NLRP3 AND CARD8 POLYMORPHISMS INFLUENCE RISK FOR ASBESTOS-RELATED DISEASES
UTICAJ NLRP3 I CARD8 POLIMORFIZAMA NA BOLESTI U VEZI SA AZBESTOM

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Summary
Background: This study aimed to investigate the association between NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms and the risk of developing pleural plaques, asbestosis, and malignant mesothelioma (MM), and to study the influence of the interactions between polymorphisms and asbestos exposure on the risk of developing these diseases.

Methods: The case-control study included 416 subjects with pleural plaques, 160 patients with asbestosis, 154 subjects with MM and 149 subjects with no asbestos disease. The NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms were determined using real-time PCR-based methods. In the statistical analysis, standard descriptive statistics was followed by univariate and multivariate logistic regression modelling.

Results: Asbestos exposure (medium and high vs low) was associated with the risk for each studied asbestos-related disease. An increased risk of pleural plaques was found for CARD8 rs2043211 AT + TT genotypes (OR = 1.48, 95% CI 1.01–2.16, p = 0.042). When the analysis was performed for MM patients as cases, and pleural plaques patients as controls, a decreased MM risk was observed for carriers of CARD8 rs2043211 TT genotype (OR = 0.52, 95% CI 0.27–1.00, p = 0.049). The interactions between NLRP3 rs35829419 and CARD8 rs2043211 genotypes did not influence the risk of any asbestos-related disease. However, when testing interactions with asbestos exposure, Kratak sadržaj
Uvod: Cilj ovog istraživanja bio je da se ispita povezanost između polymorfizama NLRP3 rs35829419 i CARD8 rs2043211 i rizika od razvoja pleuralnih plakova, azbestoze i malignog mezotelioma (MM) i da se prouči uticaj interakcija između polymorfizama i izloženosti azbestu na rizik od razvoja ovih bolesti.

Metode: Ova studija slučaja je uključivala 416 ispitanika sa pleuralnim plakovima, 160 pacijenata sa azbestozom, 154 ispitanika sa MM i 149 ispitanika bez azbestne bolesti. Polimorfizmi NLRP3 rs35829419 i CARD8 rs2043211 su određivani pomoću metoda zasnovanih na PCR u realnom vremenu. U statističkoj analizi, standardne deskriptivne i multivarijantne logistic regression modeliranje.

Rezultati: Izloženost azbestu (srednja i visoka u odnosu na nisku) bila je povezana sa rizikom za svaku proučavanu bolest povezana sa azbestom. Povećan rizik od pleuralnih plakova je ustanovljen za CARD8 rs2043211 AT + TT genotipove (OR = 1,48, 95% CI 1,01–2,16, p = 0,042). Kada je obavljena analiza za pacijente sa MM, kao i za pacijente sa pleuralnim plakovima kao kontrolne slučajove, primećen je smanjeni MM rizik za nosioce CARD8 rs2043211 TT genotipa (OR = 0,52, 95% CI 0,27–1,00, p = 0,049). Interakcije između genotipova NLRP3 rs35829419 i CARD8 rs2043211 nisu uticale na rizik od bilo koje bolesti povezane sa azbestom. Međutim, kada su testirane interakcije sa izloženošću azbestu, ustanovljen je smanjen rizik
a decreased risk of asbestosis was found for NLRP3 CA + AA genotypes (OR = 0.09, 95% CI 0.01–0.60, p = 0.014).

Conclusions: The results of our study suggest that NLRP3 and CARD8 polymorphisms could affect the risk of asbestos-related diseases.

Keywords: inflammasome, polymorphism, asbestosis, pleural plaques, malignant mesothelioma

**Introduction**

The asbestos-related diseases, including pleural plaques, diffuse pleural thickening and pleural effusion, asbestosis, and several types of cancers, such as lung cancer, malignant mesothelioma (MM) of the pleura and peritoneum, cancer of the larynx, cancer of the ovary, as well as the cancers of the buccal mucosa, the pharynx, the gastrointestinal tract, and the kidney, are still a major public health problem (1–4).

Pleural plaques and diffuse pleural thickening, which can be accompanied by pleural calcification, are among the most common non-malignant effects of asbestos and may occur even after relatively low asbestos exposure (5–11). Asbestosis, one of the most frequent diseases caused by asbestos, is an interstitial pulmonary process which, after a long latency period, slowly develops into diffuse pulmonary fibrosis. The disease continues to progress even after the cessation of exposure and the process is irreversible (10, 12, 13). Among cancers, MM is considered to be a highly aggressive and invasive malignoma that arises from the mesothelium, most commonly from pleura and less frequently from peritoneum or other serosal surfaces (14). As the onset of symptoms is often non-specific and insidious, this malignoma is very difficult to diagnose. However, an early diagnosis is extremely important for timely and more effective treatment (15). Therefore, potential new biomarkers for an earlier diagnosis of MM have been intensively investigated (16, 17).

The pathogenesis of asbestos-induced pleural diseases has been extensively investigated. Asbestos fibres are thought to provoke pleural inflammation from direct toxicity to mesothelial cell. Pleural injury can be elicited by inhaled asbestos fibres indirectly via the release of inflammatory cytokines and growth factors from within the lung (18). The mechanism of cell injury caused by asbestos and affecting the cells of the pleura and lung remains unclear. It has been suggested that inflammation may have an important role in the pathogenesis of asbestos-related diseases (19–21).

Inflammation has been proposed to be involved in the modification and/or calcification of pleural thickenings as well as in the development of asbestosis (22, 23). Chronic inflammation is also known to play an essential role in tumorigenesis and is a major contributing factor in the development of many types of cancer, including MM (22, 24). Evidence suggests that inflammation can also be associated with unfavourable clinical prognosis in cancer patients in general (24–27) as well as in MM (28).

The reactive oxygen species (ROS) and reactive nitric species (RNS) are considered to play an important role in modulating the immune response to inflammatory stimuli (29). Recent studies have led to a better understanding of molecular mechanisms underlying the pathogenesis of asbestos-related diseases, including MM. Asbestos fibres can cause genotoxic damage either directly or indirectly, via generation of reactive oxygen and nitric species (ROS and RNS). ROS may also mediate the activation of the NLRP3 inflammasome (30).

The NLRP3 inflammasome is a multiprotein cytoplasmic complex comprised of NOD-like receptors, a family of intracellular sensors that have become known as crucial components of the innate immune responses and inflammation (31, 32). The NLRP3 inflammasome complex is composed of several components, such as NLRP3, CARD8 and ASC (23, 33, 34). It is activated by numerous physical and chemical stimuli (33), including asbestos. In response to a variety of pathogens and/or danger-associated molecular patterns, the NLRP3 inflammasome activates caspase-1, which results in IL-1 secretion and consequently inflammatory response (33, 35). It has been shown that the secretion of IL-1 from alveolar macrophages induced by asbestos is mediated through the NLRP3 inflammasome (21, 23).

It has been proposed that genetic variants of inflammasome components may influence the function of the complex and that functional polymorphisms in the NLRP3 and CARD8 genes may be associated with IL-1 production and severe inflammation (23, 34).

Among the most commonly investigated inflammasome-related single nucleotide polymorphisms (SNPs) is nonsynonymous gain-of-function polymorphism NLRP3 rs35829419 (p.Gln705Lys; C > A) that leads to an overactive NLRP3 inflammasome and increased production of IL-1 (36). Another important inflammasome-related SNP is CARD8 rs2043211 (p.Cys10Ter, A>T) that results in non-functional protein and leads to loss of CARD-8 inhibition of cas-
pase-1 (23, 37). Consequently, both polymorphisms have been proposed to be associated with increased IL-1 production and proinflammatory phenotype (21, 23, 36).

Although asbestos-related diseases are among the most frequently investigated occupational diseases and the association between asbestos exposure and asbestos-related diseases has been well proved, relatively little has been known about the genetic factors that might modify the individual susceptibility to the development of these diseases (4). This study aimed to investigate the association between NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms and the risk of developing pleural plaques, asbestosis, and MM, as well as to study the influence of the interactions between NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms and asbestos exposure on the risk of developing these diseases.

Materials and Methods

The case-control study included 416 subjects with pleural plaques, 160 patients with asbestosis, 154 subjects with MM and 149 subjects with no asbestos disease.

Subjects with pleural plaques, asbestosis and no asbestos disease were occupationally exposed to asbestos and presented at the State Board for the Recognition of Occupational Asbestos Diseases in the period from 1 January 1998 to 31 December 2007. The diagnosis of pleural plaques, asbestosis or «no asbestos-related disease» was confirmed by two groups of experts from the State Board for the Recognition of Occupational Asbestos Diseases, each group consisting of an occupational physician, a pulmonologist, and a radiologist.

Patients with MM were treated at the Institute of Oncology Ljubljana in the period between 1 January 2004 and 31 December 2012. In all patients with MM, thoracoscopy or laparoscopy/laparotomy was performed. The diagnosis of MM was histologically proved by a pathologist skilled in diagnosing this cancer.

Data on smoking were obtained for all subjects during an interview using a standardized questionnaire. The number of pack-years of smoking was calculated from the duration of smoking, and the number of cigarettes smoked per day (38, 39).

To determine the exposure to asbestos, a semi-quantitative method was used. For the subjects with pleural plaques, asbestosis, «no asbestos-related disease» and for 33 patients with MM, data on cumulative asbestos exposure in fibres/cm²-years were available from the previous study (39). Based on these data, the subjects were divided into three groups: low (< 11 fibres/cm²-years), medium (11–20 fibres/cm²-years) and high (> 20 fibres/cm²-years) asbestos exposure. For patients with MM with no cumulative asbestos exposure data, accurate work history was obtained, and their asbestos exposures were compared with exposures of the group of subjects with known cumulative asbestos exposure and were accordingly divided into three groups with presumed low, medium and high asbestos exposure, as previously described (40). Reliable semi-quantitative data on asbestos exposure could be obtained only for 81 MM patients.

For the genetic analysis, genomic DNA was isolated from peripheral blood leukocytes or FTA Mini Cards (Whatman Bioscience). The NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms were determined by using a fluorescent-based competitive allele-specific polymerase chain reaction (KASPar) assay (LGC Genomics, UK) or real-time PCR-based Taqman assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions.

In the statistical analysis, standard descriptive statistics was first performed, followed by univariate and multivariate logistic regression modelling. The possible interactions between genotypes as well as between genotypes and asbestos exposure were tested by logistic regression models using dummy variables. The dominant and additive genetic models were used in the analysis. All statistical analyses were performed using IBM SPSS Statistics version 21.0 (IBM Corporation, Armonk, NY, USA).

The study was approved by the Republic of Slovenia National Medical Ethics Committee and was carried out according to the Helsinki Declaration.

Results

The study included 879 subjects altogether; among them, 645 (73.4%) were male and 234 female (26.6%). The median age of subjects was 56.70 (inter-quartile 50.00–64.90) years. Regarding smoking, 431 (49.9%) were ever smokers, and 432 (50.1%) of them never smoked. Among smokers, the median number of pack-years was 19 (inter-quartile 7.3–30). Asbestos exposure was low in 538 (67.60%) subjects, medium in 104 (13.10%) and high in 154 (19.30%) subjects.

The baseline characteristics of each subject group (subjects without the asbestos-related disease, patients with pleural plaques, asbestosis, MM) are presented in Table I. No statistically significant difference was found among the groups regarding gender and smoking (ever vs never smokers). On the other hand, there was a statistically significant difference considering age and asbestos exposure, as well as the pack-years among smokers. However, in all groups, the highest percentage of subjects had low asbestos exposure (Table I).
In univariate logistic regression analysis, a positive association was observed between age and asbestosis (OR = 1.51, 95% CI 1.18–1.94, p = 0.001), and between age and MM (OR = 2.32, 95% CI 1.79–3.01, p < 0.001). When considering patients with MM as cases and subjects with pleural plaques as controls, an increased risk of MM was found for pack-years of smoking (OR = 1.32, 95% CI 1.13–1.54, p = 0.001) (Table II). Asbestos exposure (medium and high vs low) was associated with the risk of each studied asbestos-related disease (Table II).

The genotype frequencies for NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms are shown in Table III.

The results of univariate analysis of the association between different asbestos-related diseases and NLRP3 rs35829419 and CARD8 rs2043211 geno-
Table III  Genotype frequencies of investigated polymorphisms in asbestos-exposed subjects.

| Polymorphism          | Genotype | All subjects (N=879) | No disease (N=149) | Pleural plaques (N=416) | Asbestosis (N=160) | Malignant mesothelioma (N=154) |
|-----------------------|----------|---------------------|--------------------|-------------------------|--------------------|-------------------------------|
|                       |          | N (%)               | N (%)              | N (%)                   | N (%)              | N (%)                         |
| CARD8 rs2043211       | AA       | 389 (44.5)          | 74 (50.0)          | 167 (40.3)              | 74 (46.5)         | 74 (48.1)                     |
|                       | AT       | 382 (43.7)          | 57 (38.5)          | 190 (45.9)              | 68 (42.8)         | 67 (43.5)                     |
|                       | TT       | 104 (11.9)          | 17 (11.5)          | 57 (13.8)               | 17 (10.7)         | 13 (8.4)                      |
| NLRP3 rs35829419      | CC       | 785 (89.7)          | 133 (89.9)         | 375 (90.4)              | 145 (90.6)        | 132 (86.8)                    |
|                       | AT       | 88 (10.1)           | 15 (10.1)          | 39 (9.4)                | 14 (8.8)          | 20 (13.2)                     |
|                       | AA       | 2 (0.2)             | 0                  | 1 (0.2)                 | 1 (0.6)           | 0                             |

*Missing data are presented in square brackets [*].

Table IV  The association between different asbestos-related diseases and genotypes in univariate analysis.

| Polymorphism          | Genotype | Pleural plaques vs no disease | Asbestosis vs no disease | MM vs no disease | MM vs pleural plaques |
|-----------------------|----------|-------------------------------|--------------------------|-----------------|-----------------------|
|                       |          | OR (95% CI) | P | OR (95% CI) | P | OR (95% CI) | P | OR (95% CI) | P |
| CARD8 rs2043211       | AA       | reference | | reference | | reference | | reference | |
|                       | AT       | 1.48 (0.99–2.21) | 0.058 | 1.19 (0.74–1.92) | 0.469 | 1.18 (0.73–1.90) | 0.508 | 0.80 (0.54–1.18) | 0.252 |
|                       | TT       | 1.49 (0.81–2.73) | 0.201 | 1.00 (0.48–2.11) | 1.000 | 0.77 (0.35–1.69) | 0.506 | 0.52 (0.27–1.00) | 0.049 |
|                       | AT+TT    | 1.48 (1.01–2.16) | 0.042 | 1.15 (0.73–1.80) | 0.545 | 1.08 (0.69–1.70) | 0.755 | 0.73 (0.50–1.06) | 0.099 |
| NLRP3 rs35829419      | CC       | reference | | reference | | reference | | reference | |
|                       | CA+AA    | 0.95 (0.51–1.77) | 0.861 | 0.92 (0.43–1.95) | 0.822 | 1.34 (0.66–2.74) | 0.416 | 1.42 (0.80–2.52) | 0.229 |

Table V  Multiplicative interaction between CARD8 rs2043211 and NLRP3 rs35829419 genotypes and between genotypes and asbestos exposure.

| Polymorphism          | Pleural plaques vs no disease | Asbestosis vs no disease | MM vs no disease | MM vs pleural plaques |
|-----------------------|-------------------------------|--------------------------|-----------------|-----------------------|
|                       | OR (95% CI) | P | OR (95% CI) | P | OR (95% CI) | P | OR (95% CI) | P |
| CARD8 rs2043211 and NLRP3 rs35829419 (both dominant models) | 0.74 (0.21–2.66) | 0.647 | 0.41 (0.09–1.91) | 0.254 | 0.37 (0.09–1.58) | 0.179 | 0.50 (0.16–1.61) | 0.245 |
| CARD8 rs2043211 and asbestos exposure | 0.58 (0.22–1.49) | 0.256 | 0.63 (0.22–1.79) | 0.382 | 0.75 (0.22–2.52) | 0.638 | 1.30 (0.49–3.46) | 0.601 |
| NLRP3 rs35829419 and asbestos exposure | 0.37 (0.09–1.62) | 0.188 | 0.09 (0.01–0.60) | 0.014 | 0.62 (0.11–3.62) | 0.591 | 1.66 (0.37–7.51) | 0.513 |
An increased risk of pleural plaques was found for the rs2043211 AT + TT genotypes (OR = 1.48, 95% CI 1.01–2.16, p = 0.042). On the other hand, when the analysis was done for MM patients as cases, a decreased risk of MM was found for carriers of TT genotype (OR = 0.52, 95% CI 0.27–1.00, p = 0.049). No association was found either between other asbestos-related diseases and CARD8 rs2043211 genotypes or between NLRP3 rs35829419 and different asbestos-related diseases (Table IV). In the subsequent forward conditional logistic regression analysis, the results did not change considerably after adjustment for asbestos exposure, age in and gender (Table VI).

The interactions between NLRP3 rs35829419 and CARD8 rs2043211 genotypes did not influence the risk of any asbestos-related disease. However, when testing interactions between SNPs and asbestos exposure, a multiplicative interaction was observed with NLRP3 rs35829419 (OR = 0.09, CI 0.01–0.60, p = 0.014) (Table V). A decreased risk of asbestosis was found for NLRP3 CA + AA genotypes only in subjects with medium or high asbestos exposure (OR = 0.16, CI 0.03–0.92, p = 0.040) (Table VII).
Discussion

It has been suggested that in addition to asbestos exposure, genetic factors may also affect the development of asbestos-related diseases (40–42). This study investigated the influence of NLRP3 rs35829419 and CARD8 rs2043211 polymorphisms on the risk of different asbestos-related diseases.

As expected, asbestos exposure (medium and high vs low) was associated with an increased risk of developing all asbestos-related diseases studied, which is in agreement with the results of previous studies (18, 43–46).

An important finding of this study is that subjects with CARD8 rs2043211 AT + TT genotypes had an increased risk of pleural plaques compared to those with rs2043211 AA genotype. This could be explained by observing that A to T transversion in CARD8 gene can introduce a stop codon at codon 10 (Cys10Stop) and produce a truncated CARD protein, which is unable to suppress NF-kB activity. This results in an increased production of pro-IL-1 and increased inflammation (21, 23, 36, 47, 48). Considering that asbestos fibres may provoke pleural inflammation, this result could be biologically plausible (18). According to our knowledge and available literature, the association between CARD8 rs2043211 polymorphism and pleural plaques has not been studied yet.

Another interesting result of the current study shows a decreased risk of developing MM compared to those patients with pleural plaques who had CARD8 rs2043211 TT genotype, which indicates a protective effect. As described above, the activity of truncated CARD8 protein is decreased, thus facilitating the inflammation response (23, 37) and consequently the increased risk of developing MM, which seems to be contrary to our findings. However, our results are in agreement with studies investigating the association between CARD8 rs2043211 genotypes and other diseases (48, 49). Moreover, the investigated SNP represents only a fraction of the polymorphic content of the CARD8 gene (48). Therefore, other SNPs that may be responsible for the altered CARD8 function need to be further investigated. As NLRP3 inflammasome is involved in apoptosis (49), a decreased activity of CARD8 protein may decrease apoptosis. Considering that apoptosis is a key part of the innate tumour-suppression mechanism (50), this could explain the decreased risk of MM for CARD8 rs2043211 TT genotype.

This study also revealed that NLRP3 polymorphism could modify the association between asbestos exposure and asbestosis. A decreased risk of asbestosis was found for subjects with NLRP3 CA and AA genotype. Considering that inflammation has been proposed to be involved in the development of asbestosis and the role of NLRP3 in the inflammation process, this interaction could be logical and biologically plausible. The results of our study are in agreement also with the findings of Kukkonen et al., who reported that the NLRP3 inflammasome is important in the development of fibrotic lung disease by associating the NLRP3 rs35829419 variant allele with increased risk of asbestos-related interstitial lung fibrosis (23).

To our knowledge and available literature, the influence of NLRP3 and CARD8 polymorphisms on the risk of asbestos-related diseases has been not studied yet. The results of the study suggest that NLRP3 and CARD8 polymorphisms could affect the risk of these diseases. However, further studies including more subjects and also other NLRP3 and CARD8 polymorphisms are necessary to elucidate these associations.

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Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.

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