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Polymorphisms of the IL-1 Gene Complex in Coal Miners With Silicosis

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Background *Silicosis is characterized by fibrosing nodular lesions that eventually develop into progressive pulmonary fibrosis. Pro-inflammatory cytokines, such as interleukin-1 (IL-1), play a key role in the development of silicosis by regulating mediators which are responsible for lung injury, inflammation, and potentially fibrosis. To study whether functional single nucleotide polymorphisms (SNPs) located in the regulatory elements of genes coding for the IL-1α, IL-1β, and IL-1 receptor antagonist (RA) cytokines are associated with silicosis, we examined 318 Caucasian cases confirmed histopathologically with pulmonary silicosis and 163 controls without any apparent inflammation or other pulmonary disease.*

Methods *Genotyping was carried out by polymerase chain reaction-restriction fragment length polymorphism technique.*

Results *The proportion of the IL-1RA (+2018) allele 2 genotype was increased in miners with silicosis (0.27) compared to controls (0.16). The odds of being a case were 2.15 (CI = 1.4–3.3) times higher for subjects with at least one copy of allele 2. No statistically significant differences in the allelic frequencies or genotype distributions for IL-1α (+4845) or IL-1β (+3953) were found between the control and disease groups.*

Conclusions *This is the first report showing an association between the IL-1RA (+2018) polymorphism and silicosis, and suggests that this polymorphism may confer increased risk for the development of the disease.* Am. J. Ind. Med. 39:286–291, 2001.
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KEY WORDS: silicosis; interleukin-1; polymorphism; epidemiology; genetics

INTRODUCTION

Silicosis, a chronic interstitial lung disease caused by the pulmonary response to inhaled crystalline silica, occurs in a variety of industries such as coal and metal mining, quarrying, and construction. It is manifested initially as a chronic inflammatory response which eventually leads to fibrotic changes, and is characterized by the formation of discrete, whorled, hyalinized fibrins in the lung parenchyma [Gibbs and Wagner, 1998]. Silicosis is very rarely an isolated form of pneumoconiosis in coal workers, and usually occurs against a background of simple nodular or macular coal

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workers' pneumoconiosis. Interleukin-1 (IL-1), a proinflammatory cytokine which is over-expressed in chronic inflammatory diseases, is expressed in the lungs of experimental animals following silica exposure [Driscoll et al., 1990; Davis et al., 1998; Orfila et al., 1998]. In silicosis and other pulmonary fibrotic diseases, the local release of IL-1 is thought to mediate inflammation and enhance the level of collagen production by eventually modulating the proliferation of fibroblasts [Schmidt et al., 1982, 1984].

In humans, the IL-1 family consists of three genes located on the long arm of chromosome 2 which encode for IL-1α, IL-1β, and IL-1receptor antagonist (RA) [Roux-Lombard, 1998]. Each of these genes possesses exonic polymorphisms resulting in changes in the level of cytokine expression and increased incidence of certain inflammatory diseases. For example, a rare allelic variant of the IL-1RA, referred to as +2018, has been associated with systemic lupus erythematosus, ulcerative colitis, lichen sclerosis and alopecia areata [Blakemore et al., 1994; Clay et al., 1994; Mansfield et al., 1994; Tarlow et al., 1994]. SNPs in IL-1RA exon 2 (+2018) are in linkage disequlibrium with intron 2 of the variable number tandem repeat (VNTR) [Clay et al., 1996]. Two variants in the IL-1α gene at sites −889 and +4845 are overrepresented in juvenile rheumatoid arthritis and chronic polyarthritis [McDowell et al., 1995; Jouvenne et al., 1999]. The IL-1β (+3953) variant has been found to be prevalent in severe periodonditis and psoriasis [di Giovine et al., 1995; Kornman et al., 1997].

Since IL-1 has been implicated in the pathogenesis of silicosis, the present study was conducted to test whether individuals who possess allelic variants have an altered risk for the development of the disease. We determined the allelic frequencies of polymorphisms in three regions, IL-1α (+4845), IL-1β (+3953), and IL-1RA(+2018), from autopsied lung tissues in 318 coal miners with well-characterized silicosis and 163 controls with no lung disease.

MATERIALS AND METHODS

Study Population

Associations between silicosis and IL-1 SNPs were assessed using a case–control study design derived from 6,580 autopsy cases that participated in the National Coal Workers' Autopsy Study (NCWAS); subjects were obtained between 1972 and 1996 [Green et al., 1998]. The NCWAS, which was initiated in 1969 by Congress to provide evidence for Black Lung claims, represents an estimated 12% of all deceased miners. Lung tissue from all autopsy cases (a range of 3–30 sections/case) was reviewed and graded by three pathologists from the National Institute for Occupational Safety and Health (NIOSH) for coal workers' pneumoconiosis (CWP), including silicosis and other disease status according to the criteria and schema developed by a joint committee of the NIOSH and College of American Pathologists [Kleinerman et al., 1979]. Interpathologist agreement and intrapathologist variations were established and compared before the readings were done independently by the three pathologists. All cases were randomly distributed by number coding among the three pathologists without any knowledge of the length of occupational history, demographic information or years of exposure. CWP lesions, such as macules, nodules, progressive massive fibrosis (PMF), and silicosis, were graded subjectively into three grades of severity—mild, moderate, and severe—based on profusion and size of lesions in the sections. Microscopically, silicotic nodules reflect laminated bundles of mature collagen surrounding a hyalinized central zone with necrosis or calcification. These nodular lesions, measuring up to 1 cm or more in diameter with smooth borders, or a conglomerate of these lesions were classified as silicosis. Criteria used for the selection of cases were based on the presence of one or more microscopically distinct pulmonary silicotic nodules resembling those observed in non-coal workers. Disease severity and grading were evaluated on an average of five sections per subject. Standard photographs for different grades of lesions were available for comparison. Inclusion of disease severity into the analysis of IL-1 polymorphisms did not affect results and are therefore not presented here.

Thirteen hundred of the original autopsy cohort met the criteria for inclusion as a silicosis case. All individuals included in the study were Caucasian, males, and worked as underground coal miners. Occupational exposure histories, demographic information, and smoking histories were available through a questionnaire completed by the next of kin. From these subjects, a random sample of 318 cases was selected and genotyped for at least one of the polymorphisms (IL-1α, IL-1β or IL-1RA). Additional autopsy subjects without any evidence of pulmonary disease were designated as controls and genotyped (n = 163). For any given polymorphism between 80 and 96% of the samples were successfully genotyped for both silicotics (n = 318) and controls (n = 163), which is consistent with other studies using autopsy samples. An additional 35 controls who died 55 years of age or less and had less than 16 years of exposure, were excluded due to a sampling error. This exclusion as shown in the Results section had no impact on the study.

DNA Preparation

Five micrograms thick lung tissue sections were obtained from formalin-fixed, paraffin-embedded tissue blocks stored at the NIOSH storage facility. Genomic DNA was prepared from lung tissue samples following deparaffinization in xylene and alcohol using a DNA isolation kit (QIAGEN, CA) according to the manufacturer's instructions. DNA, freshly obtained from human skin samples,

was used as an internal quality control. Genotyping was performed on genomic DNA using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique for IL-1α (+4845), IL-1β (+3953), and IL-1RA (+2018) polymorphisms as previously described [di Giovine et al., 1995; Clay et al., 1996; Jouvenne et al., 1999]. All reactions were carried out in a buffer containing 10 mM Tris–HCl, 50 mM KCl, 0.2 mM each dNTP, 1.25 units of Taq polymerase, 0.05% W-1 detergent, and 150 ng/50 μl DNA template. The $MgCl_2$ and primer concentrations varied in each type of reaction as detailed below:

IL-1α (+4845): 5′-ATG.GTT.TTA.GAA.ATC.ATC.A-AG.CCT.AGG.GCA-3′ and 5′-AAT.GAA.AGG.AGG.G-GA.GGA.TGA.CAG.AAA.TGT-3′ (0.8 μM); 1 mM $MgCl_2$; 5% DMSO; cycling: 1 min of denaturation at 95°C followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final 5 min extension at 72°C. The products were digested with 2.5 units of *Fnu* 4H1 at 37°C for 2 h. This gave products of 124 bp + 76 bp + 29 bp (allele 1) and 153 bp + 76 bp (allele 2).

Il-1β (+3953): 5′-CTC.AGG.TGT.CCT.CGA.AGA.-AAT.CAA.A-3′ and 5′-GCT.TTT. TTG.CTG.TGA.GTC.C-CG-3′ (1 μM); 2.5 mM $MgCl_2$; cycling: 95°C for 2 min followed by 35 cycles at 95°C for 1 min, 67.5°C for 1 min, 72°C for 1 min, and then a final 5 min at 72°C. The PCR product was digested with 10 units of *Taq I* restriction enzyme at 65°C for 2 h, yielding 12 bp + 85 bp + 97 bp (allele 1) and 12 bp + 182 bp (allele 2).

IL-1RA (+2018): 5′-CTA.TCT.GAG.GAA.CAA.C-CA.ACT.AGT.AGC-3′ and 5′-TAG.GAC.ATT.GCA.CCT.-AGG.GTT.TGT-3′ (1 μM); 1.75 mM $MgCl_2$; cycling: 35 cycles at 94°C for 1 min, 57°C for 1 min, 70°C for 2 min followed by 96°C for 1 min, and then a final 5 min at 70°C. Each PCR product was divided in two aliquots and digestion was performed at 37°C for 2 h with 5 units of *Alu I* and 5 units of *Msp I*. *Alu I* digestion gave products of 28 bp + 126 bp for allele 1, 154 bp for allele 2. *Msp I* digestion yielded bands of 29 bp + 125 bp for allele 2 and one band of 154 bp for allele 1.

All PCR products were electrophoresed using a 10% polyacrlyamide-TBE gel (BioRad, CA) at 150 V for 30 min and visualized by UV illumination after staining with ethidium bromide.

Statistical Methods

χ^2 tests [Rosner, 1990] were conducted to determine whether significant associations existed between each IL-1 polymorphism and disease status. Odds ratios and associated 95% confidence intervals of disease for the minor variant allele 2 carriers and those homozygous for allele 1 were also calculated. To adjust for potential confounding effects of occupational exposure (categorized as < 20, 20–30, 30–40, or > 40 years), adjusted odds ratios and associated 95% confidence intervals were also calculated with logistic regression [Hosmer and Lemeshow, 1989]. In addition to the main effects models, two-way interactions between high occupational exposure (> 30 years) and each polymorphism were tested with logistic regression. All statistical analyses were conducted with SAS statistical software [SAS, 1999].

RESULTS AND DISCUSSION

The relative frequencies of causes of death in the NCWAS population were similar to the general male population in the United States except the number of deaths due to pneumoconiosis (4.2%) and mine accidents (1.9%). Major non-pulmonary causes of death for individuals in the control and silicosis groups were ischemic heart disease, accidents, cirrhosis of the liver, and cancer. Mean age of death in controls was lower than the cases with silicosis due to the inclusion of more cases from sudden or unexpected deaths.

The distribution of age, smoking, and exposure by disease status is presented in Table I. Results indicate that age at death and occupational exposure were both substantially increased in silicosis cases as opposed to controls (67.9 vs. 63.2 and 34.3 vs. 21.3, respectively). A higher percentage of the controls was less than 60 years old (32 vs. 15%) and had less than 20 years occupational exposure (53 vs. 12%). The average number of years smoking were similar across disease categories (19.0 for the diseased cases and 20.4 for the controls) although a higher percentage of the controls was smokers (72 vs. 63%).

Table II describes the allelic distribution by disease status and presents odds ratios and confidence intervals of silicosis for allelic status. Neither IL-1α nor IL-1β demonstrated statistically significant associations with silicosis. The allelic frequencies were also similar between controls and silicosis cases, with a difference of only 2%. IL-1RA, however, revealed an association with disease, as evidenced by the odds ratio of 2.15 ($P < 0.001$), and was unaltered in magnitude by inclusion of exposure years as a co-variant in the logistic regression model. The allelic frequency of the minor variant (allele 2) was also increased in cases vs. controls (0.27 and 0.16, respectively). With the exception of IL-1α, none of the two-way interactions between polymorphisms and high occupational exposure (> 30 years) exhibited statistical significance. Although the association between IL-1α and disease status depended on length of exposure, this interaction did not appear to be biologically relevant, as the odds of disease were elevated for those with the variant and less exposure (< 30 years), but decreased (i.e., odds ratio less than one) for those with the variant and longer occupational exposure. The results in Table II could be influenced by the selection process which excluded 35 controls with the lowest values of both age and exposure. By making assumptions about the genotype in this group (i.e.,

TABLE I. Demographic Characteristics, Disease Status, and Smoking History of Autopsy Groups

Variable	Category (years)	Controls (n = 163)	Silicosis (n = 318)
Year death (mean ± SD)		63.2 ± 8.0	67.9 ± 9.0
Range		(50–87)	(27–93)
n (%)	< 60	53 (32.5)	48 (15.1)
n (%)	60–70	82 (50.3)	143 (44.8)
n (%)	> 70	28 (17.2)	127 (40.1)
Years smoking (mean ± SD)		20.4 ± 16.4	19.0 ± 18.7
Range		(0–50)	(0–70)
n (%)	smokers	118 (72.4)	201 (63.2)
n (%)	nonsmokers	45 (27.6)	117 (36.8)
Years exposure (mean ± SD)		21.3 ± 13.3	34.3 ± 10.8
Range		(1–58)	(1–55)
n (%)	< 20	88 (53.4)	37 (11.6)
n (%)	20–30	32 (19.6)	70 (22.0)
n (%)	30–40	24 (14.7)	89 (28.0)
n (%)	> 40	19 (11.7)	122 (38.4)

recalculating the odds ratio on the basis that all or none of them had the minor variant), we estimated that the bias that could be introduced from excluding these cases represents at most 18–31% of the 95% confidence interval. This would not change the overall significance. Age and exposure would be biased toward no effect, implying that the observed trend of increasing odds is an underestimate. The interaction of exposure and IL-1 SNPs would also be biased toward no effect which would not affect the direction of these results since control subjects in the lowest exposure group were not selected.

Potential for selection bias in this study may result from selection of subjects into the original autopsy cohort. Based on the original objectives of the NCWAS (i.e., identification

TABLE II. Genotype Distribution and Allele Frequencies of IL-1RA, IL-1 α, and IL-1 β

	Allelic distribution (per cent)						
Gene	1/1	1/2	2/2	Total number	Allelic frequency (1/2)	Crude OR (CI)[a]	Adjusted OR (CI)[b]
IL-1α (+4845)							
Controls	125(80.1)	30 (19.2)	1 (0.6)	156	0.90/0.10		
Silicosis	224(78.0)	59 (20.6)	4 (1.4)	287	0.88/0.12	1.13 (0.7–1.8)	0.76 (0.4–1.3)
IL-1β (+3953)							
Controls	43 (31.2)	90 (65.2)	5 (3.6)	138	0.64/0.36		
Silicosis	90 (35.6)	136 (53.7)	27(10.7)	253	0.62/0.38	0.82 (0.5–1.3)	0.75 (0.4–1.2)
IL-1RA(+2018)							
Controls	113 (72)	39 (24.8)	5 (3.2)	157	0.84/0.16		
Silicosis	149 (54.4)	101 (36.9)	24 (8.7)	274	0.73/0.27	2.16 (1.4–3.3)	2.15 (1.3–3.5)

[a]Crude OR(CI) = odds ratio (95% confidence limits) from the χ^2 test.
[b]Adjusted OR (CI) = odds ratio (95% confidence limits) from logistic regression adjusted for occupational exposure.
OR = odds ratio; CI = confidence interval.

of Black Lung claims), individuals with pre-mortem clinical diagnoses were much less likely to enter the cohort. The characteristics of this population, which represents the sampling frame for the case–control study, are therefore likely to be very different from the entire population of deceased coal miners. However, given that this study was able to identify a large number of subjects with varying degrees of silicosis and/or no lung disease, any bias in association between genotype and disease seems unlikely. The overall distribution of these polymorphisms and disease should not, however, be considered representative of an overall population.

Recent studies have focused on variations in cytokine levels among individuals and ascribed these differences, in part, to inheritable SNPs contained within the regulatory elements of cytokine genes [Pociot et al., 1992; Mandrup-Poulsen et al., 1994; Danis et al., 1995; Cork et al., 1996; Perrey et al., 1998; Engebretson et al., 1999]. These variants are associated with a higher incidence and/or severity of chronic inflammatory diseases. IL-1RA is an important endogenous regulator of inflammation and plays a crucial role in several inflammatory diseases. For example, increased frequency of allele 2 has been found in ulcerative colitis and lichen sclerosis [Clay et al., 1994; Mansfield et al., 1994]. An increase in the frequency of the IL-1RA allele 2 is also more prevalent in severe forms of alopecia areota [Tarlow et al., 1994] and systemic lupus erythematosus [Blakemore et al., 1994]. Recently, racial differences in the distribution of IL-1RA genotypes and carriage rates between Caucasians and African American controls have been reported. In this study the frequency of the allele 2 was 27.4% in Caucasian and 9.9% in African American controls [Rider et al., 2000]. The significance of this latter observation is unknown. There is increasing evidence that the severity of the inflammatory response is determined by a balance of pro- and anti-inflammatory cytokines [Tarlow et al., 1994; Casini-Raggi et al., 1995]. As IL-1RA has anti-inflammatory properties, the IL-1/IL-1RA ratio is believed to be important in the regulation of inflammation. However, an allelic association between IL-1RA and IL-1 alleles was not found with respect to silicosis.

The IL-1RA gene polymorphism does not act as a marker for the other genes of the IL-1 cluster, as polymorphic associations were not demonstrated with IL-1α or IL-1β. The contribution of a single genetic locus may not be large, and therefore it is possible that the IL-1RA association may be a genetic marker for another functional polymorphism elsewhere in chromosome 2. The genetic factors that predispose individuals to the development of silicosis are not yet well documented and there are no available data showing genetic associations between silicosis and cytokines. Therefore, we tentatively conclude that IL-1RA is a candidate susceptibility gene that may contribute to the development and pathogenesis of silicosis.

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