTBPs (Hirani et al. 2007). Therefore, LTBP-2 appears to have other roles. It was reported that LTBP-2 acts as a component of the ECM in various cell types and tissues (Moren et al. 1994; Hyytiäinen et al. 2003). It is an integral component of elastin-containing

Table 2. Predictive values and analytical parameters of LTBP-2 and NT-pro BNP in patients with heart failure with reduced ejection fraction.

	LTBP-2	NT-pro BNP	Combined
Cut off value (pg/mL)	601	152	
PPV (%)	58.50	54.00	78.00
NPV (%)	62.70	57.00	66.30
Sensitivity (%)	71.60	71.60	58.20
Specitivity (%)	51.50	62.10	83.30

LTBP, latent TGF-β binding protein; NPV, negative predictive value; PPV, positive predictive value.
The cut off value was assigned at the levels of maximal specificity and maximal sensitivity.

microfibrils, strongly binding to microfibrils and playing a structural role within elastic fibers in most cases (Shipley et al. 2000; Hirani et al. 2007). Heart failure is a condition in association with a progressive loss of contractile myocardium and development of ECM remodeling and fibrosis (Opie et al. 2006). In this study, we observed a significant elevation of LTBP-2 and obvious ECM remodeling and fibrosis accompanied by strong immunostaining of LTBP-2 in failing hearts (Figure 1). Although the present descriptive study did not provide direct evidence regarding the role of LTBP-2 in failing heart, our results together with the reported results of functional studies indicate that LTBP-2, acting as component of ECM, may directly contribute to the regulation of cardiac ECM and involved in cardiac remodeling in heart failure.

Although LTBP-2 does not form a complex with latent TGF-β1 to regulate secretion and activation of TGF-β1 (Hirani et al. 2007), TGF-β1 appears to regulate LTBP-2 expression. It has been reported that TGF-β1 dramatically increased LTBP-2 expression in human fetal lung fibroblasts, in which a member of the Ras super family and a protein kinase C are likely to be components in the signaling pathway (Ahmed et al. 1998).TGF-β1 is markedly induced and plays an important role in the pathogenesis of cardiac remodeling and fibrosis (Bujak et al. 2007). TGF-β1 may also play an important role in promoting ECM remodeling by inducing several ECM protein expression in a wide variety of cell types (Ahmed et al. 1998). Remodeling of ECM plays an important role in the progression of HF, and there are some ECM biomarkers for HF in the discovery phase, such as Galecin 3, matrix metalloproteinases and tissue inhibitors of metalloproteinases (Lok et al. 2010; Martos et al. 2009; Milting et al. 2008). Whether LTBP-2, acting as a component of ECM (Morén et al. 1994; Hyytiäinen et al. 2003), was also regulated by TGF-β1 and the underlying signaling pathways is not clear. We hypothesized that TGF-β1 might stimulate LTBP-2 expression in failing hearts. To test this hypothesis, we observed the effect of TGF-β1 on LTBP-2 in cultured rat cardiomyocytes. Our results showed that TGF-β1 indeed promoted LTBP-2 expression at both mRNA and protein levels (Figure 2). Through the use of specific inhibitors, ERK1/2 and Akt were shown to be signaling molecules responsible for elevated LTBP-2 (Figure 3). It was reported that Smads appear to be directly phosphorylated by TGF-β1 receptor, enter the nucleus where, in association with each other and other proteins, they bind to the promoters of target genes and modulate their expression (Massague et al. 1997). In our study, we also observed that TGF-β1 increased Smad3 expression in cultured rat cardiomyocytes (data not shown), but the inhibitor of TGF-β1 type 1 receptor, SB431542, could not block the TGF-β1-induced LTBP-2 increase, implying that Smad3 signaling molecule was not involved in the stimulating effect of TGF-β1 on LTBP-2. Our findings suggested that, in addition to its direct effect on cardiac remodeling (Bujak et al. 2007), TGF-β1 may also indirectly involve in cardiac remodeling by stimulating LTBP-2 production.

Another big interest to attract us is the potential of serum LTBP-2 as a biomarker in HFREF. In this study, cardiac LTBP-2 levels in the left ventricle from 34 heart failure patients with LVEF ≤40% were significantly elevated compared with the non-failing control sample (Figure 1). LVEF is a good clinical indicator of HFREF. HFREF is one of two forms of heart failure, generally defined as an LVEF ≤ 40% (Remme and Swedberg 2002). Based on our results and the role of LTBP-2 in ECM remodeling mentioned above, we assumed that serum LTBP-2 might act as a biomarker to reflect HFREF. In our enrolled 133 patients with dyspnea referred to out-patient clinic or admitted into our institution, we observed that serum LTBP-2 levels were detectable and inversely correlated with LVEF (Figure 5), and that LTBP-2 levels in the serum of patients with HFREF were significantly increased compared with those without HFREF (Figure 4). Our results suggested that higher concentrations of serum LTBP-2 were associated with a greater likelihood for a diagnosis of HFREF. For convenient to screen a large number of patients at risk of HFREF, the use of biomarkers would allow patients with HFREF who have less classical symptoms to be identified and in turn referred for echocardiographic evaluation and subsequently accurate therapy. Many studies have shown that NT-proBNP is a proven diagnostic biomarker for heart failure due to HFREF in patients with shortness of breath (McDonagh et al. 2004), NT-proBNP is related to the severity of poorer ventricular systolic function (Chen et al. 2006). We evaluated the median levels of serum LTBP-2 and serum NT-pro BNP measured in normal control subjects and patients with a final diagnosis as HFREF and HFPEF. Unlike LTBP-2, NT-proBNP had a less powerful discriminatory power in the ability to diagnose HFPEF. The reasons were unclear and might be partly due to the small sample size. In this study, we compared LTBP-2 with NT-proBNP in the diagnostic capacity to reflect the presence of HFREF. Our results showed that LTBP-2 has a similar diagnostic ability with NT-proBNP in the aspects of sensitivity, specificity, PPV, and NPV. The future of heart failure management will likely involve a multi-marker guided strategy that will incorporate the distinct but complementary information provided

by multiple biomarkers into clinical decision making (Taub et al. 2010). Thus, we analyzed the combined ability of LTBP-2 and NT-proBNP in the diagnosis of HFREF. ROC analysis showed that the combination of LTBP-2 and NT-proBNP slightly raised AUC for the diagnostic ability in separating HFREF from HFPEF (Figure 5D), with a significant increase in PPV and specificity, but a significant decrease in sensitivity (Table 2). While this work was in progress, Mueller's group reported that the plasma levels of LTBP-2 present a novel and powerful predictor of 1-year mortality in patients with heart failure, whose predictive ability is superior to NT-proBNP (Breidthardt et al. 2011).

A better understanding of mechanism of production of LTBP-2 in acute heart failure and the exact roles this biomarker may play in various stages of heart failure remains to be firmly established in further adequately powered studies. Nonetheless, the preliminary data from our study suggested that LTBP-2 may be involved in the pathophysiology of heart failure and extremely promising to act as a biomarker of significant impact in HF states.

Study limitations

First, we could not determine the source of elevated serum LTBP-2 levels in this study. It is necessary to prove that, in patients with heart failure, serum LTBP-2 mirrors what is occurring at the cardiac level. Although, we did not observe the association of myocardial expression of LTBP-2 with its serum level, a putative explanation might involve left ventricular systolic dysfunction leading to increased wall stress as a stimulus for LTBP-2 release. Another explanation might be the damaged myocardium leaking LTBP-2 into blood. Second, due to the small study population, a large multi-center population-based studies need to be performed to further test the capacity of serum LTBP-2 as a biomarker to diagnose HFREF.

Conclusion

In our experimental research, we demonstrated a significant elevation of cardiac LTBP-2 in heart failure patients with LVEF ≤40%. ERK1/2 and Akt were shown to be signaling molecules responsible for elevated LTBP-2 in NRVMs. In our clinical research, the test characteristics of LTBP-2 were similar to NT-pro BNP, and serum LTBP-2 levels may be used to confirm and rule out the presence of HFREF in patients with dyspnea. Our findings suggest that LTBP-2 may be involved in cardiac remodeling and might act as a promising biomarker in patients with acute heart failure. More exploration of LTBP-2 is needed to confirm the findings.

Declaration of interest

We certify that all authors have no financial or other conflict of interests in connection with the submitted article.This work was supported by research grants from the National 973 Program of China (2010CB529505 to S.S.H.), the National Natural Science Foundation of China (81170206 to Y.J.W.), and the Research Fund for the Doctoral Program of Higher Education of China (20091106110018 to Y.J.W).

References

Ahmed W, Kucich U, Abrams W, Bashir M, Rosenbloom J, Segade F, Mecham R, Rosenbloom J. (1998). Signaling pathway by which TGF-β1 increases expression of latent TGF-β binding protein-2 at the transcriptional level. *Connect Tissue Res* 37:263–276.

Bilchick KC, Saha SK, Mikolajczyk E, Cope L, Ferguson WJ, Yu W, Girouard S, Kass DA. (2006). Differential regional gene expression from cardiac dyssynchrony induced by chronic right ventricular free wall pacing in the mouse. *Physiol Genomics* 26:109–115.

Breidthardt T, Potocki M, Vanpoucke G, Gregoire T, Laroy W, Verleysen K, Drexler B, Mebazaa A, Kas K, Mueller CH. (2011). The novel marker Ltbp2 powerfully predicts all-cause and pulmonary death in patients with acute dyspnea. *Eur J Heart Fail* 10:S112.

Bujak M, Frangogiannis NG. (2007). The role of TGF-β signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res* 74:184–195.

Chen AA, Wood MJ, Krauser DG, Baggish AL, Tung R, Anwaruddin S, Picard MH, Januzzi JL. (2006). NT-proBNP levels, echocardiographic findings, and outcomes in breathless patients: results from the ProBNP Investigation of Dyspnoea in the Emergency Department (PRIDE) echocardiographic substudy. *Eur Heart J* 27:839–845.

Cui C, Shi Q, Zhang X, Liu X, Bai Y, Li J, Liu S, Hu S, Wei Y. (2012). CRP promotes MMP-10 expression via c-Raf/MEK/ERK and JAK1/ERK pathways in cardiomyocytes. *Cell Signal* 24:810–818.

Gabrielsen A, Lawler PR, Yongzhong W, Steinbrüchel D, Blagoja D, Paulsson-Berne G, Kastrup J, Hansson GK. (2007). Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium. *J Mol Cell Cardiol* 42:870–883.

Hirani R, Hanssen E, Gibson MA. (2007). LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol* 26:213–223.

Hyytiäinen M, Keski-Oja J. (2003). Latent TGF-β binding protein LTBP-2 decreases fibroblast adhesion to fibronectin. *J Cell Biol* 163:1363–1374.

Jeyaseelan R, Poizat C, Baker RK, Abdishoo S, Isterabadi LB, Lyons GE, Kedes L. (1997). A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *J Biol Chem* 272:22800–22808.

Lok DJ, Van Der Meer P, de la Porte PW, Lipsic E, Van Wijngaarden J, Hillege HL, van Veldhuisen DJ. (2010). Prognostic value of galectin-3, a novel marker of fibrosis, in patients with chronic heart failure: data from the DEAL-HF study. *Clin Res Cardiol* 99:323–328.

Martos R, Baugh J, Ledwidge M, O'Loughlin C, Murphy NF, Conlon C, Patle A, Donnelly SC, McDonald K. (2009). Diagnosis of heart failure with preserved ejection fraction: improved accuracy with the use of markers of collagen turnover. *Eur J Heart Fail* 11:191–197.

Massaous J, Hata A. (1997). TGF-β signalling through the Smad pathway. *Trends Cell Biol* 7:187–192.

McDonagh TA, Holmer S, Raymond I, Luchner A, Hildebrant P, Dargie HJ. (2004). NT-proBNP and the diagnosis of heart failure: a pooled analysis of three European epidemiological studies. *Eur J Heart Fail* 6:269–273.

Michel K, Roth S, Trautwein C, Gong W, Flemming P, Gressner AM. (1998). Analysis of the expression pattern of the latent transforming growth factor β binding protein isoforms in normal and diseased human liver reveals a new splice variant missing the proteinase-sensitive hinge region. *Hepatology* 27:1592–1599.

Milting H, Ellinghaus P, Seewald M, Cakar H, Bohms B, Kassner A, Körfer R, Klein M, Krahn T, Kruska L, El Banayosy A, Kramer F. (2008). Plasma biomarkers of myocardial fibrosis and remodeling

in terminal heart failure patients supported by mechanical circulatory support devices. *J Heart Lung Transplant* 27:589–596.

Miyazono K, Hellman U, Wernstedt C, Heldin CH. (1988). Latent high molecular weight complex of transforming growth factor β 1. Purification from human platelets and structural characterization. *J Biol Chem* 263:6407–6415.

Morén A, Olofsson A, Stenman G, Sahlin P, Kanzaki T, Claesson-Welsh L, ten Dijke P, Miyazono K, Heldin CH. (1994). Identification and characterization of LTBP-2, a novel latent transforming growth factor-β-binding protein. *J Biol Chem* 269:32469–32478.

Oklü R, Hesketh R. (2000). The latent transforming growth factor β binding protein (LTBP) family. *Biochem J* 352 Pt 3:601–610.

Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. (2006). Controversies in ventricular remodelling. *Lancet* 367:356–367.

Remme WJ, Swedberg K; European Society of Cardiology. (2002). Comprehensive guidelines for the diagnosis and treatment of chronic heart failure. Task force for the diagnosis and treatment of chronic heart failure of the European Society of Cardiology. *Eur J Heart Fail* 4:11–22.

Seeland U, Selejan S, Engelhardt S, Müller P, Lohse MJ, Böhm M. (2007). Interstitial remodeling in β1-adrenergic receptor transgenic mice. *Basic Res Cardiol* 102:183–193.

Shipley JM, Mecham RP, Maus E, Bonadio J, Rosenbloom J, McCarthy RT, Baumann ML, Frankfater C, Segade F, Shapiro SD. (2000). Developmental expression of latent transforming growth factor β binding protein 2 and its requirement early in mouse development. *Mol Cell Biol* 20:4879–4887.

Sinha S, Heagerty AM, Shuttleworth CA, Kielty CM. (2002). Expression of latent TGF-β binding proteins and association with TGF-β 1 and fibrillin-1 following arterial injury. *Cardiovasc Res* 53:971–983.

Taipale J, Saharinen J, Hedman K, Keski-Oja J. (1996). Latent transforming growth factor-β 1 and its binding protein are components of extracellular matrix microfibrils. *J Histochem Cytochem* 44:875–889.

Taub PR, Gabbai-Saldate P, Maisel A. (2010). Biomarkers of heart failure. *Congest Heart Fail* 16 Suppl 1:S19–S24.

Wei YJ, Huang YX, Zhang XL, Li J, Huang J, Zhang H, Hu SS. (2008). Apolipoprotein D as a novel marker in human end-stage heart failure: a preliminary study. *Biomarkers* 13:535–548.

Wei Y, Cui C, Lainscak M, Zhang X, Li J, Huang J, Zhang H, Zheng Z, Hu S. (2011). Type-specific dysregulation of matrix metalloproteinases and their tissue inhibitors in end-stage heart failure patients: relationship between MMP-10 and LV remodelling. *J Cell Mol Med* 15:773–782.