Research Article

Manganese and Extracellular Superoxide Dismutase Polymorphisms and Risk for Asbestosis

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Manganese and extracellular superoxide dismutases (SOD2 and SOD3) are part of the enzymatic defence against reactive oxygen species, which are involved in the pathogenesis of asbestosis. This study investigates whether SOD2Ala−9Val and SOD3Arg213Gly genetic polymorphisms represent risk factors for asbestosis in workers exposed to asbestos. The study included 262 cases with asbestosis and 265 controls with no asbestos-related disease. Cumulative asbestos exposure was calculated for each subject. A real-time PCR assay was introduced for genotyping. Logistic regression analysis was used to assess asbestosis risk. Asbestosis was associated with the homozygous SOD2−9Ala/Ala genotype (OR = 1.50, 95% CI 1.01–2.24), whereas the association for the SOD3Arg/Gly genotype was not significant (OR = 1.63, 95% CI 0.62–4.27). The finding that the SOD2−9Ala/Ala genotype increases the risk for asbestosis indicates that, in addition to asbestos exposure, genetic factors may also have a significant influence on the development of asbestosis.

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1. Introduction

Exposure to asbestos fibres has been shown to cause asbestosis, pleural diseases such as pleural plaques, lung cancer, malignant mesothelioma and some other types of cancers [1–3]. One of the most frequent diseases caused by inhalation of asbestos is asbestosis, an interstitial pulmonary process that slowly develops into diffuse pulmonary fibrosis [4]. Although the causal relationship between asbestos exposure and asbestosis has been well proved, little is still known about the genetic factors that may influence the development of this disease [2, 5]. At present it is suggested that both environmental and genetic factors may play an important role in the development of asbestosis [2, 6, 7]. The gene-environmental interaction studies published so far have mostly focused on the genes coding for xenobiotic metabolizing enzymes [2, 5–9].

Numerous studies have suggested the central role of reactive oxygen and nitrogen species (ROS and RNS) in fibre-induced toxicity and development of asbestosis [1, 10, 11]. The most important reactive metabolites in the pathogenesis of asbestos-related lung diseases are superoxide anion (O$_2$$\cdot^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^+$) and nitric oxide (NO) [10, 12, 13].

Human tissues contain specific enzyme systems to detoxify ROS and RNS. Superoxide dismutases (SODs) together with catalase and glutathione peroxidases constitute a primary defence against oxidative stress [10, 14, 15]. SODs catalyze the conversion of superoxide anion (O$_2$$\cdot^-$) to hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$) [14, 16]. Manganese SOD (MnSOD or SOD2) is a manganese (Mn)-containing enzyme localized in mitochondria, whereas extracellular SOD (ECSOD or SOD3), which contains copper (Cu) and zinc (Zn), is predominantly found in extracellular space [14, 15].

In humans, SOD2 is encoded by the SOD2 gene localized to chromosome 6 (region 6q25), and SOD3 by the SOD3 gene, which has been localized to chromosome 4 (region 4p-q21) [15, 17, 18]. The genes coding for SOD2 and SOD3 are polymorphic. The most common functional polymorphism of the SOD2 gene is cytosine (C) to thymine (T) substitution (201C>T, rs4880), which results in alanine (Ala) to valine (Val) amino acid change at position −9 of the mitochondrial targeting sequence (SOD2 Ala −9Val) [19, 20]. It has
been suggested that this polymorphism alters the secondary structure of the protein, and hence may affect the efficiency of transport of the SOD2 into the mitochondria, where it would be biologically available [19, 20]. Sutton et al. demonstrated that Ala-containing SOD2 is actively targeted into the mitochondrial matrix, whereas the Val-containing variant is partially arrested within the inner mitochondrial membrane [21]. The Ala-variant also resulted in 4-fold higher levels of the mature exogenous protein and SOD2 activity than the Val-variant [21]. The frequency of each allele is around 50% in Caucasian populations [22], whereas the Val allele is more frequent in Japan (87.9%) [19]. Among asbestos-related diseases, the SOD2 −9Val/Val genotype was associated with increased lung cancer risk in individuals with a low asbestos exposure score [23], whereas another study found an increased risk of mesothelioma in subjects with the SOD2 −9Ala/Ala genotype [24]. In contrast, a study by Hirvonen et al. suggested no major modifying role for SOD2 polymorphism in asbestos-related pulmonary disorders [9].

SOD3 binds lung matrix components and inhibits their fragmentation in response to oxidative stress [25, 26]. In the SOD3 gene, a cytosine (C) to guanine (G) substitution (896C>G, rs1799895) causing an amino acid change from arginine (Arg) to glycine (Gly) at position 213 has been reported (Arg213Gly) [15, 27, 28]. Although this polymorphism is rare, occurring in 2 to 6% of various populations [15, 27, 28], it causes an 8- to 15-fold increase in the concentration of plasma SOD3 levels due to impaired binding to the extracellular matrix [27, 28]. To our knowledge, the influence of SOD3 Arg213Gly polymorphism on the risk of developing asbestosis in workers exposed to asbestos has not been studied so far. Most of the data come from studies on SOD3 transgenic [29, 30] and knockout mice [31, 32] that clearly demonstrate the importance of SOD3 in lung protection. Enhanced lung damage, inflammation, and fibrosis were observed in SOD3 knockout mice compared to wild-type mice in asbestos-induced lung injuries [25, 32, 33].

To study the influence of genetic polymorphisms of some of the most important metabolic enzymes on the risk for asbestosis and possible interaction with asbestos exposure, the authors designed a series of molecular epidemiological studies [6, 7]. The basic aim of this study was to investigate the influence of the SOD2 Ala −9Val and SOD3 Arg213Gly genetic polymorphisms on the risk for developing asbestosis in workers occupationally exposed to asbestos.

2. Methods

In a nested case-control study, all 356 subjects diagnosed with asbestosis were first selected from the cohort of 2080 workers that were presented at the State Board for the Recognition of Occupational Asbestos Diseases from 1 January 1998 to 31 December 2003. For each case, one control with no asbestosis-related disease was recruited from the same cohort using gender and date of birth as matching factors.

From the selected subjects, 2 cases and 9 controls developed cancer in the period between the recognition of the occupational disease and the beginning of the study, 40 cases and 29 controls died during the same period, and 52 cases and 53 controls refused to participate, so the final number of cases was 262 (73.6%) and that of controls 265 (74.4%).

Information about smoking, including duration of smoking and the number of pack-years of smoking, was obtained for all subjects during an interview using a standardized questionnaire [3, 34].

All subjects that participated in the study worked at the Salonit Anhovo asbestos cement manufacturing plant in Anhovo, Slovenia and were occupationally exposed to asbestos [3]. For each case and control included in this study, the data on cumulative asbestos exposure were available from the previous study [3]. The methodology of cumulative asbestos exposure calculation has already been described in detail in previous papers [3, 6].

The diagnosis of asbestosis or “no asbestos-related disease” was confirmed by two groups of experts from the State Board for the Recognition of Occupational Asbestos Diseases that operate at the Clinical Institute of Occupational Medicine; each group consisted of an occupational physician, a pulmonologist, and a radiologist. The diagnostic criteria were based on the Helsinki Criteria for Diagnosis and Attribution of Asbestos Diseases [35] and on the American Thoracic Society recommendations [36].

For genetic analysis, capillary blood samples from the fingertips of all subjects were collected on FTA Mini Cards (Whatman Bioscience) and genomic DNA was isolated as previously described [6].

To determine SOD2 Ala −9Val and SOD3 Arg213Gly polymorphisms, TaqMan SNP genotyping assays were used (C_8709053_10 and C_2307506_10, resp., AB assay, Applied Biosystems, Foster City, Calif. USA). Real-time PCR was performed under universal conditions on ABI 7500 (Applied Biosystems, Foster City, Calif. USA). TaqMan SNP genotyping assays were validated on 100 samples genotyped by the PCR-RFLP method. To determine SOD2 Ala −9Val polymorphism, the region encompassing the polymorphic site was amplified and PCR products were digested with NgoM IV (New England Biolabs), as previously described [37]. To determine SOD3 Arg213Gly polymorphism, the corresponding PCR products were digested with Mwol (New England Biolabs), as previously described [38].

Among statistical methods, standard descriptive statistics were first performed. Next, t-tests for differences of means of variables between the cases and controls were calculated, and χ² tests were used to determine whether the observed differences in proportions between the study groups were statistically significant. To assess the causal relationship between asbestosis, genotypes, cumulative asbestos exposure, and standard confounders (age, sex), univariate logistic regression was first used, followed by multivariate logistic regression modelling. A possible synergistic effect between genotypes and cumulative asbestos exposure was investigated by using dummy variables. P-values less than 0.05 were considered statistically significant.
There was no statistical difference in gender between the cases and controls. Among the 262 cases there were 186 men and 76 women, and among the 265 controls 183 men and 82 women ($\chi^2 = 0.24, P = .628$). On average, cases were 61 and controls 57 years old, showing a statistical difference between them ($t = 5.18, P = .000$). No difference in smoking was observed between the cases and controls (Table 1).

As expected, the mean cumulative asbestos exposure was higher among the cases than the controls ($t = 4.78, P = .000$) (Table 1).

All of the subjects were genotyped for $SOD2$ Ala $\rightarrow$ 9Val and $SOD3$ Arg213Gly polymorphisms, although the amplification of the $SOD2$ gene failed in 4 cases and 4 controls, and that of the $SOD3$ gene in 4 cases and 2 controls.

Three different $SOD2$ genotypes (Ala/Ala, Ala/Val, and Val/Val) and two $SOD3$ genotypes (Arg/Arg and Arg/Gly) were detected. The $SOD2$ −9Ala/Ala genotype was observed in 29.0% of cases and 21.4% of controls, Ala/Val in 45.0% of cases and 53.3% of controls, and Val/Val in 26.0% of cases and 25.3% of controls. The $SOD3$ Arg/Arg genotype was found in 95.7% of cases and 97.3% of controls, and the Arg/Gly genotype was observed in 4.3% of cases and 2.7% of controls. Combining polymorphic $SOD2$ genotypes, the frequency of the −9Ala/Ala genotype versus the frequency of the Ala/Val and Val/Val genotypes was significantly higher in the cases than in the controls ($\chi^2 = 3.99, P = .046$) (Table 2). In contrast, no significant difference was found in the frequency of the $SOD3$ Arg/Arg genotype compared to the frequency of the Arg/Gly genotype ($\chi^2 = 1.00, P = .317$) (Table 2).

In subsequent logistic regression analysis, no association was observed between asbestosis and ever/never smoking (OR = 0.98, 95% CI 0.69–1.39). Knowing that cumulative asbestos exposure was skewed to the right, logarithmically transformed cumulative asbestos exposure was calculated, giving an OR of asbestosis of 3.21 (95% CI 2.43–4.23). Analyzing the association between asbestosis and $SOD2$ genotypes, the OR of asbestosis was 1.61 (95% CI 1.05–2.46) for the −9Ala/Ala genotype versus the Ala/Val genotype, 1.32 (95% CI 0.81–2.14) for the Ala/Ala genotype versus the Val/Val genotype, and 1.50 (95% CI 1.01–2.24) for the Ala/Ala genotype versus combined Ala/Val and Val/Val genotypes. The OR of asbestosis calculated for the $SOD3$ Arg/Gly genotype compared to the Arg/Arg genotype was 1.63 (95% CI 0.62–4.27).

Using multivariate logistic regression modelling, the risk of asbestosis for the $SOD2$ −9Ala/Ala genotype versus combined Ala/Val and Val/Val genotypes did not change considerably after adjustment by gender, age, smoking, and cumulative asbestos exposure (Table 3). The results showed that gender, age, and smoking did not substantially influence

| Table 1: Smoking and cumulative asbestos exposure in cases and controls. |
|---------------------------------|----------------|----------------|----------------|----------------|
|                                 | Cases (n = 262) | Controls (n = 265) | Test  | P-value |
| Smoking (ever/never smokers, n) | Mean ± SD | Range | Mean ± SD | Range | \( \chi^2 \) | P-value |
| Years of smoking               | 25.92 ± 13.37 | 0.01–60.00 | 22.90 ± 12.90 | 1.00–61.00 | t = 1.77 | .078 |
| Pack-years of smoking           | 21.92 ± 15.95 | 0.15–66.25 | 20.99 ± 16.37 | 0.05–90.00 | t = 0.44 | .659 |
| Cumulative asbestos exposure    | 37.67 ± 86.43 | 0.01–869.72 | 11.23 ± 23.47 | 0.01–149.21 | t = 4.78 | .000 |

| Table 2: Frequencies of $SOD2$ and $SOD3$ genotypes in cases and controls. |
|---------------------------------|----------------|----------------|----------------|----------------|
|                                 | Cases (n = 258) | Controls (n = 261) | \( \chi^2 \) | P-value |
| **$SOD2$ genotype**             | n   | %    | n   | %    |       |     |
| Ala/Ala                          | 75  | 29.0 | 56  | 21.4 |       |     |
| Ala/Val                         | 116 | 45.0 | 139 | 53.3 | 4.79* | .029 |
| Val/Val                         | 67  | 26.0 | 66  | 25.3 | 1.25† | .263 |
| Ala/Val + Val/Val               | 183 | 71.0 | 205 | 78.6 | 3.99† | .046 |

| **$SOD3$ genotype**             | n   | %    | n   | %    |       |     |
| Arg/Arg                         | 247 | 95.7 | 256 | 97.3 |       |     |
| Arg/Gly                         | 11  | 4.3  | 7   | 2.7  | 1.00§ | .317 |
| Gly/Gly                         | 0   | 0.0  | 0   | 0.0  |       |     |

* \( \chi^2 \) calculated for Ala/Ala versus Ala/Val
† \( \chi^2 \) calculated for Ala/Ala versus Ala/Val + Val/Val
§ \( \chi^2 \) calculated for Arg/Arg versus Arg/Gly.
the risk of asbestosis for the SOD3 Arg/Gly compared to Arg/Arg genotype either, but the involvement of cumulative asbestos exposure changed the OR from 1.63 (95% CI 0.62–4.27) to 2.07 (95% CI 0.72–5.94), indicating a possible confounding effect of cumulative asbestos exposure (Table 3). To test the possible interaction between asbestos exposure and the SOD3 genotype, a dummy variable was created by multiplying the SOD3 Arg/Gly genotype and cumulative asbestos exposure. No synergistic effect was found (OR = 0.61, P = .456). When the SOD2 and SOD3 genotypes were simultaneously introduced into logistic regression analysis, the OR of asbestosis was 1.51 (95% CI 1.01–2.25) for the SOD2 Arg/Gly genotype and cumulative asbestos exposure. No significant difference was observed due to differences in injury and consequently a higher risk of developing diseases [42]. In line with these reports and the assumption that individuals with the SOD2 –9Ala/Ala genotype may have higher SOD2 enzyme activity and therefore likely elevated levels of H2O2 and OH• radicals, which are known to be involved in the pathogenesis of asbestosis, our finding of an increased risk of asbestosis in subjects with the SOD2 –9Ala/Ala genotype may be considered biologically plausible.

In contrast to the observations of the present study, Hirvonen et al. suggested no major modifying role for SOD2 polymorphism in asbestos-related pulmonary disorders in a study based on 20 subjects with mesothelioma and 41 subjects with asbestosis or/and pleural plaques [9]. However, Landi et al. found an increased risk of mesothelioma in subjects with the SOD2 –9Ala/Ala genotype, which very likely agrees with the findings of this study [24].

A slightly elevated risk of asbestosis was also observed for the SOD3 Arg/Gly genotype, but the result was not significant. Despite the higher frequency of the SOD3 Arg/Gly genotype in subjects with asbestosis compared to the controls, no significant difference was observed due to the lack of power. However, the results of several studies demonstrated that SOD3 knockout mice show enhanced lung damage, inflammation, and fibrosis compared to wild-type mice in asbestos-induced lung injuries [25, 32, 33, 43]. In line with these reports, SOD3 polymorphism might play a role in developing asbestosis and should therefore be further investigated in association with this disease. The risk of asbestosis for the SOD3 Arg/Gly genotype increased slightly after involvement of cumulative asbestos exposure. No synergistic effect was found, but cumulative asbestos exposure acted as a confounder.

The findings of the current study were not biased by genetic heterogeneity because all of the subjects were recruited in a small geographic area with an ethnically homogenous population. There were also no differences in the cumulative exposure or diagnosis of cancer between the participants and subjects that did not want to take part in the study, died or developed a malignant disease in the period between the recognition of occupational disease and beginning of this study.

Overall, this study found evidence of an association between asbestosis and SOD2 Ala –9Val genetic polymorphism. The results of our previous studies showed

|                | SOD2 –9Ala/Ala | SOD3 Arg/Gly |
|----------------|---------------|--------------|
|                | Unadjusted    | Adjusted     | Unadjusted    | Adjusted     |
|                | OR            | 95% CI       | OR            | 95% CI       |
| Gender         | 1.49          | 1.00–2.23    | 1.61          | 0.61–4.22    |
| Age            | 1.46          | 0.97–2.19    | 1.49          | 0.56–3.96    |
| Smoking (ever/never) | 1.49 | 1.00–2.23    | 1.65          | 0.63–4.32    |
| Cumulative exposure | 1.48 | 0.96–2.28    | 2.07          | 0.72–5.94    |
a decreased risk of asbestosis in individuals with the glutathione S-transferase T1-null genotype [6] and an increased risk in subjects carrying the glutathione S-transferase P1 genotype coding for an enzyme with high conjugation capacity [7]. All of these results suggest that genetic factors may have a significant influence on the development of asbestosis. These findings should also be seriously considered which is a topic that demands further investigation and elucidation.

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