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REGULAR ARTICLE

Mesothelin (*MSLN*) methylation and soluble mesothelin-related protein levels in a Chinese asbestos-exposed population

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Abstract

Objectives This study investigated the mesothelin (*MSLN*) methylation and its relationship with soluble mesothelin-related protein (SMRP) levels in participants stratified by asbestos exposure scenarios and benign asbestos-related diseases (ARDs).

Methods The presence of benign ARDs was confirmed through chest X-ray and the asbestos exposure history was obtained using a standardized questionnaire in this study, including 262 participants. Sera SMRP were measured using MESOMARK, and *MSLN* methylation in genomic DNA extracted from whole blood was detected by real-time methylation-specific PCR. Covariates were compared with SMRP concentrations using correlation analysis and the potential covariates affecting SMRP were determined by multiple linear regression analysis, and the distribution of methylation status was analyzed by Chi-square test.

Results There was a trend toward elevation of SMRP values in healthy individuals exposed to asbestos as compared with those without asbestos exposure. The highest median level of SMRP was 1.3 nM in subjects with asbestosis, followed by cases with pleura plaque and asbestosis (1.2 nM), pleura plaque (0.9 nM), healthy subjects with occupational exposure (0.9 nM), non-occupational exposure (0.8 nM), and mixed exposure (0.8 nM). Within asbestosis cases, those with higher profusion scores had higher SMRP values than those with lower profusion scores (1.6 vs. 0.8 nM). Based on multi-regression analysis, the trend toward elevation of SMRP remained significant in subjects with occupational exposure or in those with asbestosis, as compared with healthy subjects without exposure ($p < 0.01$), although body mass index had an effect on SMRP ($p < 0.0001$). Regardless of the differences in SMRP levels among these subgroups, *MSLN* methylation ranged from 80.5 to 92.5 %, with no significant difference. The elevated level of SMRP in asbestosis with higher profusion scores could not be attributed to low *MSLN* methylation status.

Conclusions Our findings suggest that the elevation of SMRP is related to asbestos exposure and benign ARDs especially for cases with high profusion scores, which is independent of *MSLN* methylation.

Keywords Asbestos-exposed population · Asbestosis · Mesothelin · Pleural plaque · Promoter methylation

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Introduction

Asbestos was a popular raw material widely applied in automotive and construction industries around the world, and the consumption of asbestos remains large in the developing countries in the 1st decade since 2000 [1]. In China, the estimated number of workers exposed to asbestos was one million [2], and the excess mortality for these population might be substantial [3]. In face of the substantial burden of non-malignant and malignant asbestos-related diseases (ARDs), new molecular biomarkers for screening the subjects with high risk of developing ARDs are undoubtedly needed.

Soluble mesothelin-related protein (SMRP) is one of the three isoforms of mesothelin family encoded by *MSLN* gene. Accumulating evidence indicate that SMRP is a candidate molecule investigated in health surveillance of workers exposed to asbestos, and is considered as a promising biomarker useful for early diagnosis and monitoring the progress of ARDs [4–7]. Given the high false-positive of SMRP in a large-scale prospective study of SMRP for screening for ARDs in asbestos-exposed individuals [8], it is imperative to investigate the potential factors influencing the SMRP concentrations in subjects with a history of asbestos exposure. Recent studies showed that demographic variables, physiological factors, and genetic modification effects were associated with SMRP concentration [9–12]. Among these initial findings, epigenetic states relevant to asbestos exposure and/or ARDs could complement results from SMRP assay [12], so it may be an intrinsic event in regulating *MSLN* gene expression and its product levels.

Aberrant DNA methylation states of certain gene or the probed loci had been found in the pleural fluids and tissues of malignant ARDs (e.g., lung cancer and mesotheliomas) [13–15]. Furthermore, the methylation profiles could be utilized as an independent predictor of mesotheliomas patient survival as well as asbestos body burden [16]. Because of the promising role of SMRP in diagnosis of mesotheliomas, several authors provided the preliminary information on *MSLN* gene methylation and SMRP levels [11, 12], suggesting the putative role of epigenetic modification in regulating the expression of *MSLN* gene in malignant ARDs. So far, there are no experimental data exploring whether the distinct methylation profiles affect SMRP values among asbestos-exposed and non-malignant ARDs population. In the present study, serum SMRP values in subgroups stratified by asbestos exposure history and the presence of non-malignant ARDs were compared, and the methylation status of *MSLN* gene promoter CpG island in genomic DNA extracted from whole blood among these groups were examined, in order to evaluate the epigenetic modification effects on SMRP concentrations in asbestos-exposed population.

Materials and methods

Study population and blood samples

Two hundred and sixty-two subjects recruited in the present study came from a list of residents originated from Yuyao and Cixi area, China, where asbestos-product manufacturing existed for at least 60 years since 1950s. During that time, there were two kinds of plants producing asbestos-product: (1) household textile workshop (small-scale worksite); and (2) large-scaled textile manufactory. The residents involved in asbestos-product manufacturing were occupationally exposed to asbestos. The inhalation of asbestos may also occur for residents who lived surrounding the plants or had family members handling asbestos. The former was defined as occupational exposure; while the later was defined as non-occupational exposure. To clarify the exposure scenarios, all participants underwent a standardized questionnaire, including demographic data, previous occupation, asbestos exposure duration, and anamnesis. To estimate the potential asbestos exposure level, we retrieved periodic data of total dust concentrations of different workshops/manufactories available from 1984 to 2010 in these areas. The data indicated that the median dust concentration is 0.7 mg/m^3 with $IQ_{25–75}$ ranging from 0.4 to 1.0 mg/m^3 in household textile workshop (small-scale worksite); while the median dust concentration is 5.9 mg/m^3 with $IQ_{25–75}$ ranging from 2.5 to 13.2 mg/m^3 in textile manufactory. According to the corresponding median dust concentration, as well as the records of occupation and asbestos exposure duration from our standardized questionnaire, the subjects' potential exposure levels were estimated (in $mg/m^3 \times$ years) when applicable.

Each individual's posterior–anterior chest X-ray (CXR) was taken on full inspiration and standing position, using a TOSHIBA digital radiography system with settings of 120 kV, 320 mA and a 0.080 s acquisition time. The CXR digital soft copy images were evaluated using Diagnostic Monitor (EIZO RadiForce GS320) by at least three certified radiologists using the ILO guidelines. The profusion scores for small irregular opacities were compressed into four categories: normal 0/0; intermediate, 0/1 and 1/0; slightly abnormal (1/1) and clearly abnormal (1/2 through >2/1) as described previously [17].

Accordingly, the recruited participants were divided into seven groups: healthy participants without asbestos exposure (group 1, $n = 45$), apparently healthy subjects only non-occupationally exposed to asbestos (group 2, $n = 27$), apparently healthy subjects only occupationally exposed to asbestos (group 3, $n = 56$), apparently healthy subjects not only non-occupationally exposed to asbestos, but also occupationally exposed to asbestos (mixed exposure) (group 4, $n = 40$), cases with asbestosis and pleural plaque (group 5, $n = 26$), subjects only with asbestosis (group 6, $n = 17$), subjects only with pleural plaque (group 7, $n = 51$). The eGFR was calculated with the equation suitable for Chinese population as previously described [18]. Their peripheral blood and sera were collected in accordance with requirements of the Institutional Review Board on Medical Ethics, Zhejiang Academy of Medical Sciences. The blood sample were coded, and stored at −80 °C until further experiments. The written form of

informed consent from these participants was obtained in this investigation.

SMRP analysis

Serum SMRP concentrations were assayed with a commercial ELISA kit (MESOMARK, Fujirebio Diagnostics) according to the manufacturer's guidelines and SMRP values in the tested samples was determined using 6-point calibration curve (range 0–32 nM) as previously described [4, 19]. The value below the limit of detection (LOD) was reported as 0.3 nM for statistic purpose in that the LOD for SMRP assay is 0.3 nM [5, 7, 9].

Genomic DNA extraction and DNA bisulfite modification

Genomic DNA was extracted from 200 µl whole blood using BloodGen Mini kit (CWBIO Inc, China) according to the manufacture's instruction. The quantity and quality of DNA were determined by NanoDrop 2000C (Thermal Scientific, USA). Five hundred nanogram DNA was modified by sodium bisulfite and purified using EZ DNA Methylation-Gold^{TM} Kit (Zymo Research, USA). Twelve microlitre M-Elution Buffer was used to elute the DNA, and the eluted DNA was stored at −80 °C until further experiments.

Real-time methylation-specific-PCR (MSP) analysis

Modified DNA with sodium bisulfite was analyzed by real-time MSP as previously described with minor modifications [20]. One set of primers corresponding to 20 CpG sites of the genomic sequence of the *MSLN* promoter were designed using MethPrimer [11, 21, 22]. The MSLN methylation (M) and non-methylation (U) specific primer sequences were as follows: MSLN (M): (F) 5′-GGG GTA AAG TTT TTT ATT TAA TTG C-3′, (R) 5′-AAC ACC GTA AAT CCA CCG AT-3′, and the amplification length was 233 bp; MSLN (U): (F) 5′-GTT AGG GGT AAA GTT TTT TAT TTA ATT GT-3′, (R) 5′-AAA AAA CAC CAT AAA TCC ACC AAT-3′, and the amplification length was 241 bp. A total of 1 µl of modified DNA, 0.4 µl of 10 µM each primer, 0.4 µl of ROX Reference Dye II and 10 µl SYBR Premix Ex Taq (Takara, China) were used in each PCR reaction at a final volume of 20 µl. The PCR reaction was performed on ABI 7500 Fast PCR system (Applied Biosystems, USA) using 38 cycles for MSLN (M) 95 °C for 3 s, 64 °C for 30 s at cycling stage or 38 cycles for MSLN (U) 95 °C for 3 s, 62.5 °C for 30 s at cycling stage. The methylation percentage was calculated as follows: M% = 100 × (the quantity of methylated DNA/the quantity of methylated and unmethylated DNA). The sum of the quantity of methylated and unmethylated DNAs was used as the total quantity of DNA of the target genes. Methylated DNA was scored according to M% (0: M% $<$ 20.0; 1: 20.0 $<$ M% $<$ 40.0; 2: 40.0 $<$ M% $<$ 60.0; 3: 60.0 $<$ M% $<$ 80.0; 4: M% $>$ 80.0). 0, 1–3, and 4 were considered as unmethylated (U), partially methylated (U/M), and fully methylated (M), respectively. PCR products were separated by 1.5 % agarose gel electrophoresis, visualized by Gel-red staining (Biotium Inc., USA), then observed and photographed under UV illumination (Alpha Innotech, USA). Human methylated and non-methylated DNA set (Zymo Research, USA) was used as positive control and negative control.

Statistic analysis

Graphpad Prism Version 5.01 and SPSS 15.0 statistical software were utilized. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test or Kruskal–Wallis test with Dunn's multiple comparison test was carried out, depending on the distribution of the variables and the equality of variances. Spearman analysis and Pearson analysis were performed to assess the correlation between SMRP level and the individual covariate. Individual effect of multiple variables on log-transformed SMRP level was assessed using liner regression. A Chi-square or Fisher's exact test was used to examine the distribution in categorical variables. A value of $p < 0.05$ was considered significant.

Results

Characteristics of the study population

The general characteristics of the study participants by groups are shown in Table 1. The mean age of the groups was varied, with the group 1 being the youngest ($p < 0.05$) and a still significant age difference between the group 5 and group 6 or between the group 6 and group 7. There was no accurate exposure duration for participants non-occupationally exposed to asbestos, but the exposure duration was recorded for participants occupationally exposed to asbestos. The mean asbestos exposure ranged from 8.0 years (±5.3) to 10.8 years (±6.1), and the median asbestos exposure level ranged from 7 to 29.5 (in mg/m^3 × years) in the indicated groups. The mean eGFR significantly differed between group 1 and group 2 or between group 1 and group 6. The percentage of smoker was varied, with the Group 2 being the highest ($p = 0.009$).

The exposure scenarios for 94 participants who had non-malignant ARDs are shown in Table 2. Of those, 22 participants were only non-occupationally exposed to asbestos. The mean asbestos exposure duration ranged from

Table 1 General characteristics of the study participants

	Group 1 ($n = 45$)	Group 2 ($n = 27$)	Group 3 ($n = 56$)	Group 4 ($n = 40$)	Group 5 ($n = 26$)	Group 6 ($n = 17$)	Group 7 ($n = 51$)
Age year (mean ± SD)	56.7 ± 10.2	69.4 ± 7.7	65.3 ± 7.1	65.8 ± 6.3	63.8 ± 5.9	71.1 ± 5.9	64.2 ± 4.5
Gender (n) male/femalel	21/24	27/0	9/47	8/32	6/20	5/12	14/37
Body mass index, % (n)							
<18.5	2.2 (1)	3.7 (1)	7.1 (4)	5 (2)	0	11.8 (2)	11.8 (6)
18.5–24.9	44.4 (20)	81.5 (22)	62.5 (35)	47.5 (19)	57.7 (15)	35.3 (6)	62.7 (32)
25–29.9	6.7 (4)	14.8 (4)	16.1 (9)	22.5 (9)	11.5 (3)	17.6 (3)	21.6 (11)
≥30	0 (0)	0	3.6 (2)	7.5 (3)	0	5.9 (1)	0
Unavailable	46.7 (20)	0	10.7 (6)	17.5 (7)	30.8 (8)	29.4 (5)	3.9 (2)
Smoker (%)	17.8	33.3	3.6	12.5	7.7	5.9	11.8
Exposure time year (mean ± SD)	–	NA	8.6 ± 5.0	8.6 ± 7.3	10.8 ± 6.1	8.0 ± 5.3	9.5 ± 5.4
Potential exposure level mg/m^3 × year (median with $IQ_{25–75}$)	–	NA	7 (3.7–14.4)	20.7 (7–50.2)	11.2 (3.9–100.3)	29.5 (9.4–50.2)	29.5 (3.9–59)
Creatine in μmol/L (median and range)	57 (42–89)	77 (58–101)	60 (42–205)	60 (34–93)	58.5 (40–103)	67 (43–92)	61 (44–152)
Blood urea nitrogen in mmol/L (median and range)	5.2 (2.6–7.8)	5.3 (4.2–11.4)	5.4 (2.6–14.5)	6.0 (3.6–7.6)	5.4 (3.2–9.1)	6.1 (3.8–9.6)	5.7 (2.3–10.6)
eGFR in mL/min/1.73 m^2 (mean + SD)	91.6 ± 12.0	75.5 ± 7.7	84.7 ± 11.5	84.5 ± 10.0	86.8 ± 8.9	78.6 ± 9.3	85.4 ± 7.9

Group 1 healthy controls without asbestos exposure; group 2 healthy subject only non-occupationally exposed to asbestos; group 3 healthy subjects only occupationally exposed to asbestos (asbestos textile); group 4 healthy subjects both non-occupationally and occupationally exposed to asbestos (mixed exposure); group 5 asbestosis and pleural plaque; group 6 asbestosis; group 7 pleural plaque

Table 2 History of asbestos exposure in subjects with asbestosis and/or pleural plaque

	Exposure type % (n)			Exposure year (mean ± SD)		
	Only Occupational	Only Non-occupational	Occupational + non-occupational	Only Occupational	Only Non-occupational	Occupational + non-occupational
Group 5 ($n = 26$)	34.6 (9)	23.1 (6)	42.3 (11)	10.3 ± 6.3	NA	11.2 ± 6.1
Group 6 ($n = 17$)	41.2 (7)	11.8 (2)	47 (8)	8.6 ± 4.8	NA	7.5 ± 5.9
Group 7 ($n = 51$)	33.3 (17)	27.5 (14)	39.2 (20)	10.1 ± 6.4	NA	8.9 ± 4.4

Group 5: subjects with asbestosis and plaque; group 6: subjects with only asbestosis; group 7: subjects with only pleural plaque; NA not applicable

8.6 years (±4.8) to 10.3 years (±6.3) for participants only occupationally exposed to asbestos, and from 7.5 years (±5.9) to 11.2 years (±6.1) for participants with a history of mixed exposure.

The categories stratified by profusion scores in group 5 showed that the percentage of intermediate, slightly abnormal, and clearly abnormal is 50 % (13/26), 15.4 % (4/26), 34.6 % (9/26), respectively; while in group 6, the percentage of intermediate, slightly abnormal, and clearly abnormal is 47.0 % (8/17), 11.8 % (2/17), 41.2 % (7/17), respectively. The size and extent of pleural plaque in group 5 showed that nine cases have bilateral pleural plaque, and the number of cases with extent 1, 2, and 3 is 4, 4, 1, respectively; 17 cases have pleural plaque at either right or left, and the number of cases with extent 1 and 2 is 16, and 1, respectively.

SMRP, covariates, non-malignant ARDs, asbestos exposure type and year

Twenty-one sera (8.0 %) had values below the LOD (0.3 nM) of SMRP assay, and these measurements were assigned a value of 0.3 nM. Overall, mean (±SD) SMRP level was 1.13 (±0.88) nM, and median SMRP level with interquartile 25–75 was 0.87

Table 3 Influences of covariates, asbestos exposure, non-alignment ARDs on SMRP in multiple linear regression

	Parameter	Coefficient	SE	95 % CI	*P* value
Log (SMRP)	Age	0.014	0.10	−0.005 to 0.034	0.154
	Gender	0.090	0.125	−0.155 to 0.336	0.470
	Creatinine	0.005	0.004	−0.003 to 0.013	0.235
	eGFR	−0.002	0.009	−0.020 to 0.016	0.826
	BMI	−0.059	0.012	−0.083 to −0.034	<0.0001
	Smoking	0.356	0.394	0.421 to 1.134	0.367
	Cigarette/day	−0.21	0.28	−0.077 to 0.035	0.465
	Group 2	−0.042	0.177	−0.391 to 0.307	0.812*
	Group 3	0.430	0.159	0116 to 0.743	0.007*
	Group 4	0.242	0.167	−0.089 to 0.572	0.151*
	Group 5	0.708	0.193	0.328 to 1.088	<0.0001*
	Group 6	0.698	0.226	0.253 to 1.143	0.002 *
	Group 7	0.249	0.159	−0.059 to 0.558	0.112*

Group 2: healthy subjects only non-occupationally exposed to asbestos; group 3: healthy subjects only occupationally exposed to asbestos (asbestos textile); group 4: healthy subjects both non-occupationally and occupationally exposed to asbestos (mixed exposure); group 5: subjects with asbestosis and pleural plaque; group 6: subjects with asbestosis; group 7: subjects with pleural plaque

* Compared with group 1: healthy participants without asbestos exposure

(0.55, 1.54) nM. SMRP values were positively associated with age (Spearman $r = 0.2482$, $p < 0.0001$), creatinine (Person $r = 0.1458$, $p < 0.05$), while inversely associated with BMI (Spearman $r = -0.2740$, $p < 0.0001$), eGFR (Spearman $r = -0.2457$, $p < 0.0001$) (Fig. 1).

The SMRP values in 262 participants divided by groups are illustrated in Fig. 2. The highest median SMRP level was 1.3 nM as observed in group 6 and was significantly higher than group 1. Similarly, SMRP levels in group 3, group 5, and group 7 were all higher than group 1 ($p < 0.05$). To exclude the potential effects of covariates (e.g., BMI, age, gender, eGFR, creatinine, smoking) on the differences in SMRP levels between these groups, the multiple linear regression analysis was utilized (Table 3). It was found that the covariate of having BMI was independently associated with SMRP, with an adjusted squared multiple correlation coefficient (R^2) of 25 %. Nevertheless, when considering all these covariates, the level of SMRP in healthy subjects occupationally exposed to asbestos was still significantly higher than the healthy individuals without asbestos exposure ($p = 0.007$). The difference in SMRP levels between cases with asbestosis/pleura plaque and the healthy individuals without asbestos exposure was still statistically significant ($p < 0.0001$). The values of SMRP among subjects with asbestosis tend to be higher than healthy individuals without asbestos exposure ($p = 0.002$).

Furthermore, the effect of asbestos exposure type on SMRP values in those with non-malignant ARDs was analyzed. In group 5, the median level of SMRP with interquartile 25–75 was 1.0 (0.8, 1.6) nM in individuals with occupational exposure, was 1.5 (1.0, 2.3) nM in participants with non-occupational exposure, and was 1.6 (0.7, 1.6) nM in subjects with mixed exposure. In group 6, the median level of SMRP in those who occupationally exposed to asbestos was higher than those with mixed exposure [1.8 (1.0, 2.5) and 0.7 (0.4, 1.2) nM, respectively, $p = 0.0129$]. In group 7, the median level of SMRP with interquartile 25–75 was 1.0 (0.8, 1.9) nM in individuals with occupational exposure, was 0.9 (0.6, 1.7) nM in participants with non-occupational exposure, and was 0.8 (0.6, 1.2) nM in subjects with mixed exposure. Meanwhile, there were no statistically significant differences in SMRP levels in healthy asbestos-exposed subjects stratified by exposure duration (Fig. 3). The correlation analysis revealed that there was no significant correlation between asbestos exposure duration and SMRP values in these apparently healthy participants with asbestos exposure (Spearman $r = -0.001333$, $p = 0.9899$). SMRP values were also independent of the estimated asbestos exposure levels in these apparently healthy participants with asbestos exposure (Spearman $r = 0.02963$, $p = 0.7865$).

The effects of profusion scores and the extent of pleural plaque on the SMRP values among asbestosis cases were also analyzed. Our data showed that the median level of SMRP in asbestosis cases defined as intermediate in terms of profusion scores is lower than those defined as clear abnormal (0.8 vs. 1.62 nM, $p < 0.05$), but no significant difference in SMRP values was found among different extents of pleural plaque.

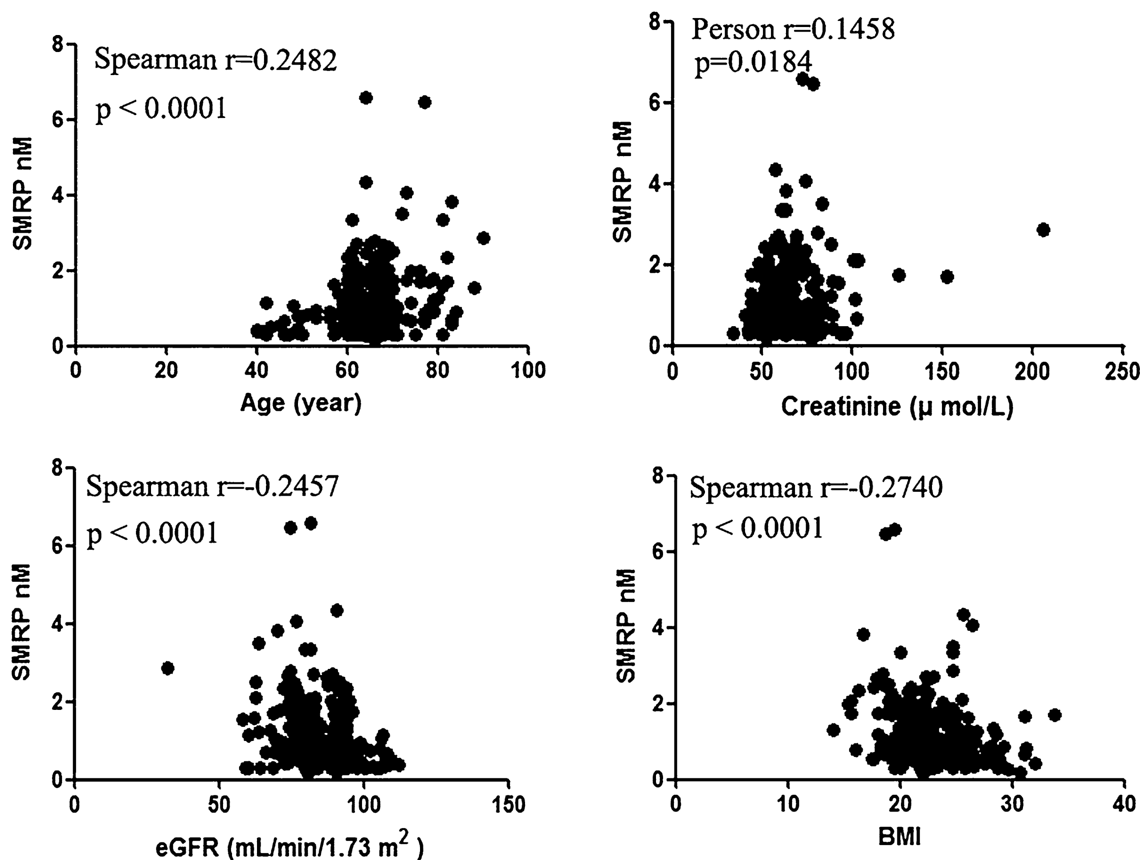

Fig. 1 Relationship between SMRP and covariates, including age, creatinine, BMI, and eGFR

SMRP media (IQ 25-75)	0.6 (0.3-0.9)	0.8 (0.5-1.4)	0.9 (0.7-1.8)***	0.8 (0.5-1.3)	1.2 (0.8-1.8)***	1.3 (0.7-2.2)**	0.9 (0.7-1.4)**

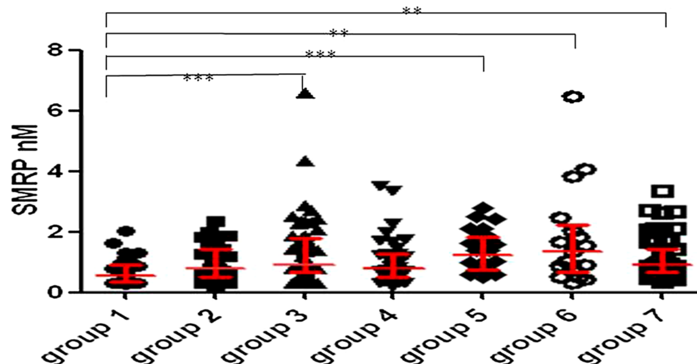

Fig. 2 SMRP levels for the seven groups. *Bars* represent median with interquartile 25–75. *Group 1*: healthy subjects without asbestos exposure; *Group 2*: healthy subjects only non-occupationally exposed to asbestos; *Group 3*: healthy subjects only occupationally exposed to asbestos (asbestos textile); *Group 4*: healthy subjects with mixed asbestos exposure; *Group 5*: asbestosis and pleural plaque; *Group 6*: asbestosis; *Group 7*: pleural plaque ***$p < 0.0001$; ** $0.0001 < p < 0.05$

Detection of *MSLN* methylation

Of 124 samples, 111 (89.5 %) samples were fully methylated in the 5′-CpG island of *MSLN* promoter, 11 (8.9 %) samples were partially methylated, and 2 (1.6 %) samples were unmethylated. Based on the definition that consider both partially methylated and unmethylated as relative low methylation status, we found that the rate of low methylation status was 10.5 %.

The rate of low methylation status was 18.2 % (4/22) in samples from healthy individuals without asbestos exposure, was 7.5 % (3/40) in samples from participants occupationally

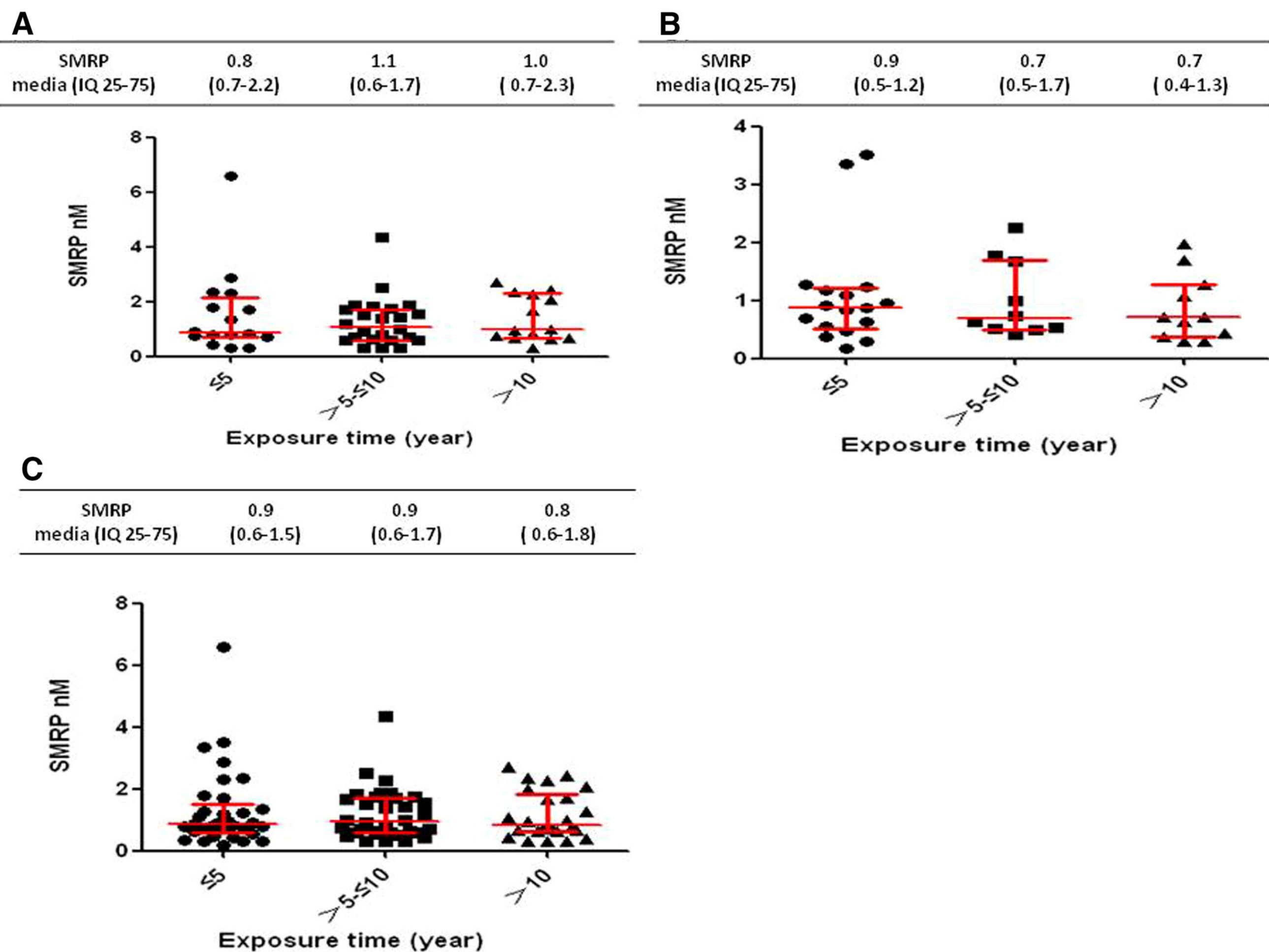

Fig. 3 SMRP levels stratified by asbestos exposure year in group 3 (**a**), group 4 (**b**), and group 3 + group 4 (**c**). *Bars* represent median with interquartile 25–75. Group 3: healthy subjects only occupationally exposed to asbestos (asbestos textile); Group 4: healthy subjects with mixed asbestos exposure

exposed to asbestos, and was 9.5 % (6/63) in samples from those with non-malignant ARDs, with no significant difference between these three subgroups ($\chi^2 = 1.775$, $p = 0.4117$). For those with asbestosis the rate of low methylation status was 5.9 % (2/34), while the rate of low methylation status was 13.8 % (4/29) in subjects with pleura plaque, with no significant difference between these two subgroups ($\chi^2 = 2.121$, $p = 0.3463$). Among 34 asbestosis cases with different categories of profusion scores, the rate of low methylation status was 5.5 % (1/18) in intermediate subgroup, was 20 % (1/5) in slight subgroup, and was 0 % (0/11) in clear abnormal subgroup, respectively.

Discussion

The awareness of potential asbestos hazards needs to be increased because of the long latency period between asbestos exposure and manifestation of ARDs [23]. Many efforts had been paid on the updated evidence-based methodology of an appropriate health surveillance program for asbestos-exposed population [24]. In this study, we revealed the following three points. First, there was a trend towards elevation of SMRP values among healthy individuals with a history of asbestos exposure, when comparing with healthy subjects without exposure. In addition to occupational exposure scenario, we investigated the SMRP values in subjects non-occupationally exposed to asbestos. According to the categories of non-occupational exposure scenarios as previously defined [25], either household contamination (secondary, para-occupational exposure) or neighborhood exposure was recorded in the present study. The household contamination determined here not only referred to the exposure resulting from asbestos fibers brought into the home through the workers' contaminated clothing [25], but also to the occasional exposure when he/she accompanied with his/her relatives during the period of manufacturing asbestos-related

product at household textile workshop. Nevertheless, these subjects found difficult to recall the exposure duration, and no quantification of exposure duration/potential exposure level was obtained in this study. In general, non-occupational exposure concentrations are lower than occupational exposure levels [25]. Our finding of low levels of SMRP in participants non-occupationally exposed to asbestos is comparable with other report, which showed that there was no significant elevation of SMRP levels in participants exposed to naturally occurring asbestos compared with subjects without asbestos exposure [26]. By contrast, the levels of SMRP in subjects occupationally exposed to asbestos were significantly higher than subjects without asbestos exposure. Furthermore, the relationship between SMRP values and asbestos exposure duration was examined in subjects occupationally exposed to asbestos. Our results support that there is no or weak correlation between SMRP levels and asbestos exposure duration [4, 19, 27]. So far, SMRP is not a desirable biomarker to discriminating the subjects exposed to asbestos from those without asbestos exposure, because the SMRP values were independent of the estimated exposure levels.

Second, our results showed that subjects with abnormal X-ray findings had statistically significantly higher SMRP levels than healthy control without asbestos exposure, and there was a trend toward elevation of SMRP in subjects with asbestosis as compared with subjects with pleura plaque. However, it was not feasible to discriminate healthy asbestos-exposed individuals from those with benign pleural and pulmonary disorders by using SMRP measurement. Previous studies indicated the possibility of SMRP to screen cases with pleural and lung disorders in asbestos-exposed population, although the efficiency of SMRP-based screening approach needs further investigation in prospective cohort studies [6, 8, 19, 26–28]. In these studies, few had evaluated the potential confounders influencing the value of SMRP [26–28]. The marginal but statistically significant correlation between SMRP values and individual physiological/demographic variable as described in the present study and others' report [9], suggesting that the covariates may likely be interrelated. The linear regression analysis revealed that the elevation of SMRP in subjects occupationally exposed to asbestos or in those with asbestosis in this study could not be attributable to the varied clinical and demographic variables (e.g., age, BMI, gender, and renal function), although SMRP levels were independently associated with BMI. In addition, smoking may not be a confounder attributing to the elevation of SMRP in those either with asbestos exposure or with benign ARDs in the present study, because the smoking rate among these subjects appeared to be lower than the healthy individuals without asbestos exposure. Indeed, smoking had no effect on SMRP values as revealed in multiple linear regression analysis. Therefore, our results support that SMRP measurement may aid in screening ARDs in workers with a history of asbestos exposure after adjusting these factors [28]. In addition, SMRP values correlated with the severity of compensable ARDs [5]. The present study showed that asbestosis cases with higher profusion scores appeared to have higher SMRP values than those with lower profusion scores. Further studies of the use of SMRP measurements in screening asbestosis with different severity to validate or test our observation are warranted.

Third, the methylation status at specific CpG sites in *MSLN* promoter between healthy individuals without asbestos exposure and subjects exposed to asbestos including those with either pleura plaque or asbestosis was compared. These CpG sites were predicted as functionally relevant regulatory sequence, and were analyzed to investigate the epigenetic modification effects on *MSLN* gene expression [11]. In that study, the authors detected DNA methylation status at 20 CpG sites in *MSLN* promoter, and found the low methylation level at 13 CpG sites was associated with high level of gene products in tumors (e.g., lung cancer, mesothelioma). It had also been found that the average tumor methylation at specific locus was 14.3 % in participants positive for SMRP (above 1.5 nM), while those negative for SMRP (below 1.5 nM) had an average tumor methylation of 23.3 % [12]. Except for tumor tissues, the methylation status of tumor suppressor genes other than *MSLN* in pleural fluid was also under investigation. Those data showed that the increased DNA methylation was significantly correlated with the extended exposure asbestos (≥30/year), suggesting the hyper-methylation was associated with asbestos-induced chronic inflammation of the pleura [14]. In addition, the aberrant methylation profile could be utilized to predict risk of cancer development among silicosis patients [29]. Because of the fact that the presence of asbestosis and plaque thickening appeared to increase the risk of tumors development [30–32], and *MSLN* gene products (e.g., SMRP) might be detected prior to the clinical manifestations of mesothelioma [27], the current study tried to reveal the relationship between methylation status and SMRP levels in asbestos-exposed individuals. The hyper-methylation in subjects with abnormal X-ray findings without pleura inflammation as determined by X-ray and physical examination were observed, nevertheless there was no significant difference in percentage of methylation at the probed CpG sites in *MSLN* promoter, as compared with the healthy participants with no history of asbestos exposure (86.2 and 81.8 %, respectively). Among asbestosis cases, no association between the methylation status and the profusion scores was observed. The comparison of the rate of hyper-methylation status between healthy subjects exposed to asbestos and healthy individuals

without asbestos exposure also showed no significant difference (92.5 and 81.8 %, respectively). The hyper-methylation of *MSLN* promoter in individuals with non-malignant ARDs is consistent with the observations that hypo-methylation could only be found in subtype of tumors with high level of *MSLN* gene product, although the decreased methylation status did not necessarily attribute to the high level of gene product [11, 12]. Despite the similar methylation status in the investigated subjects, the SMRP levels differed in subgroups as stratified by the type of asbestos exposure and the presence of non-malignant disease in the present study. It should be noted that both the median and mean SMRP levels in the investigated individuals here were below the fixed threshold (at least above 1.5 nM), which was used to screen population with high risk of mesothelioma development [11, 12].

This study observed the significantly increased levels of SMRP in subjects occupationally exposed to asbestos and in non-malignant ARDs patients when clinical and demographic variables (e.g., age, BMI, and renal function) were taken into consideration, which was independent of *MSLN* methylation status at the probed CpG sites. Meanwhile, we recognized that DNA extracted from whole blood is a mixture of different white blood cell types and plasma and may not be tissue/cell specific (e.g., mesothelial cells), and we cannot provide definitive explanation regarding the specific impact of DNA methylation in individual cell populations or pulmonary/pleural tissue on SMRP levels. Cell-free DNA secreted from damaged lung/pleural cells is desirable but is of low abundance in circulation system and the tissue collection can be problematic for subjects who do not need surgery. Human peripheral blood is relative easily obtained and the DNA extracted from whole blood in asbestos-exposed subjects was utilized to examine the relationship between mesothelin gene polymorphism and SMRP levels [10, 33, 34]. Our findings shed light on the impact of mesothelin gene methylation on SMRP levels in asbestos-exposed subjects. In addition, DNA methylation analysis utilized in our study has implications for the further design of primers targets at specific regions of mesothelin gene promoter for better interpretation of SMRP measurements in asbestos-exposed population, although the findings regarding methylation status might not fully explain the epigenetic modification effects on *MSLN* products because of the limited CpG sites analyzed here. Thus more large-scale methylation analysis are needed to investigate the aberrant epigenetic events relevant with SMRP measurements in asbestos-exposed population, which might be helpful for further research on the distinct methylation profile of asbestosis patients with or without malignant diseases, in order to identify the potential mechanism driving the carcinogenic effect in these population.

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Conflict of interest None.

References

1. Park EK, Takahashi K, Jiang Y, Movahed M, Kameda T. Elimination of asbestos use and asbestos-related diseases: an unfinished story. Cancer Sci. 2012;103:1751–5.
2. Courtice MN, Lin S, Wang X. An updated review on asbestos and related diseases in China. Int J Occup Environ Health. 2012;18:247–53.
3. Wang X, Yano E, Lin S, Yu IT, Lan Y, Tse LA, et al. Cancer mortality in Chinese chrysotile asbestos miners: exposure-response relationships. PLoS One. 2013;8:e71899.
4. Amati M, Tomasetti M, Mariotti L, Tarquini LM, Valentino M, Santarelli L. Assessment of biomarkers in asbestos-exposed workers as indicators of cancer risk. Mutat Res. 2008;655:52–8.
5. Park EK, Yates DH, Creaney J, Thomas PS, Robinson BW, Johnson AR. Association of biomarker levels with severity of asbestos-related diseases. Saf Health Work. 2012;3:17–21.
6. Rodriguez PJA, Rodriguez BE, Rodriguez RD, Alfageme MI, Quero MA, Diego RC, et al. Serum levels of soluble mesothelin-related peptides in malignant and nonmalignant asbestos-related pleural disease: relation with past asbestos exposure. Cancer Epidemiol Biomark Prev. 2009;18:646–50.
7. Creaney J, Sneddon S, Dick IM, Dare H, Boudville N, Musk AW, et al. Comparison of the diagnostic accuracy of the MSLN gene products, mesothelin and megakaryocyte potentiating factor, as biomarkers for mesothelioma in pleural effusions and serum. Dis Markers. 2013;35:119–27.
8. Park EK, Sandrini A, Yates DH, Creaney J, Robinson BW, Thomas PS, et al. Soluble mesothelin-related protein in an asbestos-exposed population: the dust diseases board cohort study. Am J Respir Crit Care Med. 2008;178:832–7.
9. Park EK, Thomas PS, Creaney J, Johnson AR, Robinson BW, Yates DH. Factors affecting soluble mesothelin related protein levels in an asbestos-exposed population. Clin Chem Lab Med. 2010;48:869–74.
10. Cristaudo A, Foddis R, Bonotti A, Simonini S, Vivaldi A, Guglielmi G, et al. Two novel polymorphisms in 5′ flanking region of the mesothelin gene are associated with soluble mesothelin-related peptide (SMRP) levels. Int J Biol Marker. 2011;26:117–23.
11. Tan K, Kajino K, Momose S, Masaoka A, Sasahara K, Shiomi K, et al. Mesothelin (MSLN) promoter is hypomethylated in malignant mesothelioma, but its expression is not associated with methylation status of the promoter. Hum Pathol. 2010;41:1330–8.
12. Nelson HH, Almquist LM, LaRocca JL, Plaza SL, Lambert-Messerlian GM, Sugarbaker DJ, et al. The relationship between tumor MSLN methylation and serum mesothelin (SMRP) in mesothelioma. Epigenetics. 2011;6:1029–34.
13. Christensen BC, Houseman EA, Poage GM, Godleski JJ, Bueno R, Sugarbaker DJ, et al. Integrated profiling reveals a global correlation between epigenetic and genetic alterations in mesothelioma. Cancer Res. 2010;70:5686–94.

14. Fujii M, Fujimoto N, Hiraki A, Gemba K, Aoe K, Umemura S, et al. Aberrant DNA methylation profile in pleural fluid for differential diagnosis of malignant pleural mesothelioma. Cancer Sci. 2012;103:510–4.
15. Hama R, Watanabe Y, Shinada K, Yamada Y, Ogata Y, Yoshida Y, et al. Characterization of DNA hypermethylation in two cases of peritoneal mesothelioma. Tumour Biol. 2012;33:2031–40.
16. Christensen BC, Houseman EA, Godleski JJ, Marsit CJ, Longacker JL, Roelofs CR, et al. Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome. Cancer Res. 2009;69:227–34.
17. Miller A, Warshaw R, Nezamis J. Diffusing capacity and forced vital capacity in 5,003 asbestos-exposed workers: relationships to interstitial fibrosis (ILO profusion score) and pleural thickening. Am J Ind Med. 2013;56:1383–93.
18. Liu X, Wang Y, Wang C, Shi C, Cheng C, Chen J, et al. A new equation to estimate glomerular filtration rate in Chinese elderly population. PLoS One. 2013;8:e79675.
19. Jakubec P, Pelclova D, Smolkova P, Kolek V, Nakladalova M. Significance of serum mesothelin in an asbestos-exposed population in the Czech Republic. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2014. doi:10.5507/bp.2014.015.
20. Yu QM, Wang XB, Luo J, Wang S, Fang XH, Yu JL, et al. CDH1 methylation in preoperative peritoneal washes is an independent prognostic factor for gastric cancer. J Surg Oncol. 2012;106:765–71.
21. Hucl T, Brody JR, Gallmeier E, Iacobuzio-Donahue CA, Farrance IK, Kern SE. High cancer-specific expression of mesothelin (MSLN) is attributable to an upstream enhancer containing a transcription enhancer factor dependent MCAT motif. Cancer Res. 2007;67:9055–65.
22. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002;18:1427–31.
23. Prazakova S, Thomas PS, Sandrini A, Yates DH. Asbestos and the lung in the 21st century: an update. Clin Respir J. 2014;8:1–10.
24. Mastrangelo G, Marangi G, Ballarin MN, Bellini E, De Marzo N, Eder M, et al. Post-occupational health surveillance of asbestos workers. Med Lav. 2013;104:351–8.
25. Goswami E, Craven V, Dahlstrom DL, Alexander D, Mowat F. Domestic asbestos exposure: a review of epidemiologic and exposure data. Int J Environ Res Public Health. 2013;10:5629–70.
26. Bayram M, Dongel I, Akbas A, Benli I, Akkoyunlu ME, Bakan ND. Serum biomarkers in patients with mesothelioma and pleural plaques and healthy subjects exposed to naturally occurring asbestos. Lung. 2014;192:197–203.
27. Felten MK, Khatab K, Knoll L, Schettgen T, Muller-Berndorff H, Kraus T. Changes of mesothelin and osteopontin levels over time in formerly asbestos-exposed power industry workers. Int Arch Occup Environ Health. 2014;87:195–204.
28. Hollevoet K, Van Cleemput J, Thimpont J, De Vuyst P, Bosquee L, Nackaerts K, et al. Serial measurements of mesothelioma serum biomarkers in asbestos-exposed individuals: a prospective longitudinal cohort study. J Thorac Oncol. 2011;6:889–95.
29. Umemura S, Fujimoto N, Hiraki A, Gemba K, Takigawa N, Fujiwara K, et al. Aberrant promoter hypermethylation in serum DNA from patients with silicosis. Carcinogenesis. 2008;29:1845–9.
30. Markowitz SB, Levin SM, Miller A, Morabia A. Asbestos, asbestosis, smoking, and lung cancer. New findings from the North American insulator cohort. Am J Respir Crit Care Med. 2013;188:90–6.
31. Reid A, de Klerk N, Ambrosini GL, Olsen N, Pang SC, Berry G, et al. The effect of asbestosis on lung cancer risk beyond the dose related effect of asbestos alone. Occup Environ Med. 2005;62:885–9.
32. Reid A, de Klerk N, Ambrosini G, Olsen N, Pang SC, Musk AW. The additional risk of malignant mesothelioma in former workers and residents of Wittenoom with benign pleural disease or asbestosis. Occup Environ Med. 2005;62:665–9.
33. Cristaudo A, Foddis R, Bonotti A, Simonini S, Vivaldi A, Guglielmi G, et al. Polymorphisms in the putative micro-RNA-binding sites of mesothelin gene are associated with serum levels of mesothelin-related protein. Occup Environ Med. 2010;67:233–6.
34. Garritano S, De Santi C, Silvestri R, Melaiu O, Cipollini M, Barone E, et al. A common polymorphism within MSLN affects miR-611 binding site and soluble mesothelin levels in healthy people. J Thorac Oncol. 2014;9:1662–8.