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Original Article

Protein profiling detection in serum of silica-exposed population by liquid-chip time of flight mass spectrometry

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Abstract: Objective: To screen serum biomarkers of silica-exposed population for early diagnosis by liquid-chip time of flight mass spectrometry, and identify some associated functions of the markers. Methods: In addition to 30 healthy donors without silica-exposed history chosen as control, All the 85 subjects diagnosed as varied phase of silicosis according to national diagnostic standard (GBZ70-2002) of pneumonoconiosis without complications were recruited and taken blood to get serum. Short peptides purified by Dynabeads RPC18, were detected and their amino acid sequences determined using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). Effects of the identified peptides on Human embryonic lung fibroblasts (MRC-5) were tested using synthesized peptides. Results: A total of 5 peaks ($P<0.01$) were detected between healthy donors and silica-exposed population, in which the expressions of 5081 Da and 5066 Da are up-regulated while the expressions of 3954 Da, 2021 Da and 1777 Da are down-regulated. 2021 Da and 1777 Da peaks were selected for MALDI-TOF-TOF-MS, the amino acid sequences of the two peaks identified were both a fragment of complement C3 called complement C3f. The level of C3f was related to silicosis activity. The synthesized peptides of C3f inhibited proliferation of I, III collagen and TGF-β_1 of MRC-5. Conclusion: This analysis in our study revealed C3f in serum of silica-exposed population may play an important position. Investigation of C3f may be a useful tool for the diagnosis and evaluation of disease activity in silicosis.

Keywords: Liquid-chip time of flight mass spectrometry, MALDI-TOF-MS, MALDI-TOF-TOF-MS, silicosis, C3f, MRC-5

Introduction

Slilicosis, with the characteristics of pulmonary interstitial fibrosis, is caused by long-term inhalation of a large number of free slilica dust. It is one of the most serious occupational diseases in China. The average annual economic losses of each pneumoconiosis case was about 34.1 thousand yuan and the direct economic losses caused by silicosis alone were more than 140 billion yuan each year. An annual increase of cases of pneumoconiosis could cause economic losses of 600 million yuan. In recent years, the prevalence of silicosis showing new features of group incidence, lower-aged tendency and high-disabled rate, which brings new challenges for prevention and treatment of silicosis. Recently, many of the valuable serum biomarkers have been found for the diagnosis of silicosis, such as the lung-specific Clara cell protein (CC16) and pulmonary surfactant D (SP-D) [1-3]. As the diagnostic indicators, they still exist disadvantages of sensitivity and specificity and different types of interstitial lung disease exist large overlap of these indicators. However, bronchoalveolar lavage fluid chemistry and cytology can only add or exclude from imaging diagnosis. The use of new technology to screen high specific and sensitive biomarkers will play an important force in prevention and treatment of silicosis. Liquid-chip time of flight mass spectrometry is one of the protein identification techniques that bases on magnetic beads separation combined with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). It can directly detect

Table 1. The age of four experimental groups ($\bar{x}\pm s$)

	Control	Phase 0	Phase 0+	Phase I
Range of age	35-62	35-60	35-58	35-62
Average age	46.2±6.4	46.1±6.3	45.1±5.8	45.9±7.0

note: female:male = 1:4, there exist no differences among groups.

clinical specimens without special treatment, such as serum, urine fluid, cerebrospinal fluid, serous effusion or others but with high sensitivity and specificity for diagnosis [4, 5]. The aim of this study is to screen serum biomarkers of silica-exposed population by Liquid-chip time of flight mass spectrometry, then estabilish an artifical neural network model for early diagnosis and identify some associated functions of the markers which may contribute to a further study of the pathogenesis of silicosis mechanisms.

Materials and methods

Subjects

Workers from a refractory plant that diagnosed as phase 0, phase 0+, phase I of silicosis according to national diagnostic standard (GBZ70-2002) of pneumoconiosis was candidate for our study, by inspecting BP, ECG, BLOOD ROUTINE, hepatic-pulmonary function and Unified high kilo-volt retroposition-orthotropia X ray plain plate (120-150 KV, 100 mA, 0.03-0.05 s) were taken, according to experiment design, total of 85 qualified subjects (30 phase 0, 30 phase 0+ , 25 phase I) without recurrent cardiovascular, hepatic, pulmonary disease were enrolled into our study. **Table 1**. The epidemic data were registered in question sheet manner. Addition, 30 healthy donors without silica-exposed history were chosen as control. As the diagnostic standards have disclaimed and the prerequisite that all subjects have the silica-exposed history, silica-exposed workers (phase 0) mean only have occupational history but not abnormal in X ray thoracic plate; suspects (phase 0+) mean the abnormal in X ray thoracic plate but insufficient to radiology criteria of I phase of pneumoconiosis; phase I of silicosis mean subject with generally grade 1 of radiological shadow which disperse at least two lung zones. 5 ml of morning blood sample was taken by punctuating ulnar vein of subjects who fasting at least 8 h, then centrifuged at 4°C at 3000 rpm for 10 min within 2 h, and aliquoted into eppendorf tube stocked at -80°C until use.

Separation and purification of serum peptides

Serum peptides were separated and purified with a purification kit of magnetic beads, using Dynabeads RPC18 (Invitrogen, American) according to the manufacturer's instructions. Briefly, 20 μl (0.25 mg) of magnet ic beads was washed 2 times using 50 μl deionized water, then suspected with 10 μl deionized water. 50 μl of each serum samples were mixed with 10 μl magnetic beads and the beads were then collected by a magnetic beads separator. After washing 3 times in an wash solution (1% TFA, trifluoroacetic acid), the bound peptides were eluted off the beads into 6 μl of 50% acetonitrile (ACN), stocked at -20°C until use.

Mass spectrometry Identification

1 μl of the eluted sample was diluted 1:10 in matrix solution matrix solution (0.3 mg/ml α-cyano-4-hydroxycinnamic acid in ethanol: acetone 2:1). Then 1 μl of the mixture was spot onto a MALDI-TOF-MS target (AnchorChipTM, Bruker Daltonics) and dried at room temperature before analysis. The mass spectra of the peptide peaks were initially detected in the automatic linear positive mode, the first ion source 25 KV, the second ion source 23.45 KV, from an average of 800 laser shots per sample. ClinProTools (ClinProt software version 2.0, Bruker Daltonics) was used to subtract baseline, normalize spectra (using total ion current) and determine peak m/z values and intensities in the mass range of 800 to 10,000 Da. The signal-to-noise (S/N) ratio should be higher than five. To align the spectra, a mass shift of no more than 0.1% was determined. The peak area was used as quantitative standardization. SPSS 11.5 for Windows was used for statistic analysis, with $P<0.05$ as statistically significant existing standards. Finally, MALDI-TOF analysis and a subsequent sequence search using the search engine Mascot (www.matrixscience.com) were performed to identify the sequences of the peptides of interest.

Synthesis of complement fragments C3f

Complement C3f, a 17 peptides, which amino acid sequence is NH2-Ser-Ser-Lys-Ile-Thr-His-

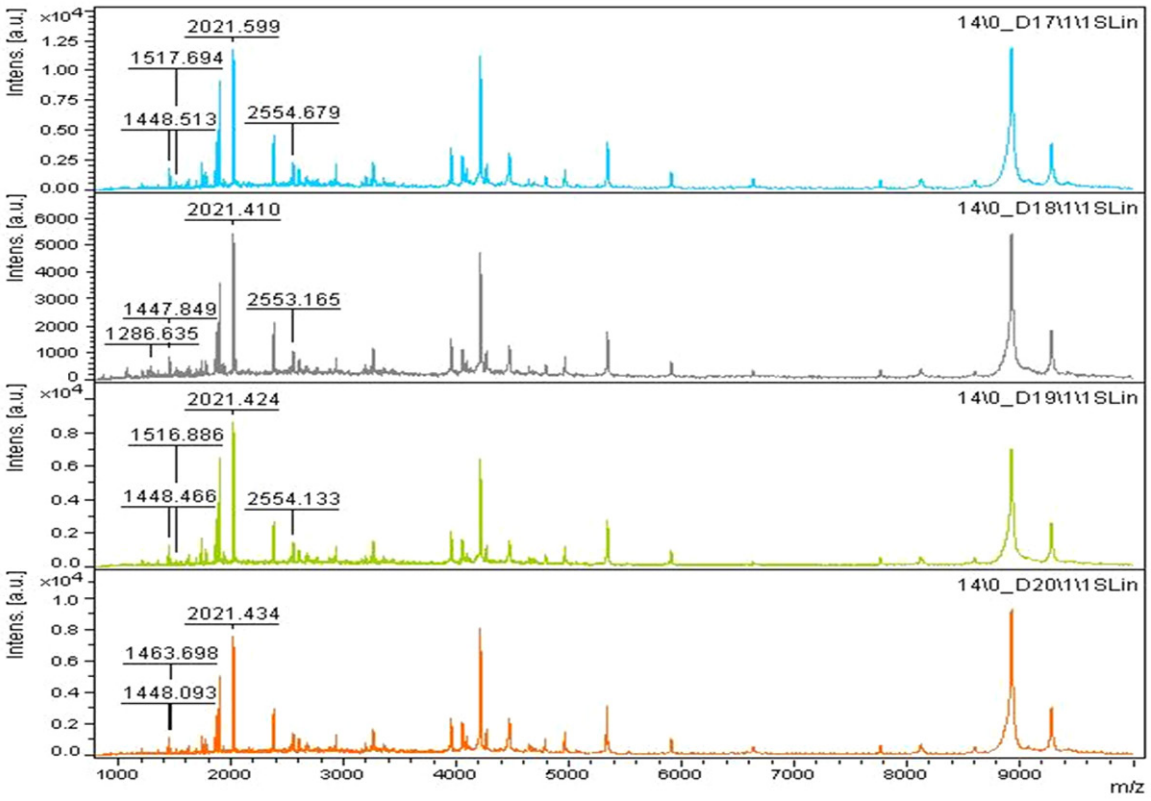

Figure 1. Analysis of experiment parallelism.

Table 2. Inter-group pairwise comparison results (SNN method)

Groups	Recognition rates	Cross-validation capabilities	Evaluation
Phase 0 vs phase 0+	64.35%	63.14%	Poor
Phase 0 vs phase I	84.38%	52.82%	Poor
Phase 0+ vs phase I	73.49%	69.07%	Poor
Control vs Phase 0	89.23%	87.66%	Better
Control vs phase 0+	98.52%	94.20%	Better
Control vs phase I	98.96%	85.53%	Better

Table 3. The significant different peaks between Silica-exposed groups and control group

Groups	Up-regulated peaks	Down-regulated peaks
Control vs phase 0	5081.8 Da	2021.4 Da
Control vs phase 0+	5066.25 Da	3954.54 Da
Control vs phase I	5081.76 Da	1777.56 Da

Arg-Ile-His-Trp-Glu-Ser-Ala-Ser-Leu-Leu-Arg-COOH. In our study, solid phase peptide synthesis method and high performance liquid analyzer were used to synthesize and purificate C3f [6], purity 99%.

Cell culture

Human embryonic lung fibroblasts (MRC-5, Cell bank of Chinese Academy of Sciences, Shanghai) were cultured in RPMI 1640 medium (Hyclone, American) containing 10% fetal bovine serum (FBS) in culture bottles (Corning, American) to a density of 5×10^7/ml, then inoculated into 12-well cell culture plate, 1 ml of each well, cultured at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Cells were washed by serum-free RPMI 1640 medium 2 times. Different concentrations (0, 15.6, 62.5, 250 ng/ml) of C3f-1640 medium were added in the 12-well plate, 3 wells of each concentration, 1 ml of each well. cultured at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Supernatants were collected to detect the Proliferation of I, III collagen and TGF-β_1 by enzyme-linked immunosorbent assay (ELISA);

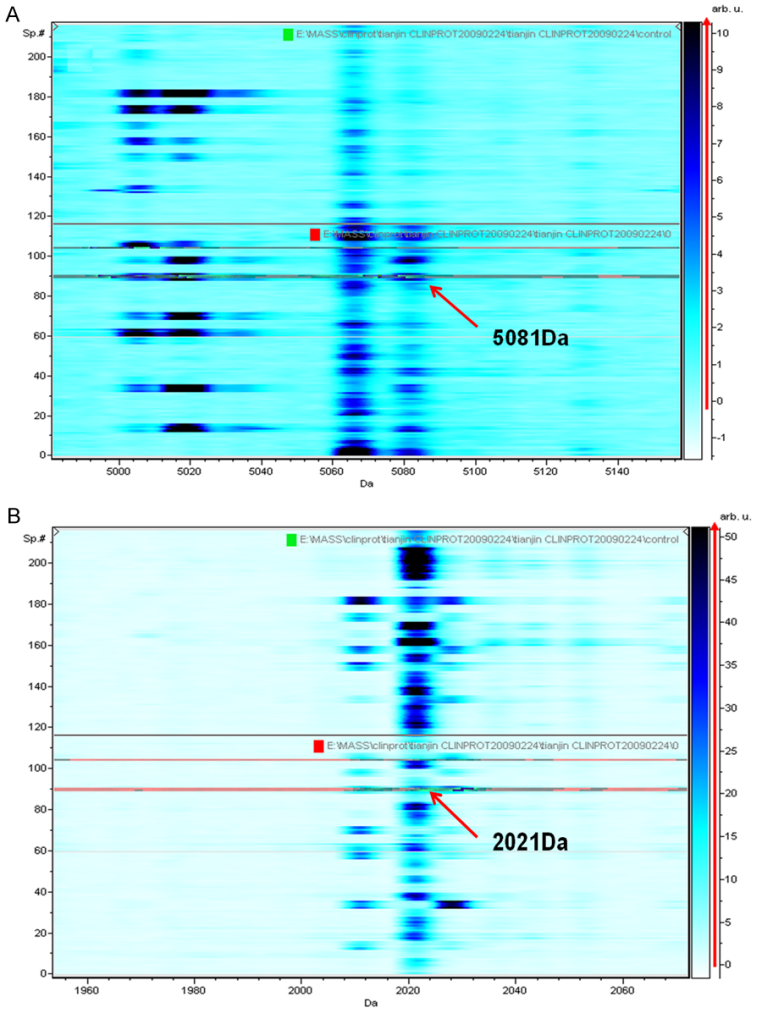

Figure 2. The gel view map of the significant different peaks between Silica-exposed phase 0 group and control group (A: Up-regulated peaks 5081 Da; B: Down-regulated peaks 2021 Da).

while cells were collected to smear on slides for detection of Proliferation of TGF-β_1 by Immunohistochemistry.

Statistical analysis

Levels of peptide peaks, clinical data, and age of the patients were expressed as the mean ± SD. Single factor analysis of variance (ANOVA) was used for comparisons between the mean values, with $P<0.05$ as statistically significant existing standards.

Results

Experimental analysis of parallel

A sample is randomly seleced in the total 115 samples for 4 parallel experiments, which indicates that the technology with good repeatability. **Figure 1**.

Neural network algorithm (SNN) for each pairwise comparison between groups

Using of SNN, we find that the recognition rates and cross-validation capabilities among phase 0 vs phase 0+, phase 0 vs phase I, phase I vs phase 0+ are poor; while control vs phase 0, phase 0+, or phase I are better. (**Table 2**).

Differential peaks between control and silica-exposed population

The SNN result reveals that differences among phase 0 vs phase 0+, phase 0 vs phase I, phase I vs phase 0+ are poor (**Table 3**); while control vs phase 0 (**Figure 2**), phase 0+ (**Figure 3**), or phase I (**Figure 4**) are better, thence we just analyze differential peaks between control and silica-exposed population.

Results of MALDI-TOF-TOF-MS

The amino acid sequences of 2021 Da and 1777 Da is identified by MALDI-TOF-TOF-MS. The results reveal that the identities of two proteins are both a fragment of complement C3 called C3f. (**Table 4**).

Correlation analysis between dust amounts and differential peaks in silica-exposed population

There exists no correlation between dust amounts and differential peaks in silica-exposed population. (**Table 5**).

The expression differences of type I, III collagen in the supernatant of MRC-5 stimulated by C3f with different concentrations

With the increase of, The expressions of type I, III collagen were progressively reduced as increasing of the concentration of C3f, each concentration points was significantly different

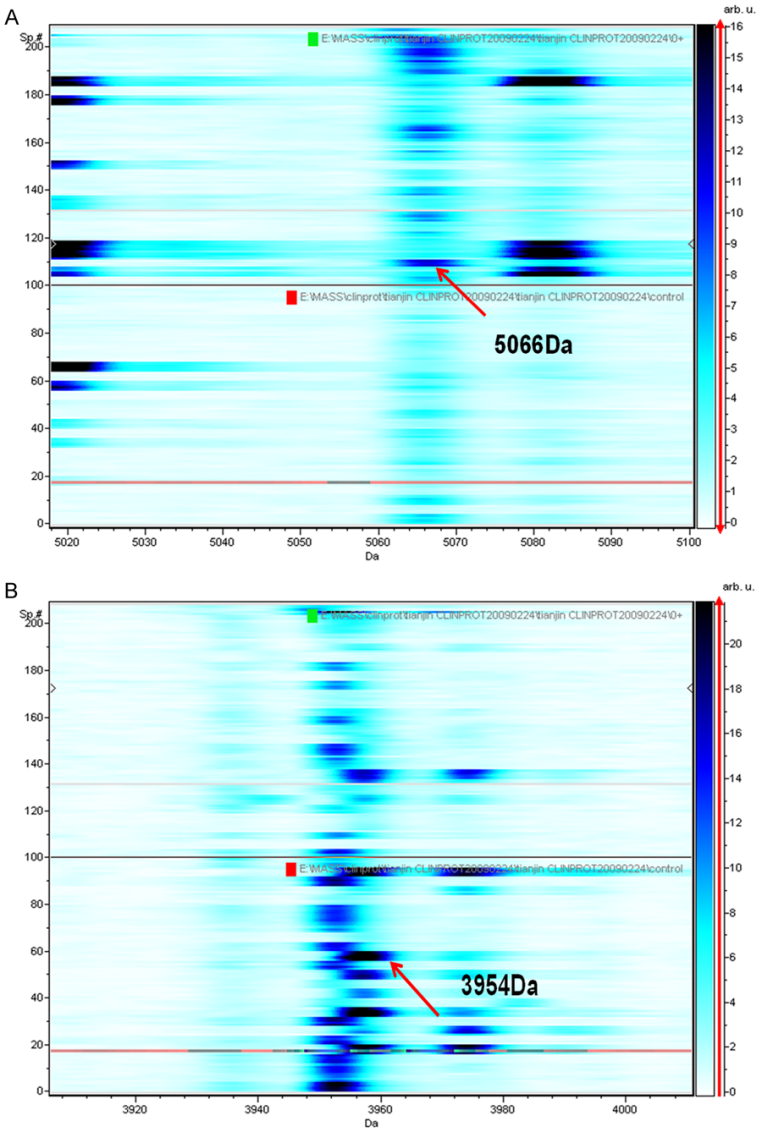

Figure 3. The gel view map of the significant different peaks between Silica-exposed phase 0+ group and control group (A: Up-regulated peaks 5066 Da; B: Down-regulated peaks 3954 Da).

(P<0.05). And the correlations between the concentration of C3f and the expression of type I, III collagen were both negative (r I = -0.800, P = 0.042; r III = -0.957, P = 0.043), which indicated that complement C3f can inhibit the formation of type I, III collagen in MRC-5. (**Table 6**).

The expression differences of TGF-β_1 in the supernatant of MRC-5 stimulated by C3f with different concentrations

The expressions of TGF-β_1 was progressively reduced as increasing of the concentration of C3f, each concentration points was significantly different (P<0.05). And the correlation between the concentration of C3f and the expression of TGF-β_1 was negative (r = -0.873, P = 0.035), which indicated that complement C3f can inhibit the formation of TGF-β_1 in MRC-5. (**Table 7**).

Immunohistochemistry results of TGF-β_1 in MRC-5

The integrated optical density of TGF-β_1 in the cytoplasm of MRC-5 was progressively reduced, each concentration points was significantly different (P<0.05). And the correlation between the concentration of C3f and the integrated optical density of TGF-β_1 was negative (r = - 0.928, P = 0.040) which indicated that complement C3f can inhibit the formation of TGF-β_1 in MRC-5. (**Table 8** and **Figure 5**).

Discussion

According to different sources of specimens, biomarkers of silicosis can be divided into three categories, including sputum, lung tissue and serum. Cells in sputum, departing from the lesions tissue, become a major source of the biomarkers. However, the amount and species of the cells are relatively random. Although lung biopsy can reflect the lesion and the reaction of proteins in organism during the courses of immune and inflammatory, trauma exist yet. During the incidence of silicosis, there exist changes of expression amount and species of many indicators in the lung tissue cells, particularly small molecule proteins, enzymes. Because the self-stabilizing function of the internal environment in organism, proteins or enzymes changed in lung tissue can be release to serum by paracrine or autocrine ways, which can cause changes in serum concentrations. Then we can conclude that serum contains more potential biomarkers, including the lung

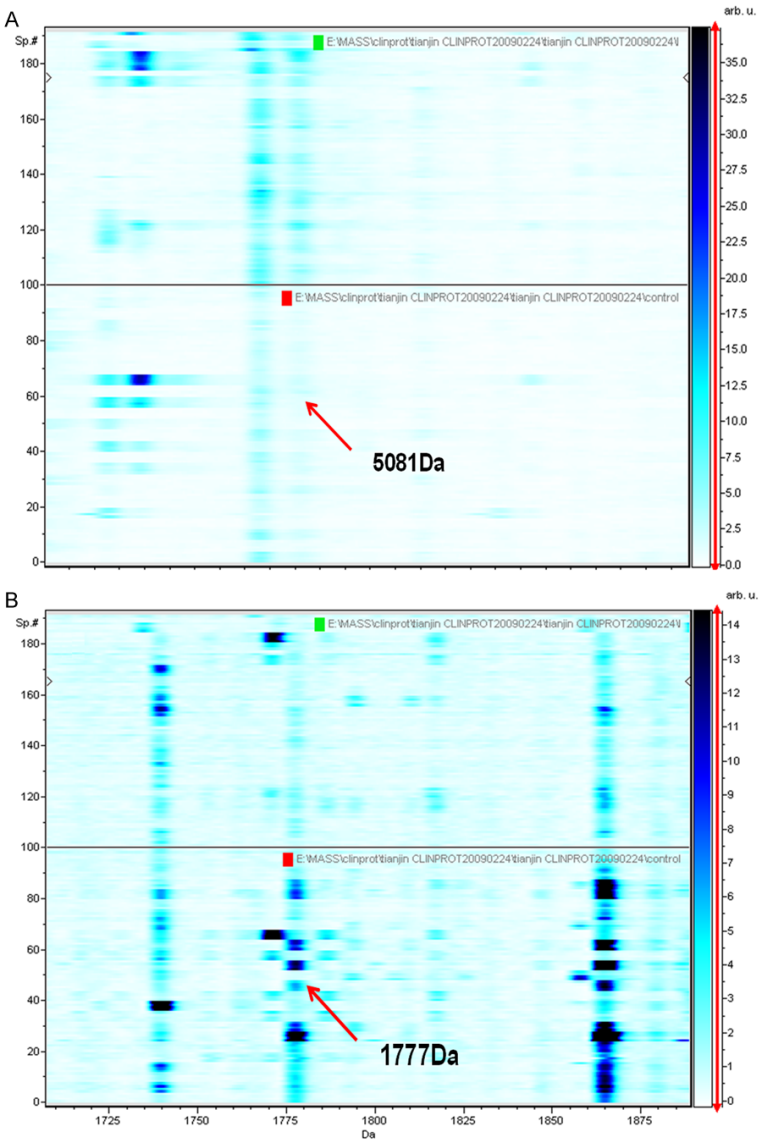

Figure 4. The gel view map of the significant different peaks between Silica-exposed phase I group and control group (A: Up-regulated peaks 5081 Da; B: Down-regulated peaks 1777 Da).

tissue and sputum biomarkers and a better clinical detection source of biomarkers.

Free SiO_2 dust is the main cause of silicosis, generally the concentration of free SiO_2 dust is higher, the condition is more serious. However, there exist no correlation between the exposure dose of dust and the intensity of peaks in our study, which may be caused by the following reasons: (1) a small number of workers have less aware of self-protection, working without wearing protective equipments, which results in relatively higher lung dust deposition; (2) there are individual differences among workers; (3) the same workers have worked in multiple workshops or factroys, which makes some statistical error exist in the exposure dose of dust; (4) the existence of rapid onset cases of silicosis, the exposure year of 15.4% of the total population are less than 2 years in our study.

Complement C3 is the molecular which has the highest concentrations of the complement system in human body, and the key elements of the complement system. In the process of complement activation, activated C3 is required in the three complement activation pathways to form C5 activating enzyme for amplification cascade reaction. C3b, formed during the activation of C3 is a component of C5 activating enzyme, which can be decomposed into iC3b and 17 peptide C3f by factor I under the help of CR1 or factor H [7]. The first two steps of decomposition, the process that C3f and C3dg are cut out, may play the most important part in the inactivation of C3b [8]. In this study we found that the peak intensities of C3f in serum of silica-exposed population, including phase 0, phase 0+ and phase I, are significantly lower ($P<0.01$), compared with the control group. That may reflect that the immune response participated by complement C3b in the pathogenesis of silicosis is relatively increased. It has been demonstrated that C3f can increase the permeability of Vascular endothelial cells, and its core molecular has growth hormone-like functions [9]. Also, C3f can improve the synthesis and secretion of TGF-β_1 in skin fibroblast [9, 10]. But the study of its specific mechanism in pathogenesis of silicosis is still blank.

Collagen is the major proteins in Interstitial lung. At present a variety of lung collagen have

Table 4. The amino acid sequence of significant different peaks by TOF/TOF($\bar{x}\pm s$)

Protein molecular weight (Da)	Identity	Amino acid sequence	Sequence matching rate	Peak values in each group ($\bar{x}\pm s$)			
				Control	Phase 0	Phase 0+	Phase I
1777	C3f	SKITHRIHWESASLL	80%	1.59±0.44	1.11±0.32*	1.22±0.12*	1.39±0.10*
2021	C3f	SSKITHRIHWESASLLR	100%	1.72±0.20	1.51±0.15*	1.56±0.20*	1.66±0.16*

Note: *compared with control, P<0.01.

Table 5. The correlation between the exprosure dose of dust and the intensitiy of peaks in silica-exposed population

Dust amount	5081 Da	5066 Da	3954 Da	2021 Da	1777 Da
130.00	7.70	13.80	11.03	8.66	0.00
259.00	2.45	2.14	5.63	83.62	0.00
346.00	3.21	8.43	8.47	8.59	0.00
1037.00	1.91	2.27	11.23	22.57	2.75
1555.00	2.94	3.70	3.37	7.44	0.00
1555.00	3.91	10.07	7.34	14.87	0.00
1744.00	2.29	3.13	4.60	31.17	0.00
1790.00	4.53	6.50	21.24	34.70	1.53
4147.00	3.68	4.69	17.37	24.37	0.00
5702.00	4.04	5.96	7.28	14.81	0.00
6739.00	5.17	8.02	7.22	11.21	0.00
7193.00	3.31	8.13	5.60	19.54	0.00
8122.00	3.90	7.30	10.30	16.32	0.00
8294.00	6.29	5.22	14.16	9.67	0.00
8294.00	2.94	3.98	9.14	21.98	1.69
8971.00	2.44	5.71	7.65	49.49	0.00
10944.00	4.18	8.25	6.24	42.42	1.45
15700.00	4.17	7.74	5.62	41.65	1.52
16416.00	1.88	3.88	8.66	22.57	5.81
16848.00	3.06	5.38	10.84	44.15	2.63
17942.00	3.51	7.99	7.04	24.84	0.00
18274.00	1.98	5.44	11.27	13.16	0.00
20250.00	2.37	5.74	12.87	68.22	2.14
21603.00	9.37	5.52	21.78	5.53	0.00
23011.00	2.80	4.15	7.10	33.02	0.00
23256.00	2.94	4.65	10.94	32.76	1.99
24883.00	3.00	6.96	21.33	29.56	3.51
26914.00	2.95	4.29	17.62	24.70	2.76
31399.00	8.27	5.12	1.79	17.28	2.58
33178.00	17.67	4.41	2.99	7.02	0.00
35885.00	0.00	4.02	9.24	40.69	4.92
41861.00	5.97	9.27	6.09	35.44	0.00
47099.00	6.07	3.76	2.92	0.00	0.00
48341.00	6.60	12.99	10.43	16.32	0.00
49248.00	2.95	5.84	9.05	15.65	1.49
49766.00	20.97	4.53	3.40	3.79	0.00
52369.00	2.96	6.49	7.42	12.25	0.00
56398.00	5.47	4.84	3.44	30.67	2.18
65117.00	3.95	6.57	20.83	21.86	0.00

71136.00	3.19	8.04	2.74	59.86	0.00
71770.00	11.59	5.10	14.92	15.29	0.00
72216.00	2.82	4.54	8.09	49.10	2.67
83750.00	6.75	6.06	5.03	24.13	11.37
89712.00	1.97	4.50	14.72	50.80	1.80
89772.00	1.79	2.48	7.02	32.16	0.00
93024.00	6.39	16.08	8.15	15.88	1.31
110808.0	3.02	5.12	3.04	77.92	0.00
114912.0	5.09	3.85	2.80	8.20	0.00
124675.0	19.46	4.81	0.00	0.00	0.00
127908.0	2.21	3.40	11.91	22.46	1.60
153446.0	2.58	4.75	15.17	24.86	2.17
156996.0	3.38	4.03	22.12	58.79	2.38
161482.0	5.57	7.70	4.90	7.74	0.00
179424.0	2.56	6.40	10.98	47.94	2.48
188438.0	4.02	7.03	8.98	21.08	1.65
204095.0	3.42	8.96	2.89	11.41	0.00
322963.0	3.65	8.98	7.47	18.57	0.00
335002.0	4.76	10.63	7.80	13.61	0.00
354499.0	3.57	5.14	4.00	31.42	2.58
354499.0	3.35	8.31	5.81	12.41	0.00
374260.0	3.30	6.27	6.19	33.27	0.00
376790.0	3.22	6.09	13.88	20.66	0.00
382320.0	3.05	3.41	18.39	8.03	0.00
439690.0	4.42	7.76	4.66	52.77	2.19
493776.0	4.27	7.92	2.80	15.55	0.00
502502.0	0.00	11.46	6.21	20.76	1.51
518465.0	26.20	5.60	0.00	3.53	0.00
521208.0	3.10	4.44	11.91	12.34	2.29
580090.0	4.68	8.09	7.67	47.06	1.45
660442.0	0.00	0.00	33.73	14.71	0.00
774691.0	0.00	10.21	3.32	40.75	0.00
790042.0	6.01	8.36	5.12	21.90	0.00
828360.0	1.97	4.39	29.16	16.28	0.00
849139.0	3.91	7.77	9.60	10.94	1.41
1065312	5.22	6.34	10.16	45.41	0.00
1427328	5.44	12.32	4.95	18.64	1.40
1580256	2.52	3.18	16.64	29.51	0.00
1717084	7.70	18.88	6.82	10.64	0.00
5504112	3.22	8.85	2.91	15.12	0.00
Correlation coefficient (r)	0.039	0.104	-0.047	-0.028	-0.016
P value	0.614	0.180	0.543	0.714	0.853

been found, in which type I, III collagen were mainly distributed in Interstitial lung [11]. Under normal physiological conditions, the contents of each type lung collagen are constant. A large number of type I, III collagen are synthesized after inhalation SiO_2, which leads to the large accumulation of collagen in the lungs and causes pulmonary fibrosis. Our study shows that complement C3f surface can reduce the formation of type I, III collagen in human embryonic lung fibroblasts (MRC-5). But this effect may not be achieved by direct stimulation, but by some other pathways.

TGF-β is a multifunctional growth factor superfamily, which has at least five kinds of isoforms

Table 6. The expression differences of type I, III collagen in the supernatant of MRC-5 stimulated by C3f with different concentrations ($\bar{x}\pm s$)

	0 ng/ml	15.6 ng/ml	62.5 ng/ml	250 ng/ml
Type I collagen	177.03±2.77	130.29±5.95*	108.16±1.42*	86.52±1.54*
Type III collagen	234.18±12.79	196.21±12.15*	164.92±3.17*	95.59±9.49*

*compared with control, P<0.01.

Table 7. The expression differences of TGF-β_1 in the supernatant of MRC-5 stimulated by C3f with different concentrations ($\bar{x}\pm s$)

	0 ng/ml	15.6 ng /ml	62.5 ng/ml	250 ng/ml
TGF-β_1	143.89±4.17	107.12±1.67*	96.23±2.04*	65.52±3.07*

*compared with control, P<0.01.

Table 8. The integrated optical density of immunohistochemistry results of TGF-β_1 in MRC-5 stimulated by C3f with different concentrations (n = 3, $\bar{x}\pm s$)

	0 ng/ml	15.6 ng/ml	62.5 ng/ml	250 ng/ml
TGF-β_1	0.34±0.032	0.30±0.014*	0.28±0.026*	0.23±0.036*

*compared with control, P<0.01.

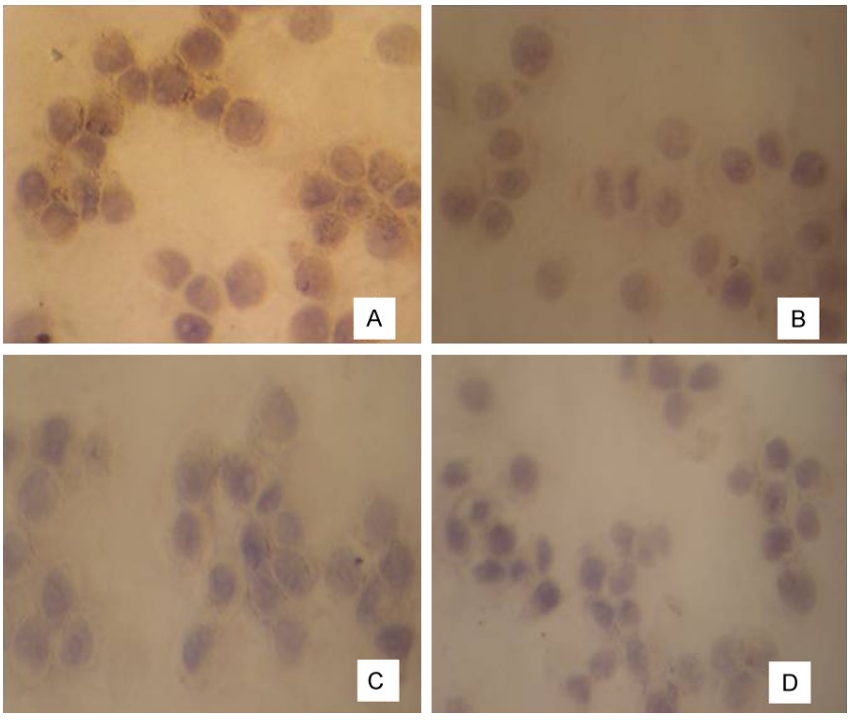

Figure 5. The immunohistochemistry results of TGF-β_1 in MRC-5 stimulated by C3f with different concentrations (PV, 200×). (A: 0 ng/ml; B: 15.6 ng/ml; C: 62.5 ng/ml; D: 250 ng/ml).

(TGFβ1-5), in which TGF-β_1 is most common. And its main functions, including regulation of cell proliferation, differentiation andembryonic development, promotion of extracellular matrix formation and suppression of the immune reaction. Immunohistochemical localization in lung tissue of silica-exposed rats showed that TGF-β_1 has both intracellular and extracellular localization [12]. Intracellular mature TGF-β_1 locates in cytoplasm of fibroblasts and alveolar macrophages infiltrating around the silicon granulomatous nodules and fibroblasts adjacent to Hyperplasia type II alveolar epithelial cells; extracellular mature TGF-β_1 locates in connective tissue matrix of the granulomas and matrix of hyperplasia type II alveolar epithelial cells and well differentiated adenocarcinoma. α-SMA is a kind of microfilaments proteins with contractile function, widely distributing in almost all the muscle-type cells, and its expression is related to the transformation of fibroblasts [13, 14]. It has been demonstrated that α-SMA-positive fibroblasts in lung tissue can be seen in the 28 days silica-exposed rats model, and this positive expression is mediated by TGF-β_1, but not the result of direct stimulation of SiO_2 [15]. In addition, the over expression of Cat K, an enzyme associated with matrix degradation, can also be restricted by TGF-β_1, which can promote formation of fiber [16]. In this study, we have found that C3f can inhibit the formation of intracellular and extracellular

TGF-β_1 in MRC-5. Therefore, we conclude that C3f may inhibit the formation of typeI, III collagen by suppressing during pathogenesis of silicosis, however it is in contrary with study in skin fibroblasts. That may be caused by differences of cells in species and sources, but its specific mechanism remains to be further discussion.

Disclosure of conflict of interest

None.

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