Plasma levels of pro-inflammatory molecules and their expressions are associated with severity of heart failure: An investigation in Chinese cohort

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Abstract
Heart failure (HF) is a syndrome with multiple clinical phenotypes affecting around 1%–2% of adult population worldwide, and about 230 million Chinese are affected by cardiovascular diseases. The important role of pro-inflammatory plasma cytokines with HF has been demonstrated in different populations. The aim of this study was to investigate importance of pro-inflammatory cytokines in Chinese HF patients. In all, 134 HF patients were enrolled in this study and further classified in to four clinical distinct groups according to New York Heart Association classification criteria (NYHA-I: n = 34, NYHA-II: n = 35, NYHA-III: n = 22 and NYHA-IV: n = 43). Sixty-eight healthy Chinese were enrolled as controls. Plasma levels of tumour necrosis factor-α (TNF-α), TNF-receptor 1 (TNFRI), TNF-receptor 2 (TNFRII), interleukin 6 (IL-6), soluble IL-6 receptor (sIL-6R), C-reactive protein (CRP), soluble cluster of differentiation 14 (sCD14) and interleukin 1 beta (IL-1β) were quantified by enzyme-linked immunosorbent assay (ELISA). Plasma levels of all parameters investigated in this study remained comparable among healthy controls and NYHA-I group. Plasma levels of TNF-α, TNFRI, TNFRII, IL-6, sIL-6R, CRP, sCD14 and IL-1β were significantly higher in NYHA-III and NYHA-IV clinical categories compared to other HF phenotype (NYHA-I and NYHA-II). Interestingly, TNFR-II levels were significantly higher in NYHA-II compared to NYHA-I. No significant difference of plasma sIL-6R was observed among various clinical categories. In conclusion, plasma levels of pro-inflammatory molecules are elevated in severe HF patients and may be used as possible biomarkers for accessing severity of HF.

Keywords
Chinese, cytokines, heart failure, pro-inflammatory molecules

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Introduction
Heart failure (HF) is a complicated clinical syndrome affecting around 1%–2% of adult population worldwide. It is a multifactorial disease defined as inability of heart to supply adequate blood and oxygen to peripheral tissue system. The clinical features include shortness of breath, dyspnoea, fatigue, tachycardia and weakened exercise tolerance. The severity of clinical manifestations in subject with HF has been classified by different agencies; however, most established and widely acceptable criteria were proposed by New York Heart Association (NYHA) as classes I, II, III and IV based on clinical symptoms, severity and physical activity of the patient.
The incidence of cardiovascular diseases and related morbidity and/or mortality has significantly increased in Chinese population. A recent report titled ‘Report on Cardiovascular Disease in China, 2011’ suggested that 230 million people were affected from cardiovascular diseases, of which about 4.2 million (0.018%) subjects suffered from HF. Another independent study suggested that about 0.9% of Chinese population within age group 35–74 years are affected by chronic HF and majority of them are female (female: 1%; male: 0.7%). Higher number of HF has been reported in urban areas of Northern China.

Several factors have been attributed to increased risk for chronic HF. Coronary heart disease (CHD) phenotype is the most frequent clinical phenotype in HF patients: more than 60% of HF patients have CHD. Various population-based investigations showed hypertension as one of the major causes of CHF in women. Obesity is also ascribed as an important risk factor for CHF and contributes 14% in women and 11% in men with HF.

There is a substantial role of host immune system in the pathogenesis of HF. Evidences on role of inflammatory immune responses in HF have been accumulating over several decades. Inflammation is biologically defined as a response of host to tissue injury. Inflammation signalling is initiated at the site of local injury and gradually spreads to the rest of the body by certain chemical mediators that mainly include cytokines and chemokines. Elevated levels of inflammatory mediators are associated with poor outcome of HF. Increased pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and interleukin 18 (IL-18) as well as chemokines such as monocyte chemoattractant protein-1 (MCP-1) and CXCL-8/IL-8 (interleukin 8) have been observed in subjects with HF. Particularly, plasma cytokines and chemokines are associated with deterioration of functional classes as graded by NYHA and cardiac performance. Similarly, pathogenic role of different cytokines has been understood with reasonable clarity using animal models. For instance, a dilated cardiomyopathy like phenotype has been observed in animal models after administration of TNF-α. All these evidences suggest a strong prognostic and diagnostic role of cytokines in progression of HF, and there is an extensive need to study the levels of these proteins in different cohorts. In this report, we examined plasma levels of various inflammatory mediators such as TNF-α and its receptors (TNF-receptors 1 and II [TNFR1 and TNFRII]), IL-6 and its receptor (soluble IL-6R), IL-1β, soluble cluster of differentiation 14 (sCD14) and C-reactive protein (CRP) in a Chinese cohort.

**Materials and methods**

**Patients**

In all, 134 HF patients were enrolled in this study. All patients were subjected to electrocardiogram, chest X-ray and echo Doppler study clinically accessed by trained clinician. Based on NYHA criteria, patients were classified in to four grades (I, II, III and IV). Standard treatment procedure was followed for treatment of HF patients as suggested by American Heart Association and European Society of Heart study. Based on clinical type and severity of CHF patient, different treatment strategies were employed including nitrates, diuretics, digitalis, angiotensin-converting enzyme (ACE) inhibitor or β-blocker. Majority of patients with NYHA-III and NYHA-IV were treated with ACE inhibitor and β-blocker or combined with diuretic/digitalis with ACE inhibitor and β-blocker. For patients with NYHA class II or III, β-blocker therapy was most used. Treatment with nitrates, diuretics and digitalis was employed in patients who belonged to NYHA-I class. Patients with autoimmune diseases, acute coronary syndromes, liver dysfunctions, chronic infections, malignancy, acute myocardial infarction, sepsis, diabetes mellitus, valvular heart disease and rheumatic heart disease were excluded from this study. Sixty-eight healthy subjects from similar geographical areas were enrolled as controls. All controls are essentially healthy and there was no history of any heart-related abnormalities. Furthermore, healthy controls (HC) with history of autoimmune diseases and hypertension were excluded from the study. The study protocol was approved by Institutional Human Ethical Committee of Weihai Wendeng Central Hospital, and written informed consent was obtained from all subjects.

**Blood samples**

About 5mL of blood samples were collected from each participant by venipuncture with BD™ vacuum tube containing ethylenediaminetetraacetic
acid (EDTA) anti-coagulant. It is mixed thoroughly and centrifuged at 400 g for 20 min for isolation of plasma. Isolated plasmas were stored at −80°C till use for quantification of cytokines.

**Plasma parameters measurement**

Various cytokines such as TNF-α, IL-6, IL-1β, sCD14 and high sensitivity CRP (hsCRP) and receptors like TNFRI, TNFRII and sIL-6R were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instruction (R&D system).

**Statistical analysis**

All statistical analysis were carried out by Graphpad prism software. Mean plasma parameters in different clinical categories were compared by one-way analysis of variance (ANOVA) followed by Tukey’s post-test. A P value less than 0.05 was taken as significant.

**Results**

**Demographic features of patients with HF**

A total of 134 subjects with HF in a Chinese cohort were included in this study and their baseline features are depicted in Table 1. The four NYHA functional classes include NYHA-I (n = 34), NYHA-II (n = 35), NYHA-III (n = 22) and NYHA-IV (n = 43). The patients’ age in all four patient groups was comparable and age-matched HC (n = 68) were included for comparison. Both males and females were included in the study with the male numbers being dominant over females in all study groups. The two major causes for onset of HF are coronary artery diseases and idiopathic dilated cardiomyopathy.8 Table 1 showed that HF patients due to coronary artery diseases constitute higher frequencies in NYHA-III (86%) and NYHA-IV (72%) as compared to NYHA-I (35%) and NYHA-II (40%) indicating that coronary artery disease is the major cause of HF in severe disease groups. On the contrary, idiopathic dilated cardiomyopathy was more frequent in NYHA-I and NYHA-II as compared to NYHA-III and NYHA-IV (Table 1). The left ventricular ejection fraction (LVEF) of NYHA-IV patients was significantly lower (25.85%) compared to NYHA-I (58.24%) and HC (62.71%).

**Plasma level of inflammatory cytokines and their receptors in HF subjects**

Cytokines exert signal through different receptors to produce distinct cellular effects, and one such event is the inflammatory responses. Thus, in our investigation, we considered both cytokines and their respective receptors to investigate possible association with HF. We observed a marginal increase in the plasma TNF-α levels in HF subjects with less severity of the disease group (NYHA-I and NYHA-II functional class) when compared to HC (Figure 1(a)). However, the levels were significantly enhanced in NYHA-III and NYHA-IV patients with less severity of the disease group (NYHA-I and NYHA-II functional class) when compared to HC (P < 0.05). TNFRI and TNFRII are two potent receptors for TNF-α signaling. We found a gradual increase in the concentrations of these two receptors directly in relation to patient’s functional class (Figure 1(b) and (c)). However, NYHA-II subjects had significantly higher levels of TNFRII compared to controls, but such association was not observed for TNFRI.

Alterations in plasma levels TNF-α and its receptors further prompted us to look at IL-6 levels and its receptor (soluble IL-6R) in HF subjects. We found that IL-6 levels were significantly

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**Table 1. Baseline characteristics of heart failure patients and HC.**

|                   | HC (n = 68) | NYHA-I (n = 34) | NYHA-II (n = 35) | NYHA-III (n = 22) | NYHA-IV (n = 43) |
|-------------------|------------|-----------------|-----------------|------------------|-----------------|
| Age (mean ± SD)   | 56.29 ± 13.82 | 55.63 ± 10.63 | 53.28 ± 9.63    | 56.22 ± 14.72    | 55.12 ± 9.71    |
| Male/female       | 43/25      | 24/10           | 25/10           | 17/5             | 32/11           |
| Cause of heart failure |           |                 |                 |                  |                 |
| Coronary artery diseases | -        | 12 (35)         | 14 (40)         | 19 (86)          | 31 (72)         |
| Idiopathic dilated cardiomyopathy | -        | 22 (65)         | 21 (60)         | 3 (14)           | 12 (28)         |
| Left ventricular ejection fraction (LVEF) (%) | 62.71 ± 3.11 | 58.24 ± 8.52 | 47.42 ± 6.92    | 34.22 ± 4.58     | 25.85 ± 8.82    |

HC: healthy controls; NYHA: New York Heart Association.
Data are presented in number (%) of participants unless otherwise specified.
higher in NYHA-III and NYHA-IV subjects as compared to NYHA-I and NYHA-II (Figure 2(a)). A steady increase in IL-6 levels was observed in relation to NYHA functional class. However, we did not find possible alteration in concentration of soluble IL-6R among four NYHA classes (Figure 2(b)).

IL-1β, another potent inflammatory cytokine, also showed a higher expression level in HF subjects as compared to HC with progressive increase in concentrations from NYHA-I to NYHA-IV class (Figure 3(c)).

**Plasma titers of CRP and sCD14 in HF subjects**

Besides cytokines, we also looked at association of other inflammatory mediators with HF severity, such as CRP and sCD14. Both inflammatory mediators are potent molecules playing important role in promoting pathogenesis of cardiovascular diseases.
We observed elevated CRP and sCD14 in patients when compared to HC. There was a steady increase in CRP levels in patients which correlated directly with deterioration of the disease (Figure 3(a)). Levels of sCD14 were markedly increased in severely affected groups (NYHA-III and NYHA-IV) as compared to less severe patients (NYHA-I and NYHA-II) (Figure 3(b)). All these data clearly suggest that, except soluble IL-6R, all other inflammatory mediators are elevated in HF subjects in direct proportion to worsening of NYHA functional classes.

**Discussion**

The functional classification of subjects with HF made by NYHA is useful to assess severity and disease prognosis. Most of the functional studies in different cohorts of HF subjects are performed after classifying them according to NYHA guidelines. Although various reports on importance of inflammatory molecules in prognosis and diagnosis of HF have been widely investigated, studies including Chinese patients are lacking. In this study, we examined levels of different inflammatory signature molecules in four distinct clinical phenotypes and correlated with disease severity in a Chinese cohort.

We observed an elevated plasma TNF-α and IL-6 levels in HF patients as compared to HC, and their levels also correlated with disease severity. Our results were corroborated by many earlier findings showing a significantly higher level of these two cytokines as compared to HC (Table 1). Earlier studies have shown an association of higher TNF-α and IL-6 levels with increased HF severity across NYHA class (NYHA-I to NYHA-IV), and our data were in accordance to these findings. Furthermore, we observed higher levels of TNFRI and TNFRII in HF patients as compared to controls, and their titres were also positively correlated with disease severity. Corroborating with earlier reports, this report also showed higher levels of TNFRI and TNFRII molecules in HF patients compared to controls and their association with severity of the disease. Although our data showed a positive association of TNF-α as well as its receptors with disease severity, this trend was not noticed when we analysed the correlation of plasma IL-6 and its receptor along with disease deterioration. A gradual increase in IL-6 levels across HF spectrum was observed with peak level reaching in patients of NYHA-IV group. On the contrary, the levels of IL-6R were similar among four NYHA classified groups. A similar finding was observed earlier that showed comparable levels of IL-6R in all clinical groups. In addition, we also found a steady increase in titre of plasma IL-1β in differential NYHA classes for HF subjects, and our data matched with earlier reports.

CRP is considered as surrogate marker for IL-1β and has been predicted to show a worse survival during acute coronary symptoms. In this report, the expression level of CRP was in line with that of
plasma IL-1β levels. The increased concentration of soluble CRP correlated with severity of disease in close relation to functional NYHA functional class.

CD14 is an identification marker for human monocytes, and sCD14 in plasma indicates the shedding of this molecule into blood. A novel study revealed a mechanism of TNF-α activation in HF subjects and indicated that HF leads to mesenteric venous congestion that causes intestinal bacterial translocation followed by endotoxin release from the bacteria into circulation. The released endotoxin activates the immune system and that leads to release of TNF-α. Simultaneously, CD14 is released to circulation due to endotoxin–monocyte interaction. As elevated TNF-α and shedding of CD14 from monocytes are interlinked, we examined the sCD14 in plasma of HF subjects and found a similar pattern as TNF-α. The increased sCD14 across the spectrum was correlated with worsening of the disease across NYHA class.

There is a need to examine biological role of different inflammatory molecules during HF as these molecules (1) mediate pathogenesis, (2) are associated with disease severity, (3) can predict development of HF in asymptomatic patients and (4) can be altered with HF therapy. However, importance of other cytokines and chemokines as biomarkers should also be explored. Upregulation of several cytokines such as TNF-α, IL-6 and IL-1β in HF subjects observed in our study as well as in several other studies indicates the use of these proteins to define adverse outcome of the disease, and they can possibly be used as potent biomarkers. However, these cytokines are present in circulation (serum and plasma) at very low levels in HF subjects thus asking for demand to use very expensive high sensitivity assay. On the contrary, the receptors for these ligands always circulate at higher levels and can be detected easily, thereby enhancing the possibility of these molecules to use as more relevant biomarkers. Quantitatively, we found the titers of TNFRI, TNFRII and soluble IL-6 receptor in ng/mL levels, whereas TNF-α, IL-6 and IL-1β levels were detected at pg/mL. Therefore, TNFRI and TNFRII are potent inflammatory biomarkers for HF which are correlated with disease severity in the Chinese cohort. However, our findings showing similar levels of soluble IL-6 receptor among different HF groups, corroborated with earlier reports, rules out the possibility of using IL-6 as a biomarker.

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