Genes involved in innate immunity associated with asbestos-related fibrotic changes

Mari K Kukkonen,1 Tapio Vehmas,1 Päivi Piirilä,2 Ari Hirvonen1

ABSTRACT

Objectives To determine whether genetic polymorphisms in several candidate genes related to innate immunity and protease–antiprotease balance modify individual susceptibility to develop asbestos-related fibrotic pleuropulmonary changes.

Methods Sixteen polymorphisms from nine genes (NLRP3, CARD8, TNF, TGFβ1, GC, MMP1, MMP9, MMP12 and TIMP2) were genotyped from 951 Finnish asbestos-exposed workers. The genotype/haplotype data were compared to signs of fibrosis and pleural thickenings using linear and logistic regression analysis adjusted for potential confounders.

Results A functional polymorphism (Q705K; rs35829419) in the NLRP3 gene was associated with interstitial lung fibrosis (p=0.013), and the TGFβ1 rs2241718 SNP with visceral pleural fibrosis (VPF) (p=0.044). In stratified analysis, the carriage of at least one NLRP3 variant allele conferred a 2.5-fold increased risk for pathological interstitial lung fibrosis (OR 2.44, 95% CI 0.97 to 6.14). Conversely, the carriage of at least one TGFβ1 rs2241718 variant allele protected against VPF (OR 0.62, 95% CI 0.39 to 0.98). The TIMP2 rs2277698 SNP and a haplotype consisting of the TGFβ1 rs1800469 and rs1800470 SNPs were associated with the degree of pleural thickening (p=0.037 and p=0.035), and the CARD8 rs2043211 SNP with the greatest thickness of pleural plaques (p=0.015).

Conclusions Our results support the hypothesis that the NLRP3 inflammasome complex 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, and the TGFβ1 rs2241718 variant allele with decreased risk of asbestos-related VPF. Polymorphisms in CARD8 and TIMP2 are proposed to modify the development and/or calcification of pleural thickenings.

INTRODUCTION

Asbestos has been mined and used widely for various insulating and construction purposes because of the thermochemical and electrical resistance, high tensile strength, and the flexibility of these naturally occurring fibrous silicate minerals. Worldwide, the most common type of asbestos used commercially is chrysotile (<90%). In Finland, however, approximately 40% of all asbestos used has been anthophyllite as it was previously produced domestically.1

Asbestos usage peaked in the mid 1970s, after which the health hazards of asbestos were recognized and the demand for asbestos started to decline in the USA and in Europe.1 In Finland, asbestos spraying was forbidden in 1976 and a complete asbestos ban was introduced in 1994. Subsequently, 54 other countries have banned or restricted the use of asbestos.3 Despite these constraints, asbestos-induced pulmonary and pleural diseases are still a significant health concern due to the enormous amounts of asbestos mined and used since the early 1900s, and the long latency period of most asbestos-associated conditions.3

The major diseases caused by asbestos are mesothelioma and lung cancer.4 In addition, asbestosis, which is interstitial lung fibrosis caused by the deposition of asbestos fibres in the lungs, is an important asbestos-related disease that greatly contributes to the mortality caused by asbestos exposure.

The WHO has recently estimated that 107 000 deaths are caused annually by asbestos-related diseases.4 Based on an analysis of all deaths caused by mesothelioma and asbestosis reported to the WHO for 1994–2010, it is calculated that approximately 1300 people die worldwide each year from asbestosis, the majority in the Americas (597) and Europe (535).5 However, countries such as China, India and Russia are not included in this estimate as they did not supply national data.

The exact mechanism by which the inhalation of asbestos fibres leads to lung tissue injury is still unclear, but it may involve a persistent inflammatory response mediated by reactive oxygen species (ROS), cytokines, growth factors, and pro-inflammatory factors, as well as changes in the proteolytic balance of the lungs.5–9 Genes involved in these pathways are potential candidate modifiers of individual susceptibility to develop lung fibrosis and other asbestos-associated disorders.
Asbestos is known to induce the production of IL-1β and TNF from alveolar macrophages, and these cytokines are therefore believed to play an essential role in the early inflammatory response following asbestos exposure. Furthermore, TNF enhances the expression of TGFβ1, a multifunctional cytokine, which may lead to immune suppression and lung fibrogenesis. Polymorphisms in both the TNF and TGFβ1 genes have previously been associated with the development of asbestosis.

TGFβ1 can down-regulate collagen degradation through matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), which play an essential role in tissue repair and remodelling, and have been proposed to contribute to the development of pulmonary fibrosis. Differential levels of several MMPs and TIMPs, such as MMP1, MMP9, MMP12 and TIMP2, have been detected in different fibrotic lung diseases. Asbestos-induced secretion of IL-1β from alveolar macrophages is mediated through the NLRP3 inflammasome, which identifies asbestos as a threat via ROS. The NLRP3 inflammasome complex consists of several components, such as NLRP3, ASC and CARD8 (also known as TUCAN), genetic variation of which may affect the function of the complex. Certain polymorphisms in the NLRP3 and CARD8 genes have been proposed to be associated with IL-1β production and severe inflammation.

Animal studies also suggest that NLRP3 could participate in ASBE. The study group consisted of 951 subjects (935 males, 16 females). Approval for the study was obtained from the local ethics committees (the Ethics Committee of the Finnish Institute of Occupational Health and the Ethics Committee for Research in Occupational Health and Safety, Hospital District of Helsinki and Uusimaa) according to the legislation in force at the time of the original study. All subjects gave informed consent to participate in the study.

**Exposure assessment**

The ASBE participants were personally interviewed by an occupational physician using a standardised questionnaire including questions on smoking habits and occupational history. They were construction workers who had installed heat and fire insulation or asbestos-containing walls and ceiling panels, used asbestos paints, putties and fillers, dismantled asbestos-containing materials, or cleaned areas where asbestos was present. They had a mean duration of asbestos exposure of 26.1 ± 9.7 years and a mean of 23.7 ± 15.0 pack-years of smoking. Most were ex-smokers (70%) or current smokers (27%), and only 3% had never smoked.

The ASBE participants filled in a self-administered questionnaire modified from the Finnish Environment and Ashma Study Questionnaire. The final questionnaire included queries on demographic characteristics, respiratory symptoms and diseases, smoking exposure, and occupational exposures with a focus on asbestos. Most of the ASBE participants were construction workers, cleaners or plumbers. The ASBE participants had a mean duration of asbestos exposure of 20.8 ± 11.4 years, and a mean of 16.1 ± 18.2 pack-years of smoking. Most were ex-smokers (50%) or current smokers (27%), and 3% had never smoked.

**Radiological examinations**

The lungs of study subjects were imaged prone in full inspiration with four different scanners: in 1996–1997 the Picker PO 2000 (Picker International, Cleveland, Ohio, USA) device was used, while in 2003–2004 the Siemens Somatom Balance (Siemens Medical, Erlangen, Germany) was used in Helsinki, the Siemens Somatom Plus 4 (Siemens Medical) in Tampere, and the GE LightSpeed 16 Advantage (GE Healthcare, Milwaukee, Wisconsin, USA) in Turku. The HRCT images were printed as hard copies and analysed blindly by two (2003–2004) or three (1996–1997) radiologists.

The radiologists visually scored the signs of interstitial lung fibrosis using an arbitrary semiquantitative scale from 0 to 5.
including one subclass between each of the five classes: 0 (normal finding), 1 (subnormal finding), 2 (mild fibrosis), 3 (moderate fibrosis), 4 (severe fibrosis) and 5 (extreme fibrosis).

VPF includes diffuse pleural thickening, rounded atelectasis and parenchymal bands. A VPF variable was constructed by adding up scores for parenchymal bands (scale 0–5), aderences at the diaphragm and sinuses as well as other aderences (scales 0–3) and rounded atelectasis (score 0–3 for up to 2 atelectases) in the ASBE study material. This sum score was then dichotomised to match the frequency distribution of the dichotomous VPF variable used in the ASBE study. Several signs of pleural changes were also recorded: the extent (cm²) and greatest thickness of pleural thickenings (ILO ‘width’: 0=no plaques, 1≤5 mm, 2=5–10 mm, 3=10 mm) and their degree of calcification (0=no, 1=spare, 2=a considerable part of the pleural thickening, 3=nearly all). The methods, including intra- and inter-reader agreement, have been described in more detail previously.

Genotyping analyses
DNA was extracted mechanically (Thermo KingFisher; Thermo Fisher Scientific, Erembodegem, Belgium) from whole blood using a BioSprint 15 DNA Blood Kit (Qiagen, Hilden, Germany) and stored at −20°C until use.

Two NLRP3 SNPs (rs35829419 and rs10925027), three CARD8 SNPs (rs2043211, rs1062808 and rs2288877), two TNF SNPs (rs1799724 and rs1800629), two TGFβ1 SNPs (rs1800469 and rs2241718), two GC SNPs (rs7041 and rs4588), one MMP12 SNP (rs652438) and one TIMP2 SNP (rs2277968) were genotyped using the OpenArray system (BioTrove, Woburn, Massachusetts, USA), a next-generation quantitative PCR platform used on TaqMan chemistry.

The TGFβ1 rs1800470 SNP was genotyped using an allelic discrimination assay on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, California, USA) with TaqMan probes. The MMP1 rs1799750 SNP was analysed with a pyrosequencing method based on an assay from the PyroMark Assay Database (Qiagen), and the MMP9 rs3918242 SNP was genotyped using a PCR-RFLP-based method essentially according to Joos et al. A detailed description of the genotyping methods is presented in online supplement 1.

For quality control, two independent readers interpreted the results and a random selection of 10% of all samples was re-tested. No discrepancies were discovered in the replicate tests for rs35829419, rs10925027, rs1062808, rs2288877, rs7041, rs4588, rs652438, rs1800470, rs1799750 or rs3918242. Minor error rates were detected for OpenArray assays rs2043211 (1%), rs1799724 (1%), rs1800629 (2%), rs2241718 (2%), rs2277968 (2%) and rs1800469 (3%).

To verify the reliability of the OpenArray platform, a random selection of 15% of samples was re-analysed for rs1800629 and rs1799724 SNPs using an RFLP-based method and a pyrosequencing-based method (rs1799724) with 100% concordant results with the earlier estimates.

Statistical analysis
Our study (n=951) has 80% power to detect ORs from 1.57 to 2.63 depending on the minor allele frequency (4–43%). The calculations, based on a two-sided α of 0.05, were performed using standard methods.

Linkage disequilibrium (LD) among the studied SNPs was examined using HaploView V.4.2. When moderate or strong linkage was detected (r²>0.5), haplotypes consisting of the SNPs in question were statistically reconstructed from population genotype data with the Markov chain method for haplotype assignments using the PHASE program (V.2.1). The associations of the haplotypes with pulmonary parameters were then examined as with the single SNPs.

The χ² test with a cut-off p value of 0.05 was used to check for deviation from Hardy–Weinberg equilibrium (HWE).

The associations between genotypes/haplotypes, fibrosis and pleural plaques were evaluated using a general linear model, while logistic regression analysis was used to evaluate potential confounders and to further study the risk for fibrotic changes and pleural plaques and their severity with a certain genotype. Covariates used in the analysis were: sex, age, pack years of smoking and years of asbestos exposure.

For further analysis, the cases were divided according to the existence and severity of radiological signs. The signs of interstitial lung fibrosis (radiological score >0) were considered subnormal if the radiological score was <2 and pathological if the score was ≥2. The degree of calcification of pleural plaques was categorised as mild if calcification was sparse and high if a considerable part or nearly all of the pleural thickenings were calcified.

All of the data analyses were performed using SPSS V18.0 (SPSS, Chicago, Illinois, USA).

RESULTS
The demographics and HRCT characteristics of the asbestos-exposed workers are summarised in table 1. All the genotype distributions of the studied gene polymorphisms were in HWE (p>0.09), except for the CARD8 rs1062808 and rs2288877 SNPs (p<0.01), which were therefore excluded from further analysis.

The NLRP3 rs35829419 SNP was associated with interstitial lung fibrosis (p=0.013) and the TGFβ1 rs2241718 SNP with VPF (p=0.044). The CARD8 rs2043211 SNP was associated with the greatest of pleural plaques thickness (p=0.015) and

Table 1: Characteristics of the asbestos-exposed workers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD) or n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.2 (7.3)</td>
</tr>
<tr>
<td>Male</td>
<td>935 (98.3%)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>135 (14.2%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>595 (62.6%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>221 (23.2%)</td>
</tr>
<tr>
<td>Pack years</td>
<td>20.4 (16.7)</td>
</tr>
<tr>
<td>Years of asbestos exposure</td>
<td>23.9 (10.7)</td>
</tr>
<tr>
<td>Intersitial lung fibrosis</td>
<td></td>
</tr>
<tr>
<td>Rs≤0</td>
<td>201 (21.1%)</td>
</tr>
<tr>
<td>0&lt;RS≤1</td>
<td>434 (45.6%)</td>
</tr>
<tr>
<td>1&lt;RS≤2</td>
<td>234 (24.6%)</td>
</tr>
<tr>
<td>1&lt;RS≤3</td>
<td>72 (7.6%)</td>
</tr>
<tr>
<td>Rs≥3</td>
<td>10 (1.1%)</td>
</tr>
<tr>
<td>Visceral pleural fibrosis (n=941)</td>
<td>131 (13.9%)</td>
</tr>
<tr>
<td>Yes</td>
<td>131 (13.9%)</td>
</tr>
<tr>
<td>No</td>
<td>810 (86.1%)</td>
</tr>
<tr>
<td>Pleural plaques</td>
<td></td>
</tr>
<tr>
<td>Greatest thickness (n=949)</td>
<td>1.88 (0.67)</td>
</tr>
<tr>
<td>Extent, cm² (n=947)</td>
<td>104.4 (72.5)</td>
</tr>
<tr>
<td>Calcification (n=939)</td>
<td>1.39 (0.94)</td>
</tr>
</tbody>
</table>

n=951 except as noted.

RS, radiological score.
common haplotype was GC (wild type–wild type, 65.2%), followed by GA (13.7%) and TA (21.1%). No associations were seen between the GC haplotypes and the studied parameters.

For the TGFB1 rs1800469 and rs1800470 SNPs, four haplotypes were identified: GT (wild type–wild type, 60.5%), GC (24.9%), AT (10.3%) and AC (4.3%). Overall, the TGFB1 rs1800469–rs1800470 haplotype was found to be associated with the degree of pleural plaque calcification (p=0.035) (data not shown). In the stratified analysis, the GC and AT haplotypes conferred increased risks (OR 1.52, 95% CI 1.09 to 2.11 for GC; OR 1.95, 95% CI 1.18 to 3.22 for AT) for pleural plaque calcification compared with the most common haplotype, GT. The risks did not notably differ between mild and high degrees of calcification (table 5).

### DISCUSSION
Although there is growing evidence of the involvement of the NLRP3 inflammasome in the development of fibrosis, there are few data on the association between the genes involved in this inflammasome complex and pulmonary fibrosis. In the current study, we found a significant association between the TGFB1 rs35829419 SNP and asbestos-induced interstitial lung fibrosis. Moreover, a truncating polymorphism (C10X; rs2043211) in another member of the complex, CARD8, was associated with the greatest thickness of pleural plaques.

The rs35829419 and rs2043211 SNPs have previously been linked to increased IL-1β production and severe inflammation. The rs35829419 SNP changes amino acid (Q705K) in the NLRP3 protein, and recent evidence suggests that it is a gain-of-function mutation leading to a constantly active NLRP3 inflammasome and increased IL-1β levels. This may induce fibrosis and therefore agrees with our findings.

Interestingly, a recent study suggests an association between the variant allele of another NLRP3 SNP (rs1539019) and coal workers’ pneumoconiosis (CWP). CWP is a lethal fibrotic lung disease caused by inhalation of airborne coal mining dust, including crystalline silica. Although the functional consequences of the rs1539019 SNP are not known, this finding is in agreement with our results and strengthens the hypothesis that the NLRP3 inflammasome complex is involved in the development of fibrotic lung diseases.

Genetic polymorphisms in TNF and TGFB1 have previously been associated with the development of asbestos-induced

### Table 2 Association between genetic polymorphisms and fibrotic pleuropulmonary changes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Gene</th>
<th>Polymorphism*</th>
<th>β Coefficient</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial lung fibrosis</td>
<td>NLRP3</td>
<td>rs35829419</td>
<td>0.078</td>
<td>0.013</td>
</tr>
<tr>
<td>Visceral pleural fibrosis</td>
<td>TGFB1</td>
<td>rs2241718</td>
<td>−0.660</td>
<td>0.044</td>
</tr>
<tr>
<td>Pleural plaques Greatest thickness</td>
<td>CARD8</td>
<td>rs2043211</td>
<td>0.076</td>
<td>0.015</td>
</tr>
<tr>
<td>Calcification</td>
<td>TIMP2</td>
<td>rs2277698</td>
<td>0.063</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Covariates used in the analysis: sex, age, pack-years and years of asbestos exposure.
*Three levels of genotypes (the wild type homozygotes, heterozygotes, variant homozygotes) were included in the model.
†Standardised β coefficient.

### Table 3 Distribution of NLRP3 and TIMP2 genotypes according to the existence and severity of radiological changes

#### Interstitial lung fibrosis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No changes*</th>
<th>Any changes OR (95% CI)</th>
<th>Subnormal‡ changes OR (95% CI)</th>
<th>Pathological‡ changes OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3 rs35829419</td>
<td>Gln705Gln</td>
<td>182 (92.4)</td>
<td>661 (88.8)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Gln705Lys or Lys705Gln</td>
<td>15 (7.6)</td>
<td>83 (11.2)</td>
<td>1.57 (0.87 to 2.82)</td>
</tr>
</tbody>
</table>

#### Calcification of pleural plaques

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No calcification*</th>
<th>Calcification OR (95% CI)</th>
<th>Mild§ calcification OR (95% CI)</th>
<th>High§ calcification OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP2 rs2277698</td>
<td>G853G</td>
<td>107 (83.6)</td>
<td>617 (77.1)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>G853A or A853A</td>
<td>21 (16.4)</td>
<td>183 (22.9)</td>
<td>1.58 (0.95 to 2.65)</td>
</tr>
</tbody>
</table>

Data are presented as n (%) except as noted.
*Reference category.
†Adjusted for age, sex, pack-years and years of asbestos exposure.
‡The changes were considered as mild if the radiological score was <2 and severe if the radiological score was ≥2.
§The calcification was considered as mild if it was sparse and high if a considerable part/nearly all of the pleural plaques were calcified.

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fibrosis in a German population. However, we could not replicate the findings concerning the TNF genotypes, possibly because of phenotypic heterogeneity. In the previous study, a strict diagnosis of asbestosis was used, whereas in the current study different fibrotic changes were studied separately. In addition, since the control subjects in the previous study were not exposed to asbestos, the analyses were not adjusted for asbestos exposure level.

Regarding the studied TGFB1 genotypes, the rs2241718 SNP was associated with asbestos-induced VPF, and two TGFB1 haplotypes consisting of the rs1800469 and rs1800470 SNPs were associated with pleural plaque calcification. In stratified analysis, the variant allele of the TGFB1 rs2241718 SNP was shown to be protective against VPF.

The TGFB1 rs2241718 SNP is located in the non-coding area near the gene and has no known functional consequences. The promoter area SNP rs1800469 is in tight linkage with the third studied signal peptide SNP rs1800470 (Leu10Pro; formerly known as rs1982073), which has been associated with increased secretion and higher TGFB1 serum levels. Moreover, the F-SNP program predicts that rs1800470 is involved in splicing regulation.

It is also possible that the causative variant is one of the several SNPs found in tight linkage ($r^2>0.9$ in the 1000 Genomes Project population) with the studied TGFB1 SNPs, located in a completely different gene. However, since TGFB1 is a downstream effector of the NLRP3-mediated innate immunity response, and also involved in the regulation of protease–antiprotease balance, it is highly likely that it has a role in the pathogenesis of external exposure and inflammation-related lung disease. The previous and current findings together imply that TGFB1 is indeed involved in the development of asbestos-related fibrosis.

We also found an association between the TIMP2 rs2277698 SNP and pleural thickenings; the rs2277698 variant allele was found to predispose to a high degree of pleural thickening calcification. The relationship between TIMP2 and pleural plaques has not been explored before, but in an animal model, the overexpression of TIMP2 has been shown to inhibit atherosclerotic plaque development and destabilisation. However, the mechanisms of the development of atherosclerotic plaques are probably very different from the development of pleural plaques.

The functional consequences of the synonymous rs2277698 SNP are unclear, but the F-SNP program predicts that it is highly likely to be involved in splicing regulation. The rs2277698 SNP is also in strong linkage ($r^2>0.9$ in the 1000 Genomes Project population) with other potential causal SNPs, some of which (rs9889410 and rs11654470) are located in an area predicted to alter transcriptional regulation.

One of the main strengths of our study is the very large, HRCT-characterised population, in which different asbestos-related abnormalities were recorded separately. Since different degrees of interstitial fibrosis were detected in 750 subjects, we...
were also able to compare the severity of these changes with the genotype data.

Our study also has potential limitations. First, since the patients were enrolled in three cities during two separate primary studies, four different CT scanners were used and seven radiologists participated in image reading. However, since the Finnish population is very homogenous and the three large cities where the patients were enrolled, are all located in southern Finland and very close to each other, we do not believe that geographical origin at the time of the examination has caused any significant bias in the data analysis. Moreover, any inconsistency in image reading causes inaccuracy and thus random noise affecting the results, leading to loss of power rather than systematic error. This increases the error variance in computations and the detected associations are therefore likely to be underestimated.

Second, the multiple comparisons performed increase the possibility of detecting false-positive associations. However, most of the methods correcting for multiple testing are very conservative, and, for example, it is unclear how many comparisons should be adjusted. In addition, based on previous findings, we had an a priori hypothesis for each polymorphism chosen, which reduces the need for correction. Nevertheless, these results should be considered with caution until replicated in another study population. The use of a large control population including asbestos-exposed subjects without pleural or pulmonary changes would also be beneficial in evaluating the reliability of our findings.

In summary, our results strengthen the hypothesis that the NLRP3 inflammasome is important in the development of fibrotic lung diseases. In particular, the NLRP3 rs33829419 variant allele is proposed to increase the risk for asbestos-related interstitial lung fibrosis and the TGFβ1 rs2241718 variant allele to protect against asbestos-related VPF. In addition, polymorphisms in CARD8 and TIMP2 are proposed to modify the development and/or calcification of pleural thickenings.

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Contributors MKK carried the main responsibility for genotyping, data analysis and preparation of the manuscript; TV participated in data collection, radiological examinations, data analysis and manuscript preparation; PP participated in data collection, planning and manuscript preparation; AH was responsible for study design and supervision of genotyping, data analysis and manuscript preparation. All authors have read and approved the final version of the manuscript.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Ethics Committee of the Finnish Institute of Occupational Health and the Ethics Committee for Research in Occupational Health and Safety, Hospital District of Helsinki and Uusimaa.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES


Online Supplement 1.

Details of the genotyping analyses

The assays spotted on the OpenArray genotyping plate for NLRP3 SNPs (rs35829419 and rs10925027), CARD8 SNPs (rs2043211, rs1062808 and rs2288877), TNF SNPs (rs1799724 and rs1800629), TGFB1 SNPs (rs1800469 and rs2241718), GC SNPs (rs7041 and rs4588), MMP12 SNP (rs652438), and TIMP2 SNP (rs2277698) were C__25648615_10, C__30713882_10, C__11708080_1_, C___3218826_10, C__15879993_10, C__11918223_10, C___7514879_10, C___8708473_10, C___7818377_1_, C___313359_30, C___8278879_10, C____785907_10, and C__15885241_10, respectively. Plate format of 16 SNPs and 144 samples per array was used. The allele calling analysis was performed by using OpenArray™ SNP Genotyping Analysis software (BioTrove Inc).

The TGFB1 rs1800470 SNP was genotyped by using an allelic discrimination assay on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with TaqMan® probes.[1] The primers and probes used in the assay were as follows: forward primer: 5´-GCG CTC TCG GCA GTG C-3´; reverse primer: 5´-CCA GGC GTC AGC ACC AGT A-3´; VIC-probe: 5´-AGC AGC GGC AGC A-3´; and FAM-probe: 5´-CAG CAG CAG CAG C-3´. The primer and probe concentrations in the PCR reaction were 1200 nM and 200 nM, respectively, and the cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 62 °C for 1 minute. Sequence Detection Software 1.4 (Applied Biosystems) was used for the allele calling analysis.

The MMP1 rs1799750 SNP was analysed with a pyrosequencing-method based on an assay from PyroMark Assay Database (Qiagen). The concentrations of the forward (5´-biotin-CCC TTA TGG ATT CCT GTT TTC-3´) and reverse (5´-CCC ATT CT TTT ACC CTC TTG-3´) primers in PCR reactions were 500 nM, and the cycling conditions were 95 °C for 5 minutes, 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds followed by a final extension of 72 °C for 5 minutes. The pyrosequencing run was performed with PSQ™96MA (Qiagen) by using Pyromark Gold Q96 Reagents (Qiagen) according to manufacturer's recommendations. Briefly, 40 µl of the PCR product was mixed with 37 µl of Binding buffer and 3 µl of Streptavidin Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden). PCR products bound to the beads were collected and denatured to single-stranded by treatment with 70% Ethanol (Aa), Denaturation Buffer, Washing Buffer, and mQ water in Pyrosequencing Washing Station. The sequencing primer 5´-GTA GTT AAA TTA TTA GAA AG-3´ was attatched to the template by incubating for 2 minutes in 80°C in annealing buffer. The Pyrosequencing run was conducted in the dispensation order of CAGCTACTAGCA. The pyrograms were generated and analyzed with PSQ 96 SNP Software 1.1.

The MMP9 rs3918242 SNP was genotyped by using a PCR-RFLP-based method essentially according to Joos et al.[2] Briefly, the concentrations of the forward (5´-TTC GTG ACG CAA AGC AGA-3´) and reverse (5´-AGC AGC CCT CTC CAC TCC T-3´) primers were 670 nM, and the cycling conditions were 95 °C for 4 minutes, 34 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 45 seconds followed by a final extension of 72 °C for 5 minutes. After digestion with SphI in 37 °C
for 3 hours the PCR product was electrophoresed on a 2% agarose gel containing EtBr and visualized under UV-light.

The pyrosequencing re-analysis of TNF rs1799724 SNP was performed with PSQ™96MA (Qiagen) by using Pyromark Gold Q96 Reagents (Qiagen) as described above for the analysis of the MMP1 SNP rs1799750. The primers and probes for the pyrosequencing protocol designed by using PyroMark Assay Design 1.0 -tool (Qiagen) were as follows: forward primer: 5´-GGT AGG AGA ATG TCC AGG GCT ATG-3´, biotinylated reverse primer: 5´-biotin-ACT CCC TGG GGC CCT CTA-3´ and sequencing primer: 5´-TCG AGT ATG GGG ACC-3´. The primer concentrations in PCR reactions were 200 nM, and the cycling conditions were: 95 °C for 5 minutes, 39 cycles of 95 °C for 15 seconds, 56 °C for 30 seconds, and 72 °C for 15 seconds followed by a final extension of 72 °C for 5 minutes. The pyrosequencing run was performed as described with MMP1 rs1799750 SNP.

References