

Polymorphisms in chemokine and chemokine receptor genes and the development of coal workers' pneumoconiosis

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Abstract

Chemokines and their receptors are key regulators of inflammation and may participate in the lung fibrotic process. Associations of polymorphisms in *CCL5* (G-403A) and its receptor *CCR5* ($\Delta 32$), *CCL2* (A-2578G) and *CCR2* (V64I), and *CX3CR1* V249I and T280M with Coal Worker's Pneumoconiosis (CWP) were investigated in 209 miners examined in 1990, 1994 and 1999. Coal dust exposure was assessed by job history and ambient measures. The main health outcome was lung computed tomography (CT) score in 1990. Internal coherence was assessed by studying CT score in 1994, 4-year change in CT score, and CWP prevalence in 1999. *CCR5* $\Delta 32$ carriers had significantly higher CT score in 1990 and 1994 (2.15 vs. 1.28, $p=0.01$; 3.04 vs. 1.80, $p=0.04$). The *CX3CR1* I249 allele was significantly associated with lower 1990 CT score and lower progression in 4-year change in CT score in *CCR5* $\Delta 32$ carriers only (p for interaction=0.03 and 0.02). *CX3CR1* V249I was associated with lower 1999 CWP prevalence (16.7%, 13.2%, 0.0% for VV, VI and II); the effect was most evident in miners with high dust exposure (31.6%, 21.7%, 0.0%). Our findings indicate that chemokine receptors *CCR5* and *CX3CR1* may be involved in the development of pneumoconiosis.

Keywords: chemokines; interaction; occupational exposure; pneumoconiosis; polymorphism.

1. Introduction

Chemokines and their receptors orchestrate leukocyte migration of monocytes, T cells and smooth muscle cells in inflammatory lesions [1]. Their role in inflammatory processes has been inferred from murine models [2] and from the expression of chemokines and of their receptors in human lesions [3]. Epidemiological studies have also helped to uncover the critical effect of chemokines and their receptors in disease susceptibility and severity, including their well-known role in progression to AIDS after HIV infection [1,3].

Few genetic epidemiology studies have documented a role for chemokine receptors and their ligands in the pathogenesis of lung inflammatory disorders. A 32-bp deletion in *CCR5* ($\Delta 32$), which causes loss of *CCR5* cell surface receptors, has been associated with asthma and sarcoidosis in some studies [4,5] but not in others [6,7]. The presence of the 64I allele in *CCR2* conferred significantly lower risk for the development of sarcoidosis in a Japanese population [8] but the effect was markedly less protective in a white population [5]. A single nucleotide polymorphism (SNP) in *CCL5* (G-403A), which causes a 8 fold increased constitutive transcription activity [9], has been associated with asthma and sarcoidosis [10-13], but the associations have not been replicated [14,15]. Similarly, a SNP in *CCL2* (A-2518G), which is associated with higher *CCL2* levels [16], has been correlated with asthma and asthma severity [17], but these associations have also not been replicated [18]. Recently, Laprise *et al.* [19] found that the expression level of *CX3CR1*, in which V249I and T280M SNPs cause enhanced leukocyte adhesiveness [20], was decreased by two-fold in bronchial tissues of asthmatic subjects in comparison to subjects with normal pulmonary function.

None of the genetic epidemiological studies mentioned above have evaluated simultaneously the role of the receptors with those of their ligands which may be necessary for the full manifestation of lung inflammatory diseases [21]. Alveolar macrophages elicit inflammation through secretion of several chemokines, some of which were investigated in lung fibrosis [22]. Mechanisms by which

CCR2 signaling (CCR2 and its ligand CCL2) may act in the lung fibrosis process include the regulation of fibrogenic cytokine expression and fibroblast responsiveness to transforming growth factor [23]. CCR5 signaling (CCR5 and its ligand CCL5) may act though their influence on macrophage functions [24,25], and downregulation of CCR5 expression in lymphocytes and macrophages associated with fibrotic stages in sarcoidosis has been reported [26]. Recently, Hasegawa et al. [27] reported enhanced expression of CX3CR1 in lung tissues and association of serum fractalkine levels with the involvement and severity of pulmonary fibrosis in patients with systemic sclerosis, another inflammatory disease.

We hypothesized that *CCR5* Δ32, *CCR2* 64I and *CX3CR1* 249I and 280M variants are associated with attenuation of airway inflammation and/or lower disease prevalence, whereas *CCL5* -403A and *CCL2* -2578G variants are associated with increased airway inflammation, except when their receptors are not expressed. We tested these hypotheses in a longitudinal study of coal workers' pneumoconiosis, an inflammatory and fibrotic lung disease. The primary health outcome for the study was the computed tomography (CT) score at the first survey, when nearly all miners were active. It is a quantitative subclinical phenotype that predicts the occurrence and the evolution to the disease [28-30]. The internal coherence of the results was tested by studying associations with CT score at the second survey and with CWP prevalence at the end of the follow-up. Looking at CT score change between 1990 and 1994 was done for 2 reasons: first as a measure of the activity of the disease between both surveys and second, because studying a difference is an indirect way to take into account unmeasured confounders. We tested the interactions of polymorphisms in chemokine and chemokine receptors with coal dust exposure (i.e., gene X environment interaction), and between polymorphisms (i.e., gene X gene interaction) on lung CT score and disease prevalence because of the relations in the same biological pathway.

2. Materials and Methods

2.1. Study sample

The study design has been described elsewhere [31]. Briefly, unrelated coal miners (n=240, aged 34-50 years in 1990, re-examined in 1994 and 1999) were recruited and contrasted by exposure and chest *x* ray including: 80 subjects heavily exposed to underground coal dusts (≥ 10 years at the coal face) with chest *x* ray classified 0/1 or 1/0; 80 subjects exposed to underground coal dusts with normal chest *x* ray classified 0/0; and 80 subjects slightly exposed with normal chest *x* ray). The three groups were matched for age and smoking habits [30]. The study sample includes 209 miners for whom genetic, biological, and health data were available; no difference regarding exposure, genotype and disease were found with those not included in the analyses (n=31). In 1990, 96% of the miners were active; the proportion of retired miners rose from 24% to 88% between 1994 and 1999. The appropriate ethical committee approved the study and written consent was obtained from all subjects.

2.2. Environment

Besides smoking, detailed information on low or high current exposure based on job description, and cumulative exposure were recorded [32]. High current exposure refers to miners working at the coal face, mining, stope or drift advance, and low exposure refers to those working at ventilation maintenance, pumping, haulage, shaft, stock equipment, or safety. Cumulative personal exposure to dust expressed as $\text{mg/m}^3 \times \text{year}$ was estimated from each person's job history and from dust measurements at various sites of the coal mine. Stratification on cumulative dust exposure used the same threshold ($71 \text{ mg/m}^3 \times \text{year}$) as previously [33].

2.3. Disease

At each survey, chest *x* rays were interpreted by two experienced readers with the International Labour Office (ILO) classification in 12 profusion grades [34]. Pneumoconiosis was defined by a grade $\geq 1/1$. Computed tomography (CT) scans were performed in 1990 and 1994, interpreted blindly to exposure and *x* ray findings. Analyses were based on the profusion score already described [30] composed as the average over six lung zones (upper, middle, lower parts of both lungs) of profusion score rated from 0 to 3 (absent, rare, intermediate, high profusion).

2.4. Genotyping

The *CCR5* $\Delta 32$ polymorphism was analyzed by PCR following the method of Petrek *et al.* [5] (see Table 1 for primer sequences) with an annealing temperature of 62°C for 30 cycles. Products were analyzed by electrophoresis on 2% agarose gels and visualized using a GelDoc 2000 Imaging System (BioRad, Hercules, CA).

The *CCL5* G-403A polymorphism was genotyped by SSCP. PCR was performed using a gamma ³²P-ATP-labeled sense primer and an unlabeled antisense primer (see Table 1 for primer sequence) with an annealing temperature of 54°C for 35 cycles. Products were electrophoresed on 6% non-denaturing SSCP gels (2.67% C) containing 10% glycerol at 10 watts for 15 hours and visualized by autoradiography.

The *CCR2* V64I polymorphism was analyzed according to Hizawa *et al.* [8] by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). Briefly, PCR was accomplished with primers (Table 1) at an annealing temperature of 59°C for 35 cycles. Amplicons were digested with BsaBI at 60°C for 2 hours and fragments were electrophoresed on 3% agarose gels and visualized on a GelDoc 2000 Imaging System (BioRad).

The *CX3CR1* V249I and T280M and *CCL2* A-2578G polymorphisms were analyzed by allelic discrimination using TaqMan Minor Groove Binder (MGB) PCR-based technology (Applied Biosystems, Carlsbad, CA) as previously described [35]. Briefly, the PCR for each SNP contained 10-200 ng of genomic DNA, 900 nM of each primer, 200 nM of each probe, and 1x TaqMan

Universal PCR master mix (Applied Biosystems) using manufacturer's recommended amplification protocol in an ABI 7700 thermocycler (Applied Biosystems). After amplification, plate reads were performed and genotypes determined based on allele clustering using instrument software.

2.5. Statistical methods

Standard statistical tests (χ^2 or Fisher exact test, analysis of variance, multiple regression analysis) were performed with the SAS statistical software. All analyses were first conducted considering each chemokine or chemokine receptor SNP separately and the main outcome CT score in 1990, when 96% of the miners were active. Associations of each SNP with CT score in 1994, change in CT score between 1990 and 1994, and 1999 CWP prevalence were also investigated as a secondary objective to test the coherence of the results, and the activity of the disease (change in CT score between 1990 and 1994). No a priori adjustment was done.

CX3CR1 haplotype analysis was performed using a maximum likelihood method for haplotype-phenotype association as implemented in the THESIAS program (<http://genecanvas.ecgene.net/>) based on the Stochastic-EM algorithm [36]. The most frequent haplotype (VT) was used as the referent. Interaction between genetic polymorphisms and exposure to coal mine dusts, or between both genetic polymorphisms (*CCL5* and its major receptor *CCR5*, or both *CCR5* and *CX3CR1* receptors) on health outcomes (CT score and pneumoconiosis prevalence) were statistically tested using multivariate linear or logistic regression models.

Significance was assessed at the 5% two sided level. We tested associations of CT score with 6 SNPs from 5 candidate genes. As *CCR5* $\Delta 32$ and *CCR2* V64I SNPs were in linkage disequilibrium, and *CX3CR1* V249I and T280M SNPs were in complete linkage disequilibrium, we considered that overall only 4 independent associations were tested for the main outcome CT score in 1990. Therefore, it is reasonable to multiply p values by 4 to apply Bonferroni correction.

3.Results

The mean age of miners in 1990 was 43 years (Table 2). More than half of the miners were current smokers and 47% were highly exposed to coal mine dusts in 1990. Sixty-eight percent were born in France, and only 2% were from non European countries. Among all coal miners, lung CT score increased by ~40% between 1990 and 1994, and the prevalence of pneumoconiosis rose from 3% to 13% between 1994 and 1999.

CT score and x ray grade were highly associated in 1990 and in 1994. Mean CT score values (SD) in 1990 were 0.68 (1.18), 2.96 (2.15) and 2.95 (2.63) in miners with x ray grade of 0/0, 0/1 and 1/0 respectively ($p<0.0001$). In 1994, mean values (SD) were 1.07 (1.39), 4.06 (3.10) and 4.21 (2.51), with the 8 pneumoconiotic miners (1/1 or more) having a mean value (SD) of 6.87 (4.19) $p<0.0001$. CT score and cumulative coal dust exposure ($\text{mg}/\text{m}^3 \times \text{year}$) were highly correlated in 1990 and in 1994: $r=0.38$, $p<0.0001$ and $r=0.32$, $p<0.0001$ respectively.

3.1.Genotype and allele frequencies

All genotype and allele frequencies for *CCR5* $\Delta 32$, *CCL5* G-403A, *CCR2* V64I, *CCL2* A-2578G, *CX3CR1* 249I and 280M fit predictions for Hardy-Weinberg equilibrium (Table 3). One miner was homozygous for the $\Delta 32$ deletion and no miner was homozygous for the *CCR2* 64I SNP. As expected, *CCR5* $\Delta 32$ and *CCR2* V64I were in linkage disequilibrium ($D'=1.0$, $r^2=0.01$); *CX3CR1* V249I and T280M SNPs were in complete linkage disequilibrium and three haplotypes were found (VT: 70.9%, IT: 11.8% and IM: 17.3%). No differences in genotype or allele distributions were observed according to the geographical origin of the miners (data not shown).

3.2.Chemokine and chemokine receptor SNPs and stages of pneumoconiosis

CCR5 $\Delta 32$ carriers had significantly higher lung CT score in 1990 (Table 4); this association remained statistically significant after Bonferroni correction. *CCR5* $\Delta 32$ was also associated with

significantly higher score in 1994 and with a higher frequency of miners with pneumoconiosis in 1994 (10.0 vs. 2.9%, $p=0.07$), but no association was found with 4-year change in CT score or with 1999 pneumoconiosis prevalence. The *CX3CR1* I249 allele was associated, at a borderline significance, with lower pneumoconiosis prevalence (trend test).

No significant association was found between *CCL5* G-403A, *CCR2* V64I or *CCL2* A-2578G polymorphisms with disease phenotypes or prevalence.

3.3. Chemokine and chemokine receptor SNPs with disease phenotypes and prevalence according to coal dust exposure

No interaction was observed between coal dust exposure and *CCR5* Δ 32, *CCL5* -403A, *CCR2* 64I, and *CCL2* -2578G SNPs on lung CT score and pneumoconiosis prevalence (data not shown).

In 1999, no miner homozygous for the *CX3CR1* I249 or M280 allele had pneumoconiosis even in those with high cumulative dust exposure (Fig. 1), and decreased pneumoconiosis prevalence with I249 or M280 allele was evident in miners with high cumulative exposure (trend test for I249 allele, $p=0.09$).

3.4. Association of the CCR5 Δ 32 SNP with disease phenotypes and prevalence according to the CCL5 -403 genotype

After stratification on *CCL5* genotype, association of *CCR5* Δ 32 SNP with 1990 lung CT score remained significant (Fig. 2a). In 1994, consistent with 1990, higher mean CT score values (SEM) were observed in *CCR5* Δ 32 carriers as compared to others: 2.45 (0.69) vs. 1.70 (0.20) in miners with the *CCL5* -403 GG genotype, and 3.72 (0.91) vs. 1.97 (0.36) in those with *CCL5* GA or AA genotype. A multivariate linear regression model showed that only *CCR5* Δ 32 was significantly associated with 1994 CT score ($p=0.008$, $p=0.1$ for *CCL5* G-403A). The progression of CT score between 1990 and 1994 was restricted to miners with *CCL5* GA or AA genotype (Fig. 2b), but no association was observed with pneumoconiosis prevalence in 1999 (data not shown).

3.5. Association of CX3CR1 V249I polymorphism with disease phenotypes and prevalence according to CCR5 Δ32 genotype

Significant associations of *CX3CR1* I249 allele with lower 1990 CT score (Fig. 3a) and lower progression in CT score between 1990 and 1994 (borderline significant; Fig. 3b) were found only in *CCR5* Δ32 carriers. Interaction between *CX3CR1* V249I and *CCR5* Δ32 SNPs on CT score was statistically significant in 1990 and from 1990 to 1994 ($p=0.03$ and 0.02 respectively). In 1999, consistent with CT score results, among miners with the *CCR5* wt/wt genotype the prevalence of pneumoconiosis was 15.6% and 11.9% in miners with *CX3CR1* VV genotype and in *CX3CR1* I249 carriers respectively, whereas the prevalence of pneumoconiosis was 20.0% and 6.7% respectively among *CCR5* Δ32 carriers.

Analyses based on *CX3CR1* haplotypes confirm these results (data not shown).

Conclusions were similar after adjustment for age, dust or smoking. Results did not change when restricting the analysis to active miners in 1990, or to retired miners in 1999.

4. Discussion

We found that, in contrast to our initial hypothesis, *CCR5* $\Delta 32$ carriers had significantly higher lung CT scores as compared with wt/wt individuals. Stratification on *CCL5* G-403A genotype which was not related to disease phenotypes alone, did not modify the associations of *CCR5* with CT score. Interestingly, we found the *CX3CR1* I249 allele was associated with lower 1999 pneumoconiosis prevalence, the effect being evident in miners with high dust exposure. Further, *CX3CR1* I249 and *CCR5* $\Delta 32$ carriers had lower CT score in 1990, slower progression in score between 1990 and 1994, and the proportion of miners with pneumoconiosis in 1999 in each group was consistent with these results.

This study is the first to consider simultaneously *CCR5* and *CCL5*, *CCR2* and *CCL2*, and *CX3CR1* polymorphisms in an inflammatory lung disease. A strength of the study was the functional relevance of each SNP under study. Observed genotype and allele frequencies were similar to those reported previously in other Caucasians populations [5-7,16,17,37,38], with a higher frequency of subjects homozygous for the *CX3CR1* M280 allele observed in our study. Other strengths of the study design include the availability of a quantitative validated phenotype measured twice 4 years apart and objective measurements of coal dust exposure, the main cause of pneumoconiosis. A limitation of the study was the relatively small sample size, which precludes detailed analyses to address simultaneously all genetic factors. Although Bonferroni correction for the independent comparisons on the main outcome (CT score in 1990) did not remove the statistical significance of the association with *CCR5* $\Delta 32$, and the internal coherence of the results with other outcomes support the findings, the limited sample size imposes caution in the interpretation of the results. Further, no family-based data were available, and replication in other studies is warranted. The association of *CCR5* $\Delta 32$, alone and after stratification on *CCL5* G-403A, with more fibrosis measured by CT score, agrees with the association previously found with sarcoidosis [5]. In contrast, Spagnolo *et al.* [39] recently found no association of *CCR5* haplotypes (including the

CCR5 Δ 32 insertion/deletion) with susceptibility to sarcoidosis, and *CCR5* Δ 32 has been associated with protection against asthma [4]. Distinct biological pathways during disease pathogenesis, and particularly the fibrotic process may partly account for the difference. As a loss of CCR5 is associated with macrophage dysfunction, we hypothesized that *CCR5* Δ 32 carriers could have impaired their cleaning capacity, leading to an increase in lung fibrosis. Further, association of *CCR5* Δ 32 SNP with fibrosis remains after stratification on *CCL5* –403 genotype. The lack of clear association between the *CCL5* –403 SNP and pneumoconiosis phenotypes and prevalence in our study was not surprising. Only one subject was homozygous for *CCR5* Δ 32, and heterozygosity in *CCR5* Δ 32 results in only a 50% decrease of CCR5 molecule expression on the cell surface [40]. Therefore, we were unable to completely evaluate the association of the *CCL5* –403 SNP with pneumoconiosis according to the *CCR5* Δ 32 polymorphism.

We also found no association of *CCR2* V64I and *CCL2* A–2578G SNPs with pneumoconiosis phenotypes and prevalence, in contrast to results previously reported for sarcoidosis and asthma [8,17]. Recently, Valentonyte *et al.* [41] found no association between *CCR2* gene polymorphisms, including the V64I SNP, and the risk of sarcoidosis in 1203 patients and their relatives. However, they found positive linkage results in the 3p21 chromosomal region, suggesting a susceptibility gene in this location. In mice, contrasting effects of *Ccr5* and *Ccr2* deficiency on pulmonary inflammatory response to influenza A virus have been reported [42], and targeted deletion of *Ccr3* (*Ccr3*^{-/-}) were found to have enhanced hyper-reactivity in an airway inflammation model [3]. Further, whereas the hypothesis that inactivation of *CCR2* or *CCR5* would ameliorate rheumatoid arthritis, it was shown in murine models that *Ccr5* null mice phenotype was similar to wild type and that collagen-induced arthritis phenotype of *Ccr2* null mice mimicked that of human disease [43]. Pneumoconiosis is another collagen-related disease, and all these results suggest that the complex network of the chemokine system needs to be evaluated in detail, as recently shown in the study of Ferreira *et al.* [44].

Within the CCR cluster, we previously identified two common SNPs in the open reading frame of the *CX3CR1* gene (V249I and T280M) [45] that associated with reduced risk of cardiovascular diseases [35,46,47]. Similarly, the present study found that the *CX3CR1* I249 allele was associated with reduced pneumoconiosis prevalence. Our study is the first to evaluate the role of polymorphisms in *CX3CR1* in an inflammatory lung disease. Fractalkine, the ligand of CX3CR1, is constitutively expressed in pulmonary endothelial and epithelial cells [48]. We hypothesize that fractalkine may be the primary signal allowing capture of CX3CR1-expressing inflammatory cells, and that *CX3CR1* I249 variant, associated with enhanced adhesiveness [20], may decrease the extravasation of monocytes, leading to attenuation of airway inflammation. Association of *CX3CR1* SNPs with reduced pneumoconiosis prevalence was more evident in miners with high exposure, *i.e.* in those having the higher recruitment of inflammatory cells in their airways. We hypothesize that the CCR5 axis may promote the migration of monocytes through the lung, and that both pairs of chemokines and their receptors may act sequentially or simultaneously to allow robust migration and fine positioning of cells expressing both chemokine receptors.

In summary, our findings suggest that chemokine receptors CCR5 and CX3CR1 may be involved in the development of pneumoconiosis, an inflammatory and fibrotic lung disorder. Further, association of the *CX3CR1* I249 allele with CT score and pneumoconiosis prevalence was more evident in miners with high cumulative exposure or in *CCR5* Δ 32 carriers, suggesting that interactions of chemokine receptor polymorphism with coal dust exposure (gene X environment) and between polymorphisms (gene X gene) may control disease susceptibility and progression. Our results also suggest the importance of considering simultaneously genetic variations in several chemokine receptors and balance with their ligands, and of combining gene with environmental parameters to better understand the aetiology of inflammatory diseases.

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Title and legends to figures**Figure 1. Association of *CX3CR1* polymorphisms with pneumoconiosis prevalence according to cumulative occupational exposure in 1999.**

Cumulative coal dust exposure: low: $\leq 71 \text{ mg/m}^3 \times \text{year}$, high: $>71 \text{ mg/m}^3 \times \text{year}$ based on job history and ambient measurements in the coal mine. Numbers of miners in each group are shown below each bar.

CWP: coal workers' pneumoconiosis.

Figure 2. Association of *CCR5* $\Delta 32$ polymorphism with 1990 (a) and difference between 1990 and 1994 (b) CT scan score according to *CCL5* –403 genotypes

Numbers of miners in each group are shown below each bar.

CT: computed tomography.

(a) Multivariate linear regression model showed that only *CCR5* $\Delta 32$ was significantly associated with CT score ($p = 0.01$, $p = 0.3$ for *CCL5* G–403A).

(b) Multivariate linear regression model: $p = 0.15$ for *CCR5* $\Delta 32$ and $p = 0.16$ for *CCL5* G–403A.

Figure 3. Associations of *CX3CR1* V249I polymorphism with 1990 (a) and difference between 1990 and 1994 (b) CT scan score according to *CCR5* $\Delta 32$ genotypes

Numbers of miners in each group are shown below each bar.

CT: computed tomography.

* $p < 0.10$, ** $p < 0.05$.