

COPD and levels of Hsp70 (HSPA1A) and Hsp27 (HSPB1) in plasma and lymphocytes among coal workers: a case-control study

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Abstract This case-control study aimed to investigate whether the levels of Hsp70 (HSPA1A) and Hsp27 (HSPB1) in plasma and lymphocytes were associated with the risk of chronic obstructive pulmonary disease (COPD) among coal workers. A total of 76 COPD cases and 48 age-matched healthy controls from a group of coal workers were included. The case group consisted of 35 COPD patients whose condition was complicated with coal workers' pneumoconiosis (CWP) and 41 COPD patients without CWP. Heat shock proteins (Hsps) in plasma and lymphocytes were detected by ELISA and flow cytometry, respectively. Multiple logistic regression models were applied to estimate the association between Hsp levels and COPD risk. Our results showed that plasma Hsp70 and lymphocyte Hsp27 levels were significantly higher and plasma Hsp27 levels were significantly lower in COPD cases than in controls ($p < 0.01$). No significant differences in lymphocyte Hsp70 levels were found between COPD cases and the matched subjects. Higher plasma Hsp70 levels (odds ratio (OR)=13.8, 95 % confidence interval (CI)=5.7–

33.5) and lower plasma Hsp27 levels (OR=4.6, 95 % CI=2.0–10.5) were significantly associated with an increased risk of COPD after adjusting for confounders. Higher lymphocyte Hsp27 levels were only associated with an increased risk of COPD with CWP (OR=6.6, 95 % CI=2.0–22.1) but not with an increased risk of COPD without CWP (OR=3.0, 95 % CI=0.9–8.9). Additionally, there were strong joint effects of different Hsps on COPD risk. These results showed that higher levels of plasma Hsp70 and lower levels of plasma Hsp27 might be associated with an increased risk of COPD among coal workers. They may have the potential to serve as monitoring markers for COPD in coal workers.

Keywords COPD · Heat shock proteins · Coal workers · Biomarker · Coal workers' pneumoconiosis

Introduction

Chronic obstructive pulmonary disease (COPD) continues to be a major cause of morbidity and mortality worldwide (Mannino and Buist 2007). It is a syndrome characterized by progressive and irreversible airflow limitation, with clinical symptoms such as chronic and progressive dyspnea, cough, and sputum production (Vestbo et al. 2013). In addition to cigarette smoking, epidemiological evidence has shown that exposure to coal mine dust is a major cause of COPD among coal workers. It has been suggested that intense and prolonged exposure to workplace dust in coal mining contributes to the development of COPD (Kuempel et al. 2009; Meijers et al. 1997). A 22-year follow-up cohort study of 25,000 British coal miners reported a consistent increase in mortality from COPD (Miller and Jacobsen 1985). The exposure-response

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relationship between coal mine dust and COPD mortality was confirmed by Kuempel et al (Kuempel et al. 1995). Although the exact pathogenesis of COPD is not completely understood, it is acknowledged that persistent oxidative damage and inflammation contribute considerably to COPD occurrence and development (Oudijk et al. 2003; Chung and Adcock 2008; MacNee and Rahman 2001). Previous studies found that patients with COPD had both local and systemic inflammatory reactions, such as elevated specific inflammatory cell numbers and altered inflammatory cytokine levels (Saetta et al. 1993; Gan et al. 2004). Scholars also observed up-regulated neutrophil peroxide anion levels and impaired antioxidant balance in the blood of COPD patients (Rahman et al. 1996; Nicks et al. 2011). Until now, the diagnosis and monitoring of COPD mainly relied on the use of a spirometry test. But this test is not recommended for COPD patients during an acute phase, because it is too difficult for them to perform. In addition, COPD is a complex syndrome with both pulmonary and extra-pulmonary effects, while the spirometry parameters only reflect the severity of airflow limitation. Research on blood sensitive protein levels will help to explain the mechanism or to monitor the development of COPD.

Heat shock proteins (Hsps) are a large group of proteins, which are highly conserved and ubiquitously expressed (Lindquist and Craig 1988). They are generally considered to be intracellular proteins with molecular chaperone functions (Hendrick and Hartl 1993). A proportion of them have been found to exist in both lesion sites and the blood stream (Zhang et al. 2010; Gruden et al. 2013; Morteza et al. 2013). Many studies have explored the potential roles of circulating Hsps as biomarkers for diabetes and acute coronary syndrome (Morteza et al. 2013; Zhang et al. 2010). Hsp70 (HSPA1A) and Hsp27 (HSPB1) are two important members of the Hsps. Hsp70 has both important intracellular and extracellular functions. Apart from its intercellular functions as a molecular chaperone and anti-apoptotic protein, Hsp70 located in extracellular medium has powerful effects on the regulation of the immune system (Asea et al. 2000, 2002; Srivastava 2002; Panjwani et al. 2002). Exogenous Hsp70 can stimulate cytokine release by acting as endogenous ligand for CD14 and toll-like receptor 4, which suggests its dual role as a chaperone and cytokine (Asea et al. 2000, 2002). Hsp27 is an important small heat shock protein. Under stress conditions, such as hyperthermia and oxidative stress, intracellular Hsp27 can act as an ATP-independent chaperone to protect against protein aggregation and can play key roles in keeping the cytoskeleton stable and resisting cell apoptosis (Rogalla et al. 1999; Hino et al. 2000; Arrigo 2007). In addition, Salari and De et al. have identified the anti-inflammatory function of extracellular Hsp27 via NF- κ B and p38 signaling pathways (Salari et al. 2013; De et al. 2000).

Previous studies indicated that altered levels of Hsps in serum and peripheral blood cells were associated with

increased COPD risk in the general population (Hacker et al. 2009; Jan Ankersmit et al. 2012; Dong et al. 2013). However, few similar studies have been conducted among coal workers. It would be useful to identify the associations between Hsp levels and the risk of COPD among coal workers. Hence, we developed this case-control study. Because long exposure to coal dust may result in coal workers' pneumoconiosis (CWP), which is an occupational lung disease characterized by pulmonary inflammation and fibrosis, we have distinguished COPD patients with CWP from COPD patients without CWP in this study (Castranova and Vallyathan 2000). The objectives of this study were (1) to assess the possible associations between levels of peripheral Hsp70 and Hsp27 and the risk of COPD and (2) to explore the potential of peripheral Hsp70 and Hsp27 as biomarkers for COPD monitoring in coal workers.

Subjects and methods

Study subjects

All subjects were male coal workers recruited from one coal mining company in Shanxi province, China. The products of this company are coke coal and meager lean coal, and the primary operation is underground coal excavation. Coal workers were usually exposed to coal mine dust for more than 30 h a week. There was no other obvious toxicant exposure in the workplace. In this study, we recruited 76 newly diagnosed COPD coal workers and 48 age-matched healthy coal workers. The COPD cases included 35 COPD patients whose condition was complicated with CWP and 41 COPD patients without CWP. All the cases and controls, who were identified by the hospital of the company, were free from other known diseases or infections in the previous 6 months. COPD was diagnosed based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Gómez and Rodriguez-Roisin 2002). CWP was diagnosed based on the Chinese Pneumoconiosis Roentgen Diagnostic Criteria, which is similar to the International Labour Organization classification (Chen et al. 2001). After the obtaining signatures indicating informed consent from the participants, face to face interviews were conducted by trained investigators using unified questionnaires, which included personal information, medical history, life habits as well as work history. Each item of the questionnaire was examined and integrated uniformly after the interviews. The amount of cigarette smoking was calculated in terms of packs-year (smoking 20 cigarettes per day for 1 year). This study was approved by the Ethics and Human Subject Committees of the coal mine's General Hospital and of Tongji Medical College, Huazhong University of Science and Technology (China).

Cumulative dust exposure data collection and spirometry tests

Total dust concentrations (mg/m^3) for each post in the workplace were available dating from the establishment of the coal mining company. All the concentrations were assessed monthly based on a gravimetric method and were reported in the form of average level per year. Cumulative exposure to dust (mg/m^3 -years) was calculated for each participant by multiplying the intensity of dust exposure by the duration of work (Chen et al. 2012). Spirometry tests were performed by trained technicians using a portable spirometer (Chestgraph, HI-101, CHEST MI Inc., Tokyo, Japan) according to the criteria of the American Thoracic Society (Miller et al. 2005).

Blood sampling and isolation of plasma and lymphocytes

For each participant, approximately 6-ml fasting venous blood was collected in heparinized tubes. Plasma and lymphocytes were separated from each sample immediately after blood collection. Plasma was isolated by centrifugating 3-ml blood at $1000\times g$ for 10 min at room temperature and then stored at -80°C until analysis. Lymphocytes were isolated and purified according to Bøyum and Serner et al. with some modifications (Bøyum 1976; Serner et al. 1992). First, peripheral blood mononuclear cells (PBMCs) were isolated from the fresh whole blood by Ficoll density gradient centrifugation. Five milliliters of a 1:1 mixture of whole blood and ice-cold physiological saline were gently applied on an equal volume of Ficoll-Hypaque (Biochemical Reagent Co, Shanghai, China) in centrifuge tubes. The tubes were then centrifuged at $400\times g$ for 30 min at room temperature. After centrifugation, the PBMCs band, the buffy coat between the plasma and the Ficoll-Hypaque layers, was removed and washed twice with phosphate-buffered saline (PBS). The PBMCs isolated by this method contained about 20 % monocytes; hence, another incubation step was conducted to obtain lymphocytes (Serner et al. 1992). The collected PBMCs were resuspended and adjusted to $1\times 10^6/\text{ml}$ with RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 1 % penicillin/streptomycin. The cell suspensions were transferred to 2 % gelatin-precoated flasks and cultured in a 37°C 5 % CO_2 incubator for 1 h to make the monocytes adhere. The non-adherent cells were collected as lymphocytes by centrifuging the suspensions at $900\times g$ for 3 min. The collected lymphocytes were then fixed in 500 μl PBS containing 4 % paraformaldehyde and stored at 4°C for detection of Hsps.

Detection of Hsp70 and Hsp27 levels in lymphocytes

Flow cytometry was used for the detection of Hsp70 and Hsp27 in lymphocytes (Xiao et al. 2003; Bachelet et al. 1998). Each fixed lymphocyte sample was divided into two to detect Hsp70 and Hsp27 separately as follows. Briefly, the

fixed lymphocytes were centrifuged at $600\times g$ for 5 min and then were resuspended in 100- μl diluted antibody solutions (1:500 dilution of rabbit-anti-human Hsp70 (SPA-812E) or 1:300 dilution of rabbit-anti-human Hsp27 (SPA-803E), Stressgen Bioreagents, Victoria, BC, Canada) in a permeabilization solution (PBS containing 1 % bovine serum albumin (BSA) and 0.04 % Triton X-100) (Wang et al. 2009). After a 30-min incubation at 4°C , cells were washed by 0.5 ml PBS containing 1 % BSA and were incubated with 100 μl of fluorescein-isothiocyanate-labeled (FITC) anti-rabbit immunoglobulin G (IgG, diluted 1:100, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for another 30 min. Lymphocytes were washed by 0.5 ml PBS containing 1 % BSA and resuspended in 0.3 ml cold PBS. Meanwhile, two negative controls without anti-human Hsp antibodies or without the secondary FITC-labeled anti-rabbit IgG were performed. The stained lymphocytes were analyzed by flow cytometry (FACS CALIBUR, Beckton Dickinson Company, San José, CA, USA). A total of 10,000 cells were counted and the mean fluorescence intensity (MFI) detected at 525 nm was used for quantitation of Hsp70 and Hsp27.

Detection of Hsp70 and Hsp27 levels in plasma

Plasma Hsp70 and Hsp27 levels were measured by ELISA using commercially available kits (EKS-715 and EKS-500, Stressgen Bioreagents, Victoria, BC Canada, now Assay Designs, Ann Arbor, USA) according to the manufacturer's recommendations. For the detection of plasma Hsp27, 1:10 diluted plasma was used. For the detection of Hsp70, plasma samples were diluted to 1:2~1:10 before detection, because pre-experiments found that Hsp70 levels in some 1:2 diluted plasma samples were still beyond the detection range of Hsp70 ELISA kits (0.20–12.5 ng/ml). The Hsp70 ELISA kit detected recombinant and native Hsp70 (HSPA1A) and did not detect other Hsp70 family members. The sensitivity of Hsp70 and Hsp27 kits were 0.09 and 0.39 ng/ml respectively according to the manufacturer. Both the inter- and intra-assay coefficients of variations of the two kits were under 10 %.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute). Shapiro-Wilk's W test was employed to assess the normality of continuous variables. The Hsp70 and Hsp27 were log-normally distributed, and other continuous variables were normally distributed, which were presented as mean \pm standard deviation (SD). Qualitative variables were presented as number and percent. Analysis of variance (ANOVA) and chi-square test were used for statistical comparisons between groups. Spearman's correlation test was performed to investigate the relationship between different levels of HSPs. Multiple logistic regression analysis was used to

calculate odds ratios (ORs) and 95 % confidence intervals (CIs) to estimate single and joint effects of Hsp70 and Hsp27 on COPD. The variables of age, amount of cigarette smoking, and cumulative coal mine dust exposure were simultaneously incorporated into the regression models to adjust for the effects of each factor. Statistical inferences were based on two statistical levels ($p < 0.05$ or $p < 0.01$ on two sides).

Results

Basic characteristics of COPD cases and controls

The characteristics of COPD cases and controls are presented in Table 1. Cases and controls were well matched for age, dust exposure duration, proportion of cigarette smoker, and amount of smoking per person. Average dust concentrations and cumulative dust exposure levels were significantly higher in the COPD group than in the matched group ($p < 0.01$). As expected, COPD cases had significantly lower levels of spirometry parameters (VC and FEV₁% pred) ($p < 0.01$). No statistically significant differences of any observed characters were observed between the COPD without CWP subgroup, and the COPD with CWP subgroup were observed ($p > 0.05$).

Hsp Levels in cases and controls

Hsp levels between controls and all the COPD patients were firstly compared. Plasma Hsp70 levels and lymphocyte Hsp27 levels were significantly higher in all the COPD cases than in the controls (44.0 vs 3.3 ng/ml for plasma Hsp70; 28.1 vs 19.2 MFI for lymphocyte Hsp27; $p < 0.01$). In contrast, plasma Hsp27 levels were significantly lower in all the COPD cases than in the controls (97.1 vs 204.7 ng/ml, $p < 0.01$). There were

no differences of lymphocyte Hsp70 levels between all the COPD cases and controls (19.3 vs 19.6 MFI, $p > 0.05$).

Hsp levels between the matched group and the two COPD subgroups were also compared (Fig. 1). Plasma Hsp27 levels significantly differed among the three groups ($p < 0.01$), while lymphocyte Hsp70 levels showed no difference between any two of the three groups ($p > 0.05$). Plasma Hsp70 levels were found to be significantly higher in the COPD with CWP patients when compared with the healthy ones or with the COPD without CWP patients. But its levels did not significantly differ between the controls and the COPD without CWP patients. Additionally, lymphocyte Hsp27 levels were significantly higher in both the two COPD subgroups than in the matched healthy group, while they showed no difference between the two COPD subgroups.

Correlations between levels of Hsp27 and Hsp70 in plasma and lymphocytes

The correlation coefficients between the levels of four Hsps are summarized in Table 2. Results showed that lymphocyte Hsp27 levels were significantly correlated with the other three Hsp levels (positively correlated with plasma Hsp70 levels and lymphocyte Hsp70 levels, and negatively correlated with plasma Hsp27 levels). Moreover, plasma Hsp27 levels were observed to be negatively correlated with plasma Hsp70 levels ($r = -0.48$, $p < 0.01$).

Association between Hsp levels and other factors and COPD risk

As shown in Table 3, we found that high levels of plasma Hsp70 were associated with a >12-fold (OR = 13.8, 95 % CI = 5.7–33.5) higher risk of COPD than subjects with low

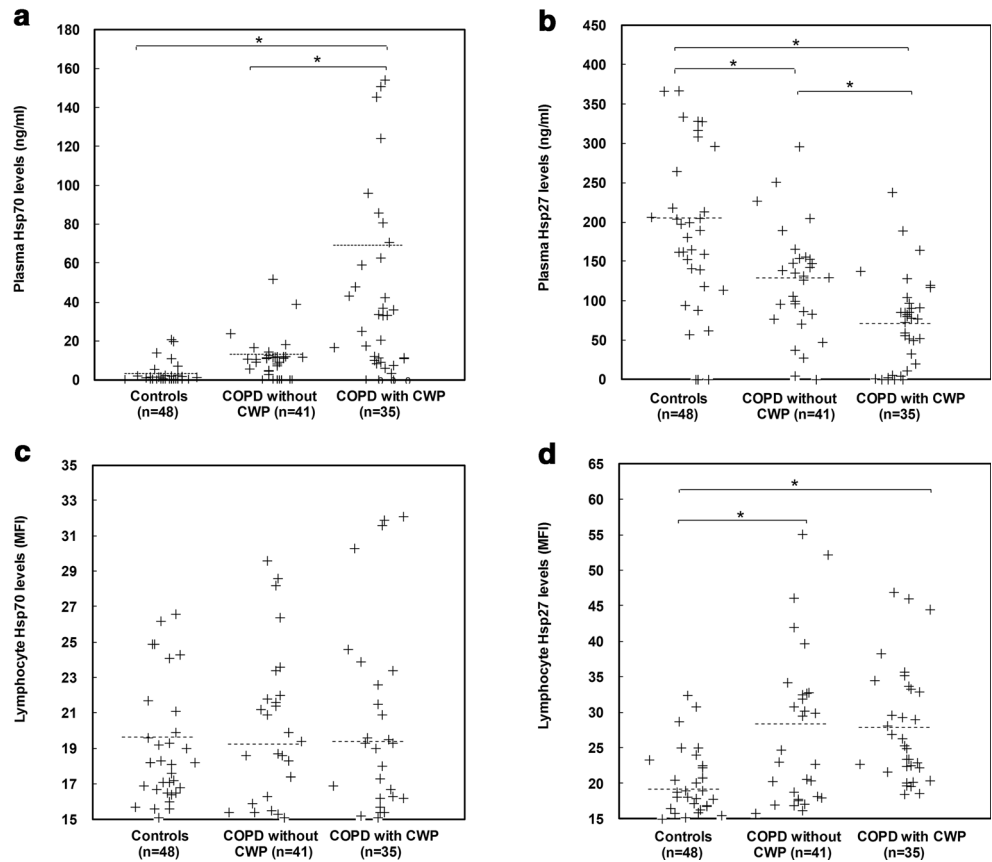
Table 1 Characteristics of COPD cases and controls

Variables	Controls ($n = 48$)	Cases		
		COPD without CWP patients ($n = 41$)	COPD with CWP patients ($n = 35$)	All COPD patients ($n = 76$)
Age (year)	68.7±6.7	69.5±6.5	68.9±6.8	69.2±6.6
Dust exposure duration (year)	27.5±9.1	28.2±10.3	28.4±5.3	28.3±8.4
Average dust concentration (mg/m ³)	20.7±8.5	26.7±4.2*	27.9±6.5*	27.3±5.4*
Cumulative dust exposure (mg/m ³ -years)	556.2±257.4	757.2±306.7*	796.9±267.7*	775.5±288.2*
Smokers (%yes)	39 (81.8)	35 (85.4)	28 (80.0)	63 (82.9)
Amount of cigarette smoking (packs-year)	26.8±26.3	32.0±25.1	26.7±24.9	29.53±24.9
VC(L)	26.3±8.4	19.7±7.4*	17.2±7.5*	18.55±7.5
FEV ₁ % pred(%)	73.0±20.1	45.8±27.3*	46.2±14.8*	46.0±22.3*

Data are presented as “mean±SD” for continuous variables and as number (percent) for categorical variables

* $p < 0.01$, compared with controls

Fig. 1 Scatter plots of Hsp70 and Hsp27 located in plasma and lymphocytes by different groups including controls, COPD without CWP patients and COPD with CWP patients. Horizontal dotted lines indicate the mean. Asterisks mark significant differences ($p < 0.01$) between different groups



plasma Hsp70 levels after adjusting for age, cumulative dust exposure, and amount of smoking. A similar effect was observed for the association between high levels of lymphocyte Hsp27 and COPD risk (OR=4.5, 95 % CI=2.1–9.7). Compared with subjects with high levels of plasma Hsp27, the risk of COPD for subjects with low plasma Hsp27 levels was increased more than three fold (OR=4.6, 95 % CI=2.0–10.5) after adjusting for confounders.

For further analysis, associations between Hsp levels and the risk of COPD with or without CWP were estimated (Table 3). Results showed that higher plasma

Hsp70 levels were associated with both an increased risk of COPD without CWP and an increased risk of COPD with CWP, with ORs of 9.5 (95 % CI=2.2–40.5) and 134.1 (95 % CI=12.6–709.7), respectively. Similarly, low plasma Hsp27 levels were found to be associated with not only an increased risk of COPD without CWP, but also with an increased risk of COPD with CWP, with ORs of 3.1 (95 % CI=1.0–9.5) and 10.7 (95 % CI=2.6–44.4), respectively. However, high lymphocyte Hsp27 levels were observed to be associated only with the risk of COPD with CWP (OR=6.6, 95 % CI=2.0–22.1), but not with the risk of COPD without CWP (OR=3.0, 95 % CI=0.9–8.9). Logistic regression results showed no significant associations between lymphocyte Hsp70 levels and COPD.

To understand the impact of cigarette smoking on the associations between Hsps and COPD, we conducted a stratified analysis. All participants were divided into two subgroups of cigarette smokers (current and previous smokers) or non-smokers. The associations between HSP and COPD obtained by multiple logistic regression models among smokers were similar to those of all participants. As the number of non-smokers (22 subjects) in this study was not big enough to establish a regression model, we did not show these results.

Table 2 Correlations between Hsp70 and Hsp27 levels in plasma and lymphocytes

Variables	Plasma Hsp70	Plasma Hsp27	Lymphocyte Hsp70	Lymphocyte Hsp27
Plasma Hsp70	–	–0.48 ^a	–0.09	0.38 ^a
Plasma Hsp27		–	–0.15	–0.39 ^a
Lymphocyte Hsp70			–	0.61 ^a
Lymphocyte Hsp27				–

The correlation coefficients between Hsp27 and Hsp70 levels were calculated by Spearman’s rank correlation test

^a Correlation is significant at the 0.05 level

Table 3 Multiple Logistic regression analysis for the association between Hsp levels and the risk of COPD without CWP, COPD with CWP, and all COPD among coal workers

Variables	COPD without CWP		COPD with CWP		All COPD	
	<i>n</i> (%)	OR (95 % CI)	<i>n</i> (%)	OR (95 % CI)	<i>n</i> (%)	OR (95 % CI)
Age^a						
~60	8 (9.0)	1.0	7 (8.4)	1.0	11 (8.9)	1.0
61~70	34 (38.2)	0.6 (0.1–3.3)	37 (44.6)	0.4 (0.0–3.9)	52 (41.9)	0.9 (0.2–3.5)
71~	47 (52.8)	0.5 (0.1–2.8)	39 (47.0)	0.2 (0.0–1.8)	61 (49.2)	0.5 (0.1–1.8)
Amount of cigarette smoking^b						
Never	15 (16.9)	1.0	16 (19.3)	1.0	22 (17.7)	1.0
Low (>0 and ≤25 pack-year)	28 (34.5)	1.4 (0.3–6.0)	28 (33.7)	1.9 (0.4–8.9)	40 (32.3)	1.2 (0.4–3.4)
High (>25 pack-year)	46 (51.7)	2.2 (0.6–8.5)	39 (47.0)	1.5 (0.4–6.0)	62 (50.0)	1.3 (0.5–3.4)
Cumulative coal mine dust exposure^c						
Low (>0 and ≤500 mg/m ³ -year)	30 (33.7)	1.00	24 (29.0)	1.0	33 (26.6)	1.0
Middle (>501 and ≤800 mg/m ³ -year)	30 (33.7)	2.3 (0.7–7.5)	32 (38.6)	12.3 (2.2–69.3)	45 (36.3)	4.6 (1.7–12.1)
High (>801 mg/m ³ -year)	29 (32.6)	5.9 (1.7–20.6)	27 (32.5)	20.4 (3.5–118.8)	46 (37.1)	6.7 (2.5–18.0)
Plasma Hsp70^d						
Low (≤11.2 ng/ml)	69 (77.5)	1.0	51 (61.5)	1.0	76 (61.3)	1.0
High (>11.2 ng/ml)	20 (22.5)	9.5 (2.2–40.5)	32 (38.6)	134.1 (12.6–709.7)	48 (38.7)	13.8 (5.7–33.5)
Plasma Hsp27^d						
High (>120.3 ng/ml)	49 (55.1)	1.0	38 (45.8)	1.0	55 (44.4)	1.0
Low (≤120.3 ng/ml)	40 (45.0)	3.1 (1.0–9.5)	45 (54.2)	10.7 (2.6–44.4)	69 (55.7)	4.6 (2.0–10.5)
Lymphocyte Hsp70^d						
Low (≤18.2 MFI)	52 (58.4)	1.0	49 (59.0)	1.0	72 (58.1)	1.0
High (>18.2 MFI)	37 (41.6)	1.1 (0.4–3.0)	34 (41.0)	0.7 (0.2–2.1)	52 (41.9)	1.1 (0.6–2.3)
Lymphocyte Hsp27^d						
Low (≤22.3 MFI)	61 (68.5)	1.0	49 (59.0)	1.0	72 (58.1)	1.0
High (>22.3 MFI)	28 (31.5)	3.0 (0.9–8.9)	34 (41.0)	6.6 (2.0–22.1)	52 (41.9)	4.5 (2.1–9.7)

Multiple logistic regressions were employed to analyze the associations of risk factors and COPD risk in coal workers. Continuous variables were categorized according to their distribution, and the dividing values were listed following the corresponding variables

^a Adjusted for amount of cigarette smoking, cumulative coal mine dust exposure among subjects including healthy controls and patients with corresponding disease

^b Adjusted for age and cumulative coal mine dust exposure among subjects including healthy controls and patients with corresponding disease

^c Adjusted for age and amount of cigarette smoking among subjects including healthy controls and patients with corresponding disease

^d Adjusted for age, cumulative coal mine dust exposure and amount of cigarette smoking among subjects including healthy controls and patients with corresponding disease

Joint effects of different Hsps on COPD risk

Results of the joint effects of Hsp70 and Hsp27 on COPD by regression analysis are shown in Table 4. The ORs of three joint forms were estimated by multiple logistic regressions after adjusting for age, cumulative dust exposure, and amount of cigarette smoking. Lymphocyte Hsp70 was excluded because no significant associations between its levels and risk of COPD were observed in the above analysis (Table 3). All three joint forms were associated with a higher risk of COPD compared with the form of certain single Hsps. The adjusted OR of the joint form “high plasma Hsp70 and low plasma Hsp27” (OR=24.9, 95 % CI=9.5–91.0) was higher than the other two joint ORs.

Discussion

In the present study, we found that plasma Hsp70 and lymphocyte Hsp27 levels were significantly higher and that plasma Hsp27 levels were significantly lower in COPD patients than in controls. After adjusting for confounders including amount of smoking and coal dust exposure, there was an increased risk of COPD when plasma Hsp70 levels increased or when plasma Hsp27 levels decreased. Higher lymphocyte Hsp27 levels were only associated with an increased risk of COPD with CWP, but not with an increased risk of COPD without CWP.

COPD is a systemic, oxidative, stressful and inflammatory disease with both pulmonary and extra-pulmonary effects

Table 4 Multiple logistic regression analysis for the association between joint Hsp levels and COPD risk among coal workers

Variables	All COPD	
	<i>n</i> (%)	OR (95 % CI)
Plasma Hsp70 and plasma Hsp27		
Low plasma Hsp70 or high plasma Hsp27	90 (72.6)	1
High plasma Hsp70 and low plasma Hsp27	34 (27.4)	29.4 (9.5–91.0)
Plasma Hsp70 and lymphocyte Hsp27		
Low plasma Hsp70 or low lymphocyte Hsp27	95 (76.6)	1
High plasma Hsp70 and high lymphocyte Hsp27	29 (23.4)	22.9 (7.2–72.6)
Plasma Hsp27 and lymphocyte Hsp27		
High plasma Hsp27 or low lymphocyte Hsp27	100 (80.7)	1
Low plasma Hsp27 and high lymphocyte Hsp27	24 (19.4)	27.0 (6.8–106.9)

Multiple logistic regressions were employed to analyze the associations between joint Hsp levels and risk of COPD among all the participants after adjusting for age, cumulative coal mine dust exposure, and amount of cigarette smoking; Hsp levels were categorized according to medians (plasma Hsp70, 11.2 ng/ml; lymphocyte Hsp27, 22.3 MFI; plasma Hsp27, 120.3 ng/ml)

(Agusti et al. 2003). Previous studies have suggested that inhaled coal mine dust particles could induce a stress response and start the chronic inflammation, which are associated with the development of COPD (Gosset et al. 1991; Morfeld et al. 2001; Júnior et al. 2009). This concurs with our finding that elevated cumulative coal mine dust exposure was associated with an increased COPD risk. Stressful cellular environments were found to induce extracellular Hsp70 release (Vega et al. 2008; Lancaster et al. 2004). Previous *in vitro* studies found that extracellular Hsp70 could modulate immune responses, such as the production of inflammatory cytokines and inducible nitric oxides (Panjwani et al. 2002; Asea et al. 2000). Campisi et al. thought extracellular Hsp70 might have an improved immune response to pathogenic challenges, especially those involved in proinflammatory responses (Campisi et al. 2003). In this study, although the increase of plasma Hsp70 levels in patients with COPD without CWP was not significant as compared with the controls, plasma Hsp70 levels were significantly higher in patients with COPD with CWP. Moreover, plasma Hsp70 levels were associated both with an increased risk of COPD without CWP and with an increased risk of COPD with CWP. We speculated that the higher plasma Hsp70 levels in COPD patients might result from the stressful cellular environments caused by inhalation of coal mine dust, and the upregulated plasma Hsp70 might play an important role as an inflammatory regulator in the development of COPD. In the relationship between

intracellular and extracellular Hsps, the general view was that intracellular Hsps could be released into an extracellular environment and induce a range of proinflammatory responses (Pockley 2003). The sources of elevated plasma Hsp70 in COPD patients might be necrotic lung cells or peripheral blood mononuclear cells such as lymphocytes (Njemini et al. 2006). In the present study, our results showed no significant correlation between lymphocyte Hsp70 and plasma Hsp70, and no differences of lymphocyte Hsp70 levels were found between COPD cases and controls. It might be possible that the source of elevated plasma Hsp70 was from necrotic lung cells, but further studies of this mechanism are needed to prove this view. From the results above, we considered that higher plasma Hsp70 levels might represent a danger signal for COPD among coal workers, but this inference needs further epidemiology and mechanism evidences.

Extracellular Hsp27 can act as a signaling molecule in stress conditions, but was found to be decreased in some diseases (Salari et al. 2013). In our previous research among coal workers, plasma Hsp27 levels were found to be lower in lung cancer patients when compared with healthy ones (Wang et al. 2010). Analogous changes of Hsp27 levels in coronary artery disease were found by Seibert and his colleagues, and lower serum Hsp27 levels were associated with the presence of coronary heart disease in humans (Seibert et al. 2013). In the present study, plasma Hsp27 levels were lower in coal workers with COPD compared to those in the matched group. Its downregulation may be associated with the progress of COPD. Previous studies have found the important pathogenic role of protease-antiprotease imbalance in the development of COPD, while extracellular Hsp27 was found to be degraded by enzymes and might reflect a proteolytic imbalance (Abboud and Vimalanathan 2008; Martin-Ventura et al. 2006). In this view, plasma Hsp27 might be a potential biomarker for COPD monitoring among coal workers. Our results showed an increase of lymphocyte HSP27 levels in all COPD cases when compared with controls, and lymphocyte Hsp27 levels were found to be negatively associated with plasma Hsp27 levels. The increase of lymphocyte Hsp27 levels seemed to have a regenerative feedback effect in peripheral lymphocytes when plasma Hsp27 levels in COPD patients were low. An *in vitro* study also found that heat shock stress could cause an increase of Hsp27 levels in lymphocytes (Njemini et al. 2006). However, it was noted that the OR of high lymphocyte Hsp27 levels on COPD without CWP was high but without statistical significance (OR=3.0, 95 % CI=0.9–8.9). Therefore, we thought that lymphocyte 27 levels might have the potential to be a monitoring marker for COPD. To further evaluate the potential of Hsps as monitoring markers, we estimated the joint effects of Hsp70 and Hsp27 on COPD. The higher OR value of the joint form “high plasma Hsp70 and low plasma Hsp27” reminded us that the combination of plasma Hsp70 levels and plasma Hsp27 levels

might be a more effective and sensitive way to monitor COPD.

The advantage of the current study was that plasma and lymphocyte Hsp levels were considered together. Moreover, more than one Hsp was included. The combination might be more powerful in clarifying the relationship between Hsps and COPD among coal workers. The main limitation was that this study was a cross-sectional epidemiology study. Our findings needed larger prospective cohort studies and mechanism studies to be further proved.

Conclusion

In summary, this case-control study showed that higher levels of plasma Hsp70 and lower levels of plasma Hsp27 might be associated with an increased risk of COPD among coal workers. They may have the potential to be monitoring markers for COPD in coal workers.

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