Original

Differential gene expression associated with inflammation in peripheral blood cells of patients with pneumoconiosis

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Abstract: Objectives: To study expression changes in inflammation-related genes in peripheral blood of patients with pneumoconiosis and to explore the possibility of these genes as pneumoconiosis biomarkers. **Methods:** Peripheral blood samples of patients with pneumoconiosis patients and controls were collected, and total RNA of the blood cells were extracted and reverse transcribed to cDNA. Screenings of deferentially expressed genes associated with inflammation between patients with pneumoconiosis and controls were performed using real-time quantitative PCR array and the expressions of the three most upregulated genes were confirmed by real-time PCR. **Results:** The expression of 11 genes was significantly altered in patients with pneumoconiosis compared with those of the control. Among these 11 genes, 8 genes were upregulated and 3 were downregulated. Preliminary results indicated that interleukin 6 (IL-6) mRNA expression in patients with pneumoconiosis was higher than that in controls (P=0.019). The level of IL6 mRNA expression in the patients was higher than that in non-smoking controls, but it was neither affected by type and stage of pneumoconiosis nor by time of contact with dust. **Conclusions:** IL6 was possibly involved in the development of pneumoconiosis.
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Key words: Pneumoconiosis, Peripheral blood cells, Real-time quantitative PCR array, Inflammation

Introduction

Pneumoconiosis is a diffuse fibrosis in lung tissue caused by occupational and long-term inhalation of production dust and its deposition in the lungs. *The National Occupational Disease Report*, published by the National Health and Family Planning Commission of PRC, suggested that coal workers' pneumoconiosis (CWP) and silicosis account for 60.28% and 34.96% of the total number of new cases of pneumoconiosis in China in 2013, respectively. Silicosis is extensive lung fibrosis due to long-term inhalation of crystalline silica dust. The pathogenesis of silicosis and the role of alveolar macrophages in this process remain unclear[1]. Under normal circumstances, alveolar macrophages remove foreign substances and play a protective role[2,3]. Substantial evidence shows that long-term exposure to silica particles can cause macrophage apoptosis. In this process, macrophages may release large amounts of cytokines to induce immune response and inflammation and reduce the number of macrophage cells[4-7].

CWP is caused by long-term inhalation of coal dust in the lungs, followed by chronic inflammation associated with the accumulation of alveolar macrophages and the activation of vascular endothelial cells[8]. Reactive oxygen species and cytokines may play an important role in lung diseases due to coal exposure. Inflammatory cell recruitment (including of neutrophils and monocytes) and adhesion play an important role in lung inflammation[9]. In addition, T helper cells and their secreting cytokines are involved in the pneumoconiosis. For example, IL-1 can regulate Th17 in silica dust-induced lung inflammation[10]. sTNFR2 (Soluble tumor necrosis factor receptors 2) is associated with silicosis severity and early exposure to silica dust[11]. IL-1 can regulate Th1/Th2 balance to inhibit inflammation and fibrotic response[12]. In silica dust-induced lung fibrosis, the IL-6 receptor mediates the Th2 cytokine network. Th2-type cytokines (IL-4, IL-5, and

IL-6) and the signal molecules (STAT3: Signal transducer and activator of transcription 3 and SOCS 3: Suppressor of cytokine signaling 3) are significantly increased in the airway of mice exposed to silica dust, whereas Th1-type cytokines (IL-1 and TNF-α) are not[13]. TGFβ-1 (Transforming growth factor beta 1) and MCP-1 (monocyte chemotactic protein 1) are significantly higher in the serum of patients with progressive CWP than in nonprogressive CWP, indicating the relevance of their progression[14]. Inhalation of dust can induce the formation of inflammasome NLRP 3 (NACHT, LRR, and PYD domains-containing protein 3). NLRP3 can activate caspase-1 and promote proinflammatory factors IL-1b and IL-18[15]. However, the specific mechanism of these cytokines and the network of inflammatory factors in silicosis and CWP remain unclear.

There is currently no effective treatment of pneumoconiosis, only prevention and combinational methods[16,17]. Therefore, early detection is very important. Screening inflammation-associated genes from the peripheral blood favors the early discovery of gene expression changes in inflammatory genes in patients with pneumoconiosis. This is important for secondary prevention. Therefore, we used real-time PCR array technology to screen genes correlated with inflammation in the peripheral blood of patients with pneumoconiosis. Further, we studied the genes in population and found a potential biomarker gene associated with pneumoconiosis.

Subjects and Methods

Subjects

All patients with pneumoconiosis were diagnosed and staged according to the diagnostic criteria of pneumoconiosis (GBZ 70-2009, which is one of national occupational health standard in China). The pneumoconiosis was staged on the basis of the chest X-ray manifestations according to the GBZ 70-2009. The chest X-ray has a general intensive small shadow level 1, with a distribution range of at least 2 lung regions was classified as stage I of pneumoconiosis; the overall intensity of the small shadow of level 2, with a distribution range >4 lung area; or a small shadow of total density 3, with a distribution range of up to 4 lung regions was classified as stage II of pneumoconiosis. Stage III of pneumoconiosis must meet one of the following conditions: a. A big shadow occurs, with length ≥20 mm and short diameter ≥10 mm; b. there is the overall intensity of the small shadow of level 3, with a distribution range >4 lung areas and the small shadow occurs together; c. There is a small shadow of the overall density of level 3, with a distribution >4 lung areas, and a large shadow occurs.

According to smoking status, the control group was divided into group A which was "smokers" and group B which was "nonsmokers or ex-smokers." Patients with pneumoconiosis who were nonsmokers or ex-smokers belonged to group C.

All experiments involving clinical specimens were approved by the Ethics Committee of the Henan Institute of Occupational Disease Prevention. All blood samples from patients and controls were obtained after informed consent. No previous history of bronchial asthma, chronic bronchitis, chronic obstructive pulmonary disease, or other respiratory diseases were reported. Idiopathic pulmonary fibrosis and other interstitial lung disease that could cause connective tissue disease were excluded. No history of exposure to dust or other harmful factors was reported.

Blood collection and RNA Extraction

In each subject, 1.5-mL peripheral vein blood was collected with EDTAK2 anticoagulant. Further, RNA was extracted using blood RNA-extraction kit (Qiagen Biotechnology Co.) according to the manufacturer's instructions. Total RNA was determined and then saved.

Real-time quantitative PCR array

Real-time quantitative PCR array containing inflammation-related genes (PAHS-097Z, Qiagen) was used for screening. The PCR reaction system is as follows: 1,350 μL 2 × RT2 SYBR Green Master mix, 102 μL cDNA synthesis reaction, 1,248 μL RNase free water, for a total volume of 2,700 μL. PCR reaction conditions were: 95°C, 10 min; 95°C, 15 s and 60°C, 1 min, ×40 cycles. After completion of the reaction, the melting curve was analyzed, 95°C, 15 s, 60°C-95°C, 1 min.

Data analysis

We used the software RT2 Profiler PCR Array Data Analysis version 3.5. Ct values of *ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0* were housekeeping genes. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression in the control group. This was calculated as follows: ΔCt of pneumoconiosis = Ct target gene in pneumoconiosis group - Ct reference genes in the pneumoconiosis group. ΔCt of control group = Ct of target gene in control group - Ct reference genes. ΔΔCt = Ct pneumoconiosis group - Ct control group, $2^{-\Delta\Delta Ct}$ is the relative mRNA expression of target gene of pneumoconiosis group relative to the control group.

Preliminary population study

Total RNA from peripheral blood cells of 44 cases of patients with pneumoconiosis and 49 cases of the control were extracted with Trizol (Invitrogen). cDNAs were synthesized using a Promega RT kit followed by real-time quantitative PCR reactions. Three replicates were set for each sample set. PCR primer sequences are shown in Table 1.

Table 1. Real-time quantitative PCR primer sequences

Gene symbol	Primer sequences	GenBank	Length (bp)
GAPDH	5'-GGAGAAGGCTGGGGCTCAT-3' (F)	NM_001924.3	240
	5'-TGGGTGGCAGTGATGGCA-3' (R)		
IL6	5'-CAAATTCGGTACATCCTC-3' (F)	NM_000600.3	257
	5'-CTGGCTTGTTCCTCACTA-3' (R)		
IRF2	5'-ATTTGCCAAGTTGTAGAGG-3' (F)	NM_002199.3	116
	5'-TCAGTCGTTTCGCTTTCT-3' (R)		
PTGS2	5'-TGCAACACTTGAGTGGCTAT-3' (F)	NM_000963.3	139
	5'-AAGGTGTCAGGCAGAAGG-3' (R)		

Table 2. Differentially expressed inflammation genes associated with pneumoconiosis

Symbol	$2^{-\Box Ct}$		Fold change PG/CG	Fold regulation
	Pneumoconiosis Group (PG)	Control Group (CG)		
AIM2	0.011646	0.026957	2.315	2.315
CASP5	0.011249	0.024702	2.196	2.196
CXCL1	0.036172	0.109636	3.031	3.031
CXCL2	0.043616	0.099359	2.278	2.278
IL6	0.000101	0.000329	3.271	3.271
IRF2	0.040274	0.131652	3.269	3.269
PTGS2	0.095127	0.319702	3.361	3.361
PYDC1	0.000166	0.000338	2.032	2.032
IFNB1	0.00013	0.000059	0.455	−2.198
MOK	0.001377	0.000643	0.467	−2.142
TNFSF11	0.000326	0.00015	0.460	−2.172

Statistical analysis

Experimental data were analyzed by SPSS 21.0 statistical software. After verifying the data through data transformation (logarithm of the relative expression amount in base 10) with normal distribution, we used independent-sample t-test and ANOVA analysis to analyze the gene expression data of the pneumoconiosis group and the control group. Test level α=0.05.

Results

Screening of differentially expressed inflammation genes in patients with pneumoconiosis

A total of 5 pneumoconiosis cases confirmed by the Occupational Disease Prevention Institute included 1 Stage I asbestosis, 1 stage I of CWP, 1 stage III silicosis, 1 stage II potters' pneumoconiosis, and 1 case of stage II welders' pneumoconiosis. The age was 53.2 ± 7.0 years (45-59 years). The sex ratio was 1.5 (male:female). The healthy controls were two examiners from a hospital in Henan province, aged 50 and 60 years, and the age difference between the pneumoconiosis group and control group was not statistically significant (P=0.773). Pneumoconiosis cases for verification tests were from inpa-

tients of the same hospital diagnosed with pneumoconiosis, with a total of 44 cases, male, aged 53.3 ± 13.4 years. It included 20 cases of silicosis, 19 cases of CWP, 2 cases of pneumoconiosis welders, and 3 cases of cement pneumoconiosis. There were 12 cases of stage I, 12 cases of stage II, and 20 cases of stage III. The nonexposure time was 10.5 ± 11.8 years (0-44 years); the control group was 49 healthy persons in a hospital in Henan Province with an average age of 53.4 ± 15.8 years. Age between the two groups was not statistically significant (P=0.746).

Fold changes in inflammation-related gene expression in five cases of patients with pneumoconiosis versus two cases of healthy control were evaluated. Genes with expression ≥2 or ≤0.5 were considered deferentially expressed genes. The results showed that 11 genes, among 84 detected inflammation-related genes, in five types of pneumoconiosis were significantly changed, among which 8 were upregulated genes and 3 were downregulated genes (Table 2, Figs. 1 and 2).

Differences in inflammatory genes were compared between male and female patients. In three male cases, AIM2, CASP5, CXCL1, IL12B, IL6, IRF2, PTGS2, and PYDC1 were increased. IFNG and MOK were decreased. In two female cases, AIM2, CXCL1, CXCL2, IL6, IRF2,
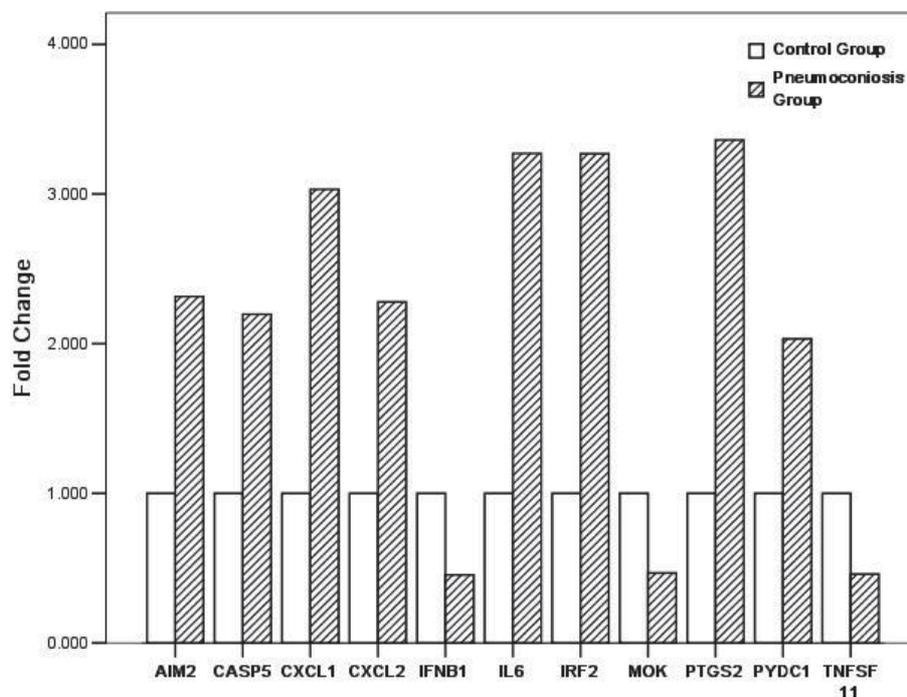
Fig. 1. Histogram of fold difference of inflammation-related genes and significantly differentially expressed genes among pneumoconiosis group/healthy controls
Note: Group 1 is pneumoconiosis group, Control is control group

and PTGS2 were increased and IFNB1 and TNFSF11 were decreased in two types of pneumoconiosis.

Gene expression differences among different types of patients with pneumoconiosis

The experimental data of five cases of patients with pneumoconiosis were compared with five types of pneumoconiosis, namely "asbestosis group," "the CWP group," "the silicosis group," "the potters' pneumoconiosis group," and "the welders' pneumoconiosis group." In the CWP group, 19 gene expressions changed, including those of 16 genes with increased expression (AIM2, CARD6, CASP5, CXCL1, CXCL2, IL6, IRF2, PTGS2, PYDC1, IL12B, CASP1, CCL2, IL1B, MAPK12, MAPK13, and TAB2) and 3 genes with decreased expression (BCL2, IFNB1, and IFNG). In the asbestosis group, 9 genes changed in gene expression, with 6 genes increasing (CXCL2, CXCL1, IL6, IRF2, NFKB1A, and PTGS2) and 3 genes decreasing (BCL2, IFNB1, and TNFSF11). In the welders' pneumoconiosis group, 21 genes increased in expression (AIM2, CARD18, CASP1, CASP5, CCL5, CXCL1, CXCL2, IL12B, IL33, IL6, IRF2, NLRC4, NLRP5, PTGS2, CCL7, IFNG, MAPK11, MYD88, NLRC5, NLRP4, and PYDC1) and 6 genes decreased in expression (CIITA, FADD, NLRX1, NOD2, P2RX7, and PANX1). The potters' pneumoconiosis group had 7 increased genes (AIM2, CASP5, CXCL1, IL6, IRF2, PTGS2, and TNFSF4) and 3 decreased genes (FADD, IFNB1, and NOD2). In the silicosis group, there

were 5 genes with increased expression (CXCL1, IL6, IRF2, NLRP4, and PTGS2) and 5 genes with decreased expression (MAPK11, MAPK12, MOK, TIRAP, and TNFSF11).

Preliminary population study of IL6, IRF2, and PTGS2

After the summary of microarray results, we found that the expression of IL6, IRF2, and PTGS2 in the distribution of pneumoconiosis group and the control group did not meet normal distribution. After data transformation, the distribution was consistent with normal distribution. The results showed that the relative expression of IL6 (relative expression quantity, RQ) in the pneumoconiosis group and the control group were 0.638 ± 0.376 and 0.492 ± 0.366, respectively. Relative expression of IL6 in the pneumoconiosis group was statistically significantly higher than that of the control group ($t=2.381$, $P=0.019$). IRF2 RQ values of expression in pneumoconiosis group and the control group were 1.655 ± 0.734 and 1.425 ± 0.496, respectively. The difference in RQ value was not statistically significant ($t=0.956$, $P=0.341$). The RQ values of PTGS2 in the pneumoconiosis and control groups were 2.769 ± 2.000 and 2.466 ± 1.326, respectively. The difference in RQ value was not statistically significant ($t=0.251$, $P=0.803$; Fig. 3A).

Then, we investigated whether pneumoconiosis disease, staging, nonexposure time, and smoking status could affect IL6 mRNA expression. According to different types of pneumoconiosis grouping, IL6 expression be-
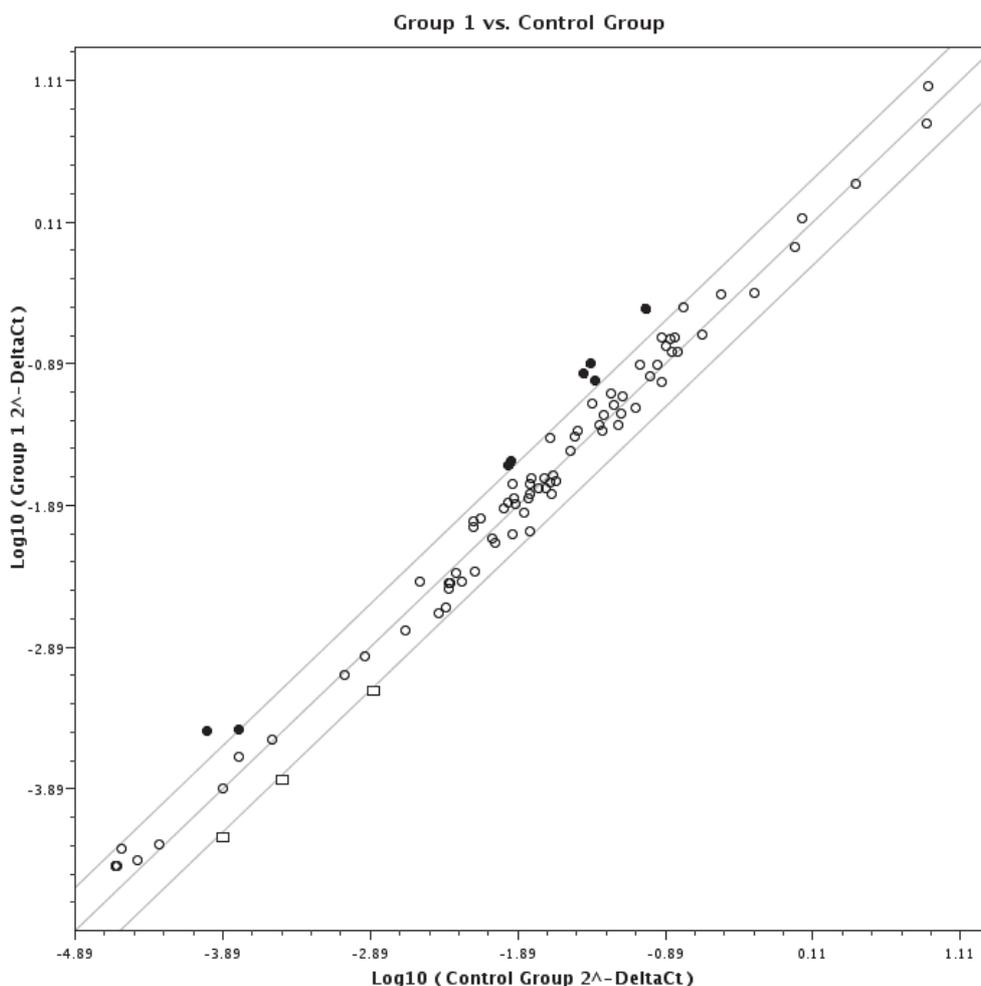
Fig. 2. Scatter plot of of fold difference vs gene difference of 84 inflammation-related genes (pneumoconiosis group/healthy control group).
Note: In figure with three gray lines, the middle of representative of the ratios of 1 point in the region on both sides of two gray lines included (black) represents difference between the two groups with no genetic expression, the red dot represents differentially expressed genes upregulated in pneumoconiosis group, and the green dots represent differentially expressed genes downregulated in the pneumoconiosis group.

tween the groups silicosis, CWP group, welders' cement pneumoconiosis group, and the control group was not statistically significant (F=2.392, P=0.074; Fig. 3B). According to the different stages of pneumoconiosis, IL6 expression differences between stage I, II, and III pneumoconiosis and the control group was not statistically significant (F=2.518, P=0.064; Fig. 3C). According to frequency distribution of nonexposure time of patients with pneumoconiosis, the patient leaves his post exposure to dust, which was divided into A group "0-2 years," Group B "3-4 years," Group C "5-10 years," and D group ">11 years." *IL-6* expression between each group and the control group was not statistically significant (F=1.425, P= 0.233; Fig. 3D). We found IL6 expression was statistically significant (F=3.598, P=0.032) between each pair (Fig. 3E). We found that Group C (pneumoconiosis

group) IL6 mRNA expression levels were significantly higher than those of group B (non-smokers in the control group; P<0.05). We found types of pneumoconiosis disease, staging, nonexposure time, and smoking status had no effect on the expression of IRF2 mRNA and PTGS2 mRNA.

Discussion

In the early stages of pneumoconiosis, a large number of inflammatory cells infiltrate into the lung, cause alveolar inflammation, and release various cytokines[18,19]. These cytokines interact with corresponding receptors, transduce the signal into cells, and regulate the expression of cellular genes that may cause inflammation [20,21]. Current mechanistic research often focuses on a single factor or
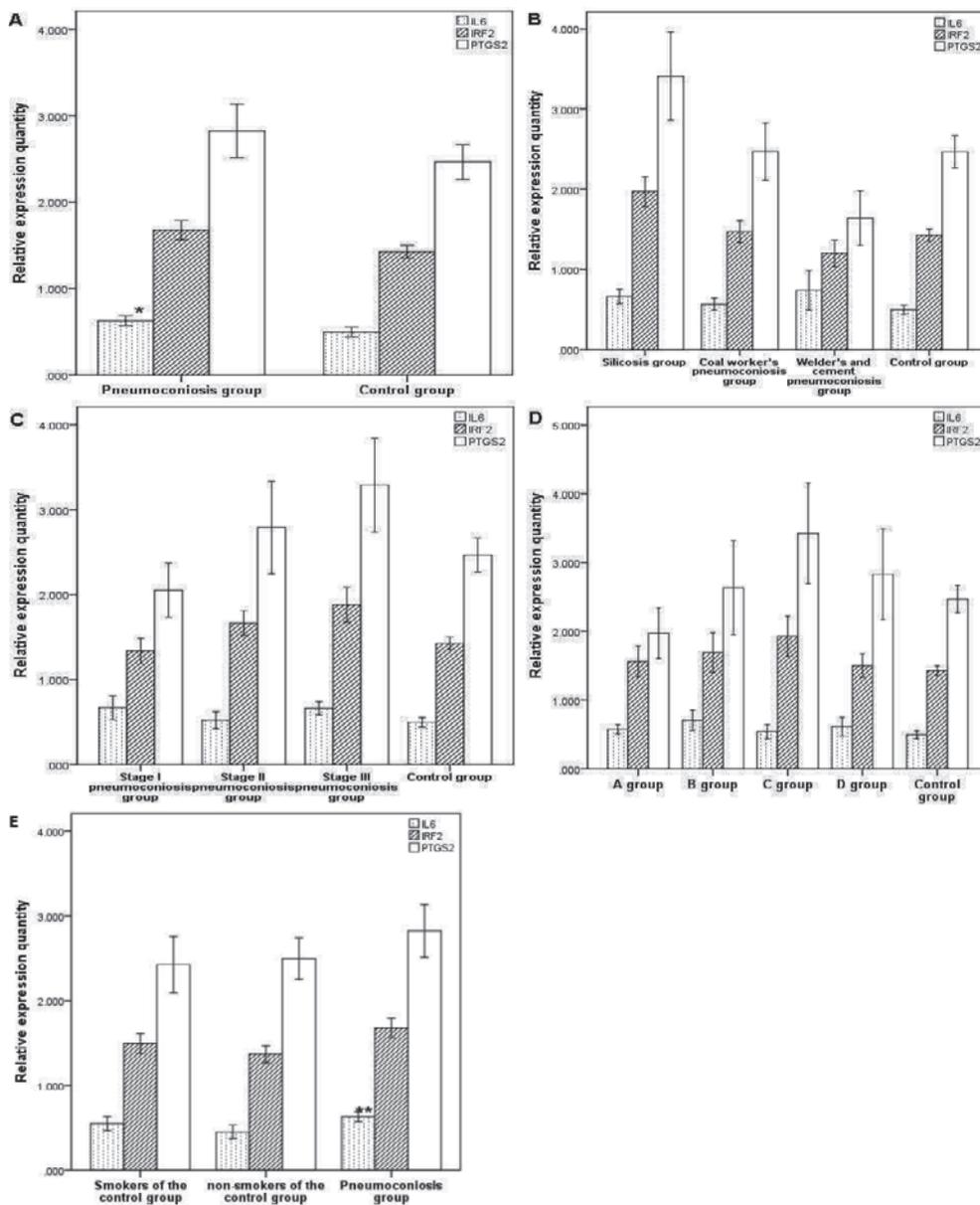
Fig. 3. Comparison of three genes expression between pneumoconiosis groups and control group under different classifications.

Herein, relative expression quantity was calculated as follows: ΔCt of pneumoconiosis=Ct target gene in pneumoconiosis group - Ct reference genes in the pneumoconiosis group. ΔCt of control group=Ct of target gene in control group - Ct reference genes. $\Delta\Delta$Ct=Ct pneumoconiosis group - Ct control group, $2^{-\Delta\Delta Ct}$ is the relative mRNA expression of target gene of pneumoconiosis group relative to the control group. The target gene was IL-6, IRF2 and PTGS2, respectively.

A. pneumoconiosis group and control group; B classification of different diseases; C classification of different stages; D Classification of different time of the patient leave his post exposure to dust, A group represent "0-2 years", B Group represent "3-4 years", C Group represent "5-10 years", and D Group represent "more than 11 years". E smoking status classification * compared to the control group, P<0.05; ** and non-smokers of the control group, P<0.05.

single path, but the network regulation of various cytokines remains unclear.

Applying inflammation-related gene PCR Array, this study was conducted the comparison of inflammatory genes of peripheral blood between patients with pneumoconiosis and healthy controls, screening 11 inflammatory

genes (accounting for 13.1% of detected genes) associated with five types of pneumoconiosis. Inflammation-related gene PCR Array (PAHS-097Z) for each 96-well plate contains PCR primers for 84 inflammation-related genes and five housekeeping genes, a genomic DNA contamination quality control, 3 RT quality control, and 3 PCR efficiency quality control. The inflammation-related gene PCR Array (PAHS-097Z) can analyze the expression profile of inflammatory cytokines and their receptors genes, including genes of cytokines associated with metabolism, cytokines receptor interaction-related genes, and genes associated with acute phase reaction, inflammation, and the humoral immune response. We found that AIM2, CASP5, CXCL1, CXCL2, IL6, IRF2, PTGS2, and PYDC1 (9.5%) had increased expression. IFNB1, MOK, and TNFSF11 (3.6%) had decreased expression. These candidate genes included interleukin, the CXC chemokine family, interferons, and the tumor necrosis factor.

Among these deferentially expressed genes, IL6 mRNA and protein levels are elevated in alveolar macrophages and bronchial lavage fluid of fibrosis animal models, patients with idiopathic pulmonary fibrosis, nodular pulmonary fibrosis patients, and patients with silicosis[22-25]. IL-6 protein level was elevated in the serum of CWP and silicosis[17,18,26]. However, IL-6 mRNA change has not been reported in patients with pneumoconiosis. In various pneumoconiosis, COX-2 (cyclocygenase-2, also known as PTGS2) expression is significantly increased in the cerebral cortex of CWP rats and in macrophages of patients with pneumoconiosis, which plays a role of anti-fibrosis[19,20], but its expression in blood of patients with pneumoconiosis and pulmonary fibrosis has not been reported. Therefore, we validated IL6, IRF2, and PTGS2 expression in blood from patients with pneumoconiosis and controls with matched age.

Preliminary populations findings showed that IL6 mRNA expression in peripheral blood of patients with pneumoconiosis was significantly higher than in that of controls, and in line with the screening results (3.271 times) and reports[23,24]. IRF2 and PTGS2 results were inconsistent with the screening results (3.269 times and 3.361 times, respectively). The reason may be the reduced sample size, different housekeeping genes in screening, and verification, or individual differences. Although immunohistochemistry staining shows widespread COX2 expression in idiopathic interstitial pneumonia or fibrosis[27]; however, expression of pneumoconiosis remains unclear and needs further study. In addition, because pneumoconiosis is caused by occupational exposure to dust, the vast majority are male patients. Apart from the female patients in screening, only two female cases were gathered. One showed mixed pneumoconiosis from metal dust and silica dust. The other showed lung infections. Therefore, they were not included in the verification test. Only male patients were included.

In summary, this study used real-time quantitative PCR array technology to compare the expression of inflammation-related genes in peripheral blood of pneumoconiosis and healthy control and screen 11 deferentially expressed genes. Real-time quantitative PCR arrays can accurately detect mRNA levels of hundreds of genes at one-time, avoiding much follow-up verification. It is a powerful tool for performing pneumoconiosis biomarker-related studies. Initial validation results showed that cytokine IL6 mRNA expression levels may be associated with the development of pneumoconiosis. The next step is to collect more pneumoconiosis and control samples to study the expression of nonselected genes in the screening and to find new candidate genes associated with pneumoconiosis. The preliminary results of this study may provide experimental basis for pneumoconiosis candidate genes, help to find new biomarkers in population with dust exposure, in different pneumoconiosis stages, different types of pneumoconiosis in the future, and further provide a reliable scientific basis for the effective implementation of secondary prevention (early detection, diagnosis, and treatment).

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