ERJ Express. Published on July 12, 2006 as doi: 10.1183/09031936.06.00132505

Genetic Linkage Analysis of Pulmonary Fibrotic Response to Silica in Mice

(73 letters)

Yoshinori Ohtsuka, Xing-Tao Wang, Junpei Saito, Takashi Ishida,

Mitsuru Munakata

From Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, Fukushima, 960-1295 Japan

Running title: genetic linkage analysis of murine silicosis (45 letters)
Body of Text Word Count; 2816 Words

Correspondence should be sent to:
Yoshinori Ohtsuka, MD, PhD
Department of internal medicine, Iwamizawa Rosai Hospital
4-jyo, Higashi 16-chome 5, Iwamizawa, Hokkaido 068-0004 Japan

TEL: +81-126-22-1300
FAX: +81-126-22-1304
Mail address: yohtsuka@kvf.biglobe.ne.jp

Supported by research grants from the Ministry of Education, Science and Culture, Japan (#13470129)

Abstract

Inter-individual variations in the development of silicosis even in the same environments have been reported, which suggest the contribution of genetic factors in its etiology.

Question of the study Is there significant genetic influence on the development of silicosis? Which genetic loci are responsible for the response?

Materials and Methods Eight strains of inbred mice were used to examine the genetic influence on the lung fibrotic response to silica exposure. After breeding intercross between the most susceptible strain and the resistant one, a genome-wide linkage analysis of quantitative trait loci (QTLs) was performed. Hydroxyproline was applied as an index, and genotypes of 167 marker genes were analyzed by the fragment analysis using a capillary-type sequencer.

Results There was significant inter-strain difference among 8 strains of mice on the mean concentration of hydroxyproline contents. Breeding studies were proceeded between the most susceptible strain C57BL/6J and the most resistant CBA/J. A genome-wide linkage analysis of silica-exposed intercross cohort identified significant QTLs on chromosome 4 and suggestive QTLs on chromosomes 3 and 18 respectively.

The answers to the question Genetic factors play significant roles in lung fibrotic responses to silica and we found one significant QTL and 2 suggestive QTLs.

Key word: C57BL/6J, CBA/J, hydroxyproline, quantitative trait locus, intercross

(197 words).

INTRODUCTION

Silicosis is an occupational lung disease caused by inhalation of dust containing free crystalline silica. Free silica is still an occupational fibrogenic hazard in spite of the technical adequacy of the protective technique. In the United States, it was estimated that 200,000 miners and 1.7 million non-mining workers had occupational exposures to inhaled silica [1]. In advanced stage, silicosis complicates tuberculosis, respiratory failure due to progressive massive fibrosis, emphysema, and lung cancer. Considerable attention has been paid on these adverse effects of silica dusts.

Concerning the development of silicosis, extrinsic factors such as duration, amount of exposure, and content of free crystalline silica have been known as critical determinants of the progression of silicosis [2]. However, some of the intrinsic (genetic) factors that may influence susceptibility to silicosis have been reported. Associations of specific human leukocyte antigen (HLA) haplotype with silicosis have been reported in Japanese population [3]. In a German population, HLA haplotype association with coal worker's pneumoconiosis has also been reported [4]. Associations of gene polymorphisms proinflammatory cytokines (e.g. tumor necrosis factor-alpha (TNF-alpha), interleukin-1 receptor antagonist (IL-1-RA)) have been reported [5-7]. In addition to these epidemiological studies, we clarified the importance of genetic background on the variation in inflammation and fibrotic response in murine silicosis model [8,9].

The objective of this study was to use inbred mice to identify the genetic basis for susceptibility to silica-induced pulmonary fibrosis. Inbred mice provide a well-characterized animal model for understanding genetic and environmental factors that determine susceptibility to complex disease processes. Furthermore, the mouse is accepted as an

excellent model of human disease processes because there is extensive linkage homology between the mouse and human genomes.

Using inbred mice, we examined whether there was significant genetic influence on the inflammatory and the fibrotic response among 8 strains of mice. And we also performed genome-wide search for responsible genes for silicosis in our model. This article reports that we found significant genetic influence on the fibrotic response among 8 strains of mice compared to TiO_2 instilled control, and the linkage analysis of F2 identified significant QTL on chromosome 4 and suggestive QTLs on chromosomes 3 and 18 respectively. Some of the results of this study have been previously reported in the form of abstracts [9].

Materials and methods

Animals:

Male (6-8 wk of age) mice of the following inbred strains were purchased from Sankyo Labo Service (Tokyo, Japan) and Clea Japan Inc. (Tokyo Japan): A/J, AKR/J, Balb/cJ, C3H/HeJ, C57BL/6J, C57BL/10J, CBA/J, and DBA/2J. Breeding studies between the most susceptible strain and the resistant strain of mice were conducted. The following crosses were generated: male F1 were bred with the females of the most susceptible strain to produce backcross progeny; F1 were intercrossed to produce F2 progeny. Progeny were weaned at 4week of age, separated according to sex and housed in micro-isolation cages until they reached the appropriate age for experimentation (6-8week). The mice were handled in accordance with Helsinki convention and the standards established by the Animal Welfare Acts set forth by Fukushima Medical University guidelines.

Study design:

(For inter-strain comparison)

Six to eight - week old mice of 8 strains were anesthetized with pentobarbital by the intra-peritoneal route and their tracheal were exposed surgically. Then 0.2g/kg of prepared silica (50mg/ml saline) were instilled intratracheally in the same way as described in previously [8]. The control animals in each strain were given 0.2g/kg of inert TiO_2 (50mg/ml saline) were instilled intratracheally. Twenty-eight days after intratracheal silica instillation, we evaluated pathological fibrosis indices by Ashcroft's estimates [10], and hydroxyproline content per lung.

(For linkage analysis)

Six to eight week-old intercross (F2) mice were anesthetized and exposed to silica as described earlier. As control, three mice of the most susceptible strain were given 0.02ml of saline intratracheally in each experiment. After silica instillation, mice were treated in the same way as described earlier. Four weeks after silica instillation, collagen deposition of the right lung in each mouse was assayed and kidneys were taken for DNA extraction.

Collagen Assay:

Collagen deposition was estimated by determining the total hydroxyproline content of the left lung that was stored at –80 degree centigrade. Hydroxyproline was measured according to Woessner's method [11] as reported previously [8]. Results were calculated as micrograms hydroxyproline per lung, and expressed as the ratio of the mean value of control mice in each experiment.

Morphological Evaluation of Lung Sections:

The right lungs were fixed, embedded in paraffin, sectioned horizontally to include most of the parenchyma at 4 μm, and stained with hematoxylin-eosin. The sections were scanned to determine the degree of fibrosis in 30 fields per one sample at magnification x100 and analyzed semi-quantitatively according to Ashcroft's method [10]. An observer unaware of the treatment read all slides microscopically. Values of the samples were expressed as a fibrosis index, which was the mean grade of fibrosis per one field.

DNA extraction and Genotyping

DNA was extracted from a kidney of each phenotyped animal and prepared for polymerase chain reaction (PCR). PCR reactions were run in 96-well plates with 15.0μl total volume: 1μl DNA (4ng), 1.5μl 10x reaction buffer, 1.5μl 25mM $MgCl_2$, 1.5μl 2.5mM deoxynucleotide triphosphates (equal mixture of deoxy adenosine, deoxycyitidine, deoxyguanosine, and deoxythymidine triphosphates), 0.3μM 10mM primer Mix Applied Biosystems (Foster city, CA, USA), 0.6U Taq polymerase, and brought to volume with distilled water. Primers for simple sequence-length polymorphisms (SSLPs) that differed between C57BL/6J and CBA/J progenitors were chosen from the website [12].

The amplification of the 15 μl PCR reaction were 10 cycles (94°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec) precede by denaturation step of 12 min of at 94°C, and followed by 20 cycle (89°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec) and 10min at 72°C. Pooled PCR product mix was mixed with 310 Genetic analyzer standard solution (Gene Scan 400HD ROX 0.5μl, deionized formamide 12μl), and denatured at 95°C for 2minutes. This sample was quickly chilled on ice until loading on the machine. The treated PCR products were analyzed by ABI PRISM 310 Genetic Analyzer with Gene Scan program (Applied BIosystems, Foster, CA, USA).

Statistical analysis of inter-strain comparison:

The effects of exposure (silica vs. titanium oxide) and strain on pulmonary responses were assessed by two-way analysis of variance (ANOVA) [13]. Tukey's test was used for *a posteriori* comparisons of means [13]. In *a posteriori* comparison of hydroxyproline content among silica-exposed C57BL/6J, CBA/J, and B6CBAF1/J mice, Dunnett T3 test was used because of the different number of mice in each group [13]. The strain distribution patterns (SDPs) for lung responses to silica were compared with the

SDPs for silica by nonparametric Spearman rank correlation [13]. Significance was accepted at $P < 0.05$.

Linkage Analysis:

We completed a scan of the entire genome by genotyping 25 phenotypically high-responders (n=13) and nonresponder (n=12) mice from a total population of 117 F2 mice [14]. Interval analyses were performed each SSLP marker and at 10-centimorgan (cM) intervals between SSLPs. The dominance properties of each putative QTL were evaluated by performing interval analyses using free, additive, recessive, and dominant regression models. The regressions and significance of each association (likelihood ratio chi square statistic) were calculated by the Map Manger QTX program, which is distributed electronically [15]. Putative QTLs were further analyzed by including the entire F2 cohort within the chromosomes identified by selective genotyping. Permutation tests were performed on the phenotype and the genotype data to establish empirically the significance thresholds of all QTL mapping results (Map Manager QTX and the following the methods of Churchill and Doerge [16]). For the genome scan, 10,000 permutations were performed to establish significant and suggestive linkage threshold values. These values correspond to the genome-wide probabilities proposed by Lander and Kruglyak [17].

RESULT

Hydroxyproline content per lung and pathological fibrosis indices were used as indicators of lung fibrosis. ANOVA of both indicators indicated significant effects of strain ($p<0.001$, $p<0.0025$, respectively) and exposure ($p<0.0005$, $p<0.0005$), and interaction of strain and exposure effects on the mean ratio of hydroxyproline in silica-exposed groups ($p<0.0005$) and pathological fibrosis indices ($p<0.0005$) compared to TiO_2 exposed mice.

Strain distribution pattern in hydroxyproline contents among 8 strains of silica-exposed mice was shown in Fig.1. The hydroxyproline content in C57BL/6J was the highest, and that in CBA/J was the lowest among 8 strains of mice. Multiple comparisons by Tukey's test revealed significant interstrain differences in the fibrotic response. Significant interstrain differences were detected between C57BL/6J and Balb/cJ ($p<0.05$), and between C57BL/6J and CBA/J ($p<0.05$). The hydroxyproline content of all other silica-exposed strains except CBA/J mice were significantly higher than that of silica-exposed CBA/J ($p<0.05$).

Strain distribution pattern in pathological fibrosis indices among 8 strains of silica-exposed mice was shown in Fig.2. The pathological fibrosis index in A/J was the highest, and that in CBA/J was the lowest among 8 strains of mice. Multiple comparisons by Tukey's test revealed significant interstrain differences in the fibrotic response. Significant interstrain differences were detected among A/J, AKR/J, and CBA/J ($p<0.05$). The pathological fibrosis indices of all other silica-exposed strains except CBA/J mice were significantly higher than that of silica-exposed CBA/J ($p<0.05$).

Because phenotype chosen for linkage analysis investigation needs to be highly repuroducible and quatitaitve [18], we have chosen hydroxyproline as a marker of pulmonary fibrosis. From the study results of hydroxyproline, we named C57BL/6J as susceptible and CBA/J as resistant strain. To obtain insight about the mode of inheritance of susceptibility to silica exposure, F1 mice derived from a cross between susceptible C57BL/6J mice and resistant CBA/J mice were phenotyped for their response to silica exposure. Compared with TiO_2 exposed control animals, the range of responses to silica exposure by F1 situated between that of C57BL/6J progenitor and CBA/J progenitor (Fig. 3). The mean response of F1 was significantly different from those of C57BL/6J and CBA/J ($p<0.05$).

A genome-wide linkage analysis was initiated to identify the chromosomal location of the quantitative trait loci that control susceptibility to intratracheal silica exposure. In order to minimize experimental error [18], saline was used for control of silica instillation in the linkage analysis study. The first step in this analysis was to select phenotypically extreme 25 F2 responders (50 meioses) were genotyped at 167 SSLP markers spaced to provide complete coverage of the mouse genome with 95% confidence [12,14]. Interval analyses with free regression model revealed 8 suggestive QTLs on chromosome 3 (D3mit57.1), 4 (D4Mit9.1), 5 (D5Mit309.1, D5Mit277.1), 6 (D6Mit274.1), 10 (D10Mit230.1), 11 (D11Mit289.1), 14(D14Mit98.1), and 18 (D18Mit177.1) (Fig. 4).

Each of the putative QTLs was analyzed further by including F2 cohort (n= 117 animals, 234 meioses). Only the chromosome 4 QTL revealed statistically significant linkage as determined empirically by permutation test (Fig. 5). The amount of total trait variance explained by this QTL (at *D4Mit9.1*) was approximately 9%. The QTLs on

chromosome 3, 18 exceeded the threshold for suggestive linkage after remaining F2 animals were considered in the analysis. The amount of total trait variance explained by this QTLs (at *D3Mit319* and *D18Mit177.1*) were approximately 7% and 6%, respectively. Further interval analyses were not done, due to the unavailability of commercial SSLPs between C57BL/6J and CBA/J.

DISCUSSION

A clear pathological association between exposure to silica particles and silicosis has been well clarified since antiquity. Epidemiological studies have also shown that duration and amount of exposure as well as content of free crystalline silica are most critical determinants of the progression of silicosis [2]. However, inter-individual variations in the development of silicosis even in the same environments have been reported. For example, specific human leukocyte antigen (HLA) haplotype associations with silicosis have been reported in Japanese population [3]. However, all inter-individual variability cannot attribute solely to MHC (major histocomaptibility complex). Therefore genetic factors including MHC must be examined in the murine model of silicosis. From these backgrounds, we have made hypothesis that genetic background contributes significantly to the variation in inflammation and pulmonary fibrosis induced by inhalation of silica in the mouse.

Firstly, to address this question, we studied the relative susceptibility to silica exposure in inbred strains of mice. The inbreeding process results in genetic homogeneity at almost all loci, and different inbred strains of mice may be homogeneous for different alleles at the same loci. Generally, strains with greater evolutionary divergence will have a greater degree of polymorphism than strain that is closely related. Therefore these characteristics enable investigations into genetic basis for a physiological and /or toxicological response of phenotype. That is, if a difference in a chosen phenotype is found after a screen of a number of inbred strains to provide sufficient variation across the species, then it may be concluded that one or more loci contributes to the genetic variance observed among strains [18]. The present study evaluated fibrotic response after exposure

to silica intratracheal instillation in 8 strains of mice that are chosen based on their differing lineage and common usage in genetic studies. The significant interstrain variation (genetic) in the fibrotic response to silica exposure supports the hypothesis that susceptibility to silica exposure in inbred mice has a significant genetic component.

Secondly, to determine whether susceptibility to silica exposure is inherited as a dominant or recessive trait, hydroxyproline content were determined in silica-exposed B6CBAF1/J mice. Althoug hydroxyproline content of B6CBAF1/J mice was different from that of silica-exposed C57BL/6J and CBA/J mice, the phenotype of F1 showed full pehnotype of the dominat trait and the mode of inheritance seems to be complex pattern in this model. Further characterization of the mode of inheritance will need formal segregation analyses in segregated backcross and intercross populations derived from C57BL/6J and CBA/J mice as previously done by Kleeberger et.al [19].

Thirdly to further explore a genetic basis for susceptibility to silicosis, we performed QTL analysis of a large F2 mice population generated from the CBA/J and C57BL/6J strains. In this study, we found a significant QTL for susceptibility to silicosis in chromosome 4 and two suggestive QTLs in chromosome 3 and 18.

Several studies have used inbred strains of mice in identifying susceptible genes for pulmonary fibrosis. Haston CK et al. identified two genetic loci, chromosome 17 (major histocomaptibility complex) and chromosome 11 in radiation induced lung fibrosis model [20,21]. These QTLs nearly overlapped QTLs identified for ozone and particular matter susceptibility [22,23]. Haston et al. proposed a common genetic influence in mouse models of lung fibrosis [24].

In this study, we found significant QTL in chromosome 4, with a peak likelihood ratio occurring at the microsatellite marker *D4Mit9.1* (located at 44.5cM distal to the centromere) and two suggestive QTLs located in chromosomes 3 and 18. These QTLs were different from other QTLs in mouse model of bleomycin and radiation induced lung fibrosis. These results suggest different mechanisms may play roles in pathophysiological mechanisms in the development of lung fibrosis. Differing from bleomycin and radiation, crystalline of silica is an inorganic dust and causes granulomatous reaction in the lung. This factor might induce different pathophysiological mechanism in the lung and yield different results.

Several number of candidate genes within chromosome 4 QTL, including the oncogene *jun*, insulin-like growth factor binding protein like 1 *Igfbp1*, phospholipase A2 activating protein *plaa*, and cyclin-dependent kinase *cdkn2a*. Oncogene *jun* has transcriptional regulatory roles with p21, and is involved in cell mitogenic responses and growth [22]. Insulin-like growth factor binding proteins (IGFBPs) as well as Insulin growth factors (IGF) has been proposed as pathogenic in lung fibrosis. Some of IGFBPs work as modulating factors of IGF and have also been detected in idiopathic pulmonary fibrosis tissues [23]. Though the function of *Igfbp-1* in our result has not been clarified yet, it might have a potential to be one of important candidate genes. Other candidate genes, such as *plaa* and *cdkn2*, are also transcriptional factors and have important roles in cell cycle machinery. Candidate genes around QTL on chromosome 18 are *fgf1*, sprouty homolog 4 (Drosophilia) *Spry4*, and *pas9* (pulmonary adenoma susceptibility –9). Functions of *fgf-1* (fibroblast growth factor –1) have been implicated in the hepatic fibrotic process [24]. *Spry4* is an intracellular FGF receptor antagonist, was expressed in epithelial cells of fetal

lung under control of a doxycycline-inducible system. As these factors are suggested in silicosis, we need to study their function firstly in silicosis models.

In our previous study, the inheritance of phenotypic trait suggests that this silicosis model is not single genetic model and is rather one of the polygenetic models [6]. In this study, sum of total trait variance explained by three QTLs is 23%. These results suggest the important contribution of genetic factor in response to instilled silica.

In summary, we have found that significant interstrain variation exists in the pulmonary responses to intratracheal instillation of silica. From this, we conclude that there is a significant genetic effect on fibrotic response to silica. And the genome-wide linkage analysis of F2 identified significant QTL for silicosis on chromosome 4 and suggestive QTLs on chromosomes 3 and 18 respectively. This is the first demonstration of candidate loci for the susceptibility to silicosis. Several interesting candidates genes (e.g. *fgf*, *igfbpl-1*) have been observed within the regions of interest. These genes with the polymorphisms need to be further analyzed. Future approaches will be the comparative sequencing of the candidate genes in 8 strains of strains. To refine the identified QTLs will give us an understanding of the mechanism of host response to the silica exposure and provide a potential means to identify genetically susceptible individuals who may be at risk to adverse effects of silica exposure.

References

1. National Institute for Occupational Safety and Health. Work-related lung disease surveillance report. Cincinnati (OH): DHHS (NIOSH): 1999; Publication No.: 2000-105.

2. American thoracic society. Adverse effect of crystalline silica exposure. *Am J Respir Crit Care Med* 1997: 155:761-5.

3. Honda K, Kimura A, Dong RP, Tamai H, Nagato H, Nishimura Y, Sasazuki T. Immunogenetic analysis of silicosis in Japan. *Am J Respir Cell Mol Biol* 1993: 8:106-11.

4. Rihs HP, Lipps P, May-Taube K, Jager D, Schmidt EW, Hegemann JH, Baur X. Immunogenetic studies on HLA-DR in German coal miners with and without coal worker's pneumoconiosis. *Lung* 1994: 172: 347-54.

5. Corbett E, Mozzato-Chamay N, Butterworth AE, Cock KM, Williams BG, Churchyard GJ, Conway DJ. Polymorphisms in the tumor necrosis factor-α gene promoter may predispose to severe silicosis in black south african miners. Am J Respir Crit Care Med 2002: 165: 690-3.

6. Yucesoy B, Vallyathan V, Landsittel DP, Sharp DS, Weston A, Burleson GR, Simeonova P, McKinsstry M, Luster MI. Association of tumor necrosis factor-a and interleukin-1 gene polymorphisms with silicosis. Toxicol Appl Pharmacol 2001: 172: 75-82.

7. Yucesoy B, Vallyathan V, Landsittel DP, Sharp DS, Matheson J, Burleson F, Luster MI. Polymorphisms of the IL-1 gene complex in coal miners with silicosis. Am J Industrial Med 2001: 39: 286-91.

8. Ohtsuka Y, Munakata M, Ukita H, Takahashi T, Satoh A, Homma Y, Kawakami Y. Increased susceptibility to silicosis and TNF-alpha production in C57BL/6J mice. *Am J Respir Crit Care Med* 1995: 152: 2144-2149.

9. Ohtsuka Y, Wang X, Saito J, Ishida T, Munakata M. Genetic linkage analysis of susceptibility to silica exposure in mice. *Am J Respir Crit Care Med*: 2004: 169: A526.

10. Ashcroft T., J. M. Simpson, V. Timbrell. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 1988: 41: 467-70.

11. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 1961: 93:440-7.

12. Informative markers for across – mouse microsatellite studies-" (http://www.cidr.jhmi.edu/mouse/mouse_dif.html). Date last accessed: November 1 2005.

13. Zar J.H. 1996. Biostatical Analysis. New Jersey, Prentice Hall, 389-92.

14. Silver LM. Mouse genetics. Concept and applications. New York: Oxford University Press: 1995.

15. Manly KF, Cudmore Jr RH, Meer JM. Map Manager QTX, cross-platform software for genetic mapping. *Mammalian Genome* 2001: 12: 930-932. http://www.mapmanager.org/mmQTX.html Date last accessed: November 1, 2005.

16. Churchill GA, and Doerge RW. Empirical threshold values for quantitative trait mapping. *Genetics* 1994: 138: 963-971.

17. Lander ES, and Kruglyak L. 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995: 11: 241-7

18. Kleeberger SR, Ohtsuka Y. Gene-particulate matter-health interactions. *Toxicol Appl Pharmacol* 2005:207(2Suppl):276-81.

19. Kleeberger, S. R., R.C. Levitt, L.Y. Zhang, M. Longphre, J. Harkema, A. Jedlicka, S. M. Eleff, D. DiSilvestre, K.J. Holroyd. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. Nat Genet 1997: 17: 475-8.

20. Haston CK, Travis EL. Murine susceptibility to radiation-induced pulmonary fibrosis is influenced by a genetic factor implicated in susceptibility to bleomycin-induced pulmonary fibrosis. *Cancer Research* 1997: 57:5286-91.

21. Haston CK, Wang M, Dejournett RE, Zhou X, Ni D, Gu X, King TM, Weil MM, Newman RA, Amos CI, Travis EL. Bleomycin hydrolase and a genetic locus within the MHC affect risk for pulmonary fibrosis in mice. *Hum Mol Genet* 2002: 11: 1855-63.

22. Kleeberger SR, Levitt RC, Zhang LY, Longphre M, Harkema J, Jedlicka A, Eleff SM, DiSilvestre D, Holroyd KJ. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nat Genet* 1997: 17: 475-8.

23. Ohtsuka Y, Brunson KJ, Jedlicka AE, Mitzner W, Clarke RW, Zhang LY, Eleff SM, Kleeberger SR. Genetic linkage analysis of susceptibility to particle exposure in mice. *Am J Repir Cell Mol Biol* 2000: 22: 574-81.

24. Haston C K, Zhou X, Gumbiner-Russo L, Irani R, Dejournett R, Gu X, Weil M, Amos CI, Travis EL. Universal and radiation-specific loci influence murine susceptibility to radiation-induced pulmonary fibrosis. *Cancer Research* 2002: 62: 3782-8.

25. Shaulian E, Schreiber M, Piu F, Beeche M, Wagner EF, Karin M. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 2000: 103:897-900.

26. Pilewski JM, Liu L, Henry AC, Knauer AV, Feghali-Bostwick CA. Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. *Am J Pathol* 2005: 166:399-407.

27. Yu C, Wang F, Jin C, Huang X, Miller DL, Basilico C, McKeeha WL. Role of fibroblast growth factor type 1 and 2 in carbon tetrachloride-induced hepatic injury and fibrogenesis. *Am J Pathol* 2003: 163:1653-62.

Figure Legends

Figure 1. The ratio of Hydroxyproline content of lung in 8 stains of mice 28 days after silica intra-tracheal instillation. Values are mean ± SE; number above bars, number of mice per group. *: There is significant increase in hydroxyproline compared to its own controls. $p<0.05$. Lines below this bar graph mean the results of post ad hoc multiple comparison tests. For example, the line, which begins from B6, shows that there are no significant differences between B6 and strains underlined. B6; C57BL/6J, B10; C57BL/10J, C3H; C3/HeJ, D2; DBA/2J, AKR; AKR/J, Balb; BALB/CJ, CBA; CBA/J

Figure 1

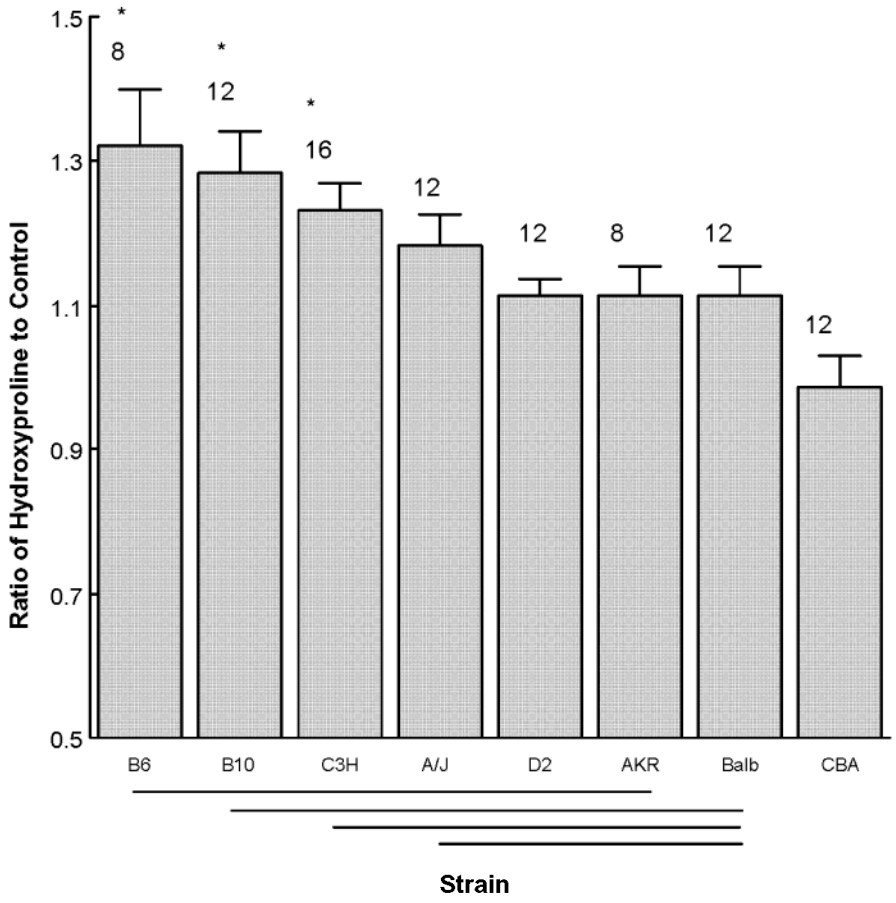

Ratio of Hydroxyproline to Control
1.5
1.3
1.1
0.9
0.7
0.5
*
8
*
12
*
16
12
12
8
12
12
B6
B10
C3H
A/J
D2
AKR
Balb
CBA
Strain

Figure 2. Fibrosis indices in 8strains of mice 28 days after silica intra-tracheal injection. Values are mean ± SE; number above bars, number of mice per group. . *: There is significant increase in fibrosis index compared to its own controls. $p<0.05$. Lines below this bar graph mean the results of post ad hoc multiple comparison tests. For example, the line, which begins from A/J, shows that there are no significant differences between B6 and strains underlined.

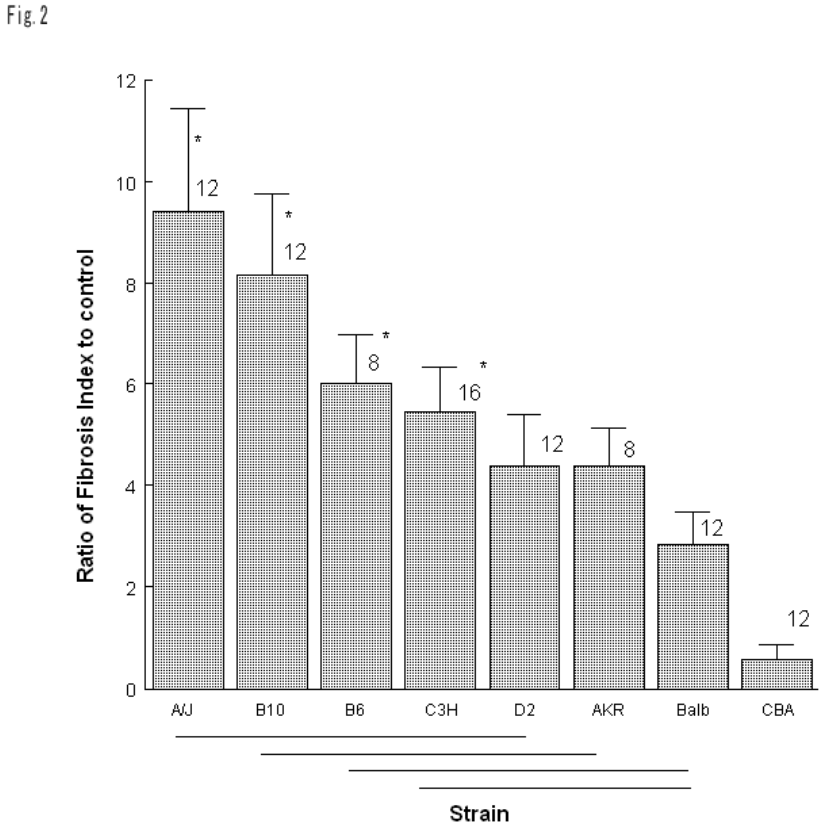
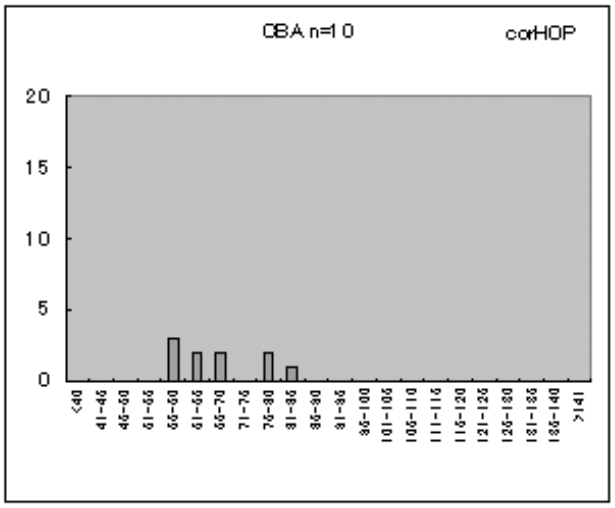
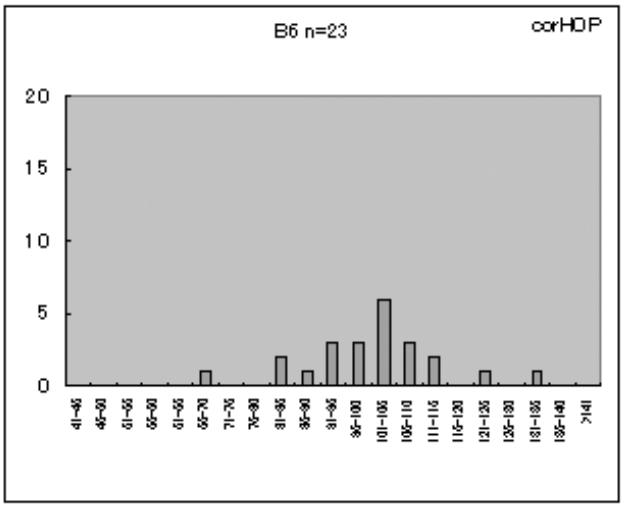
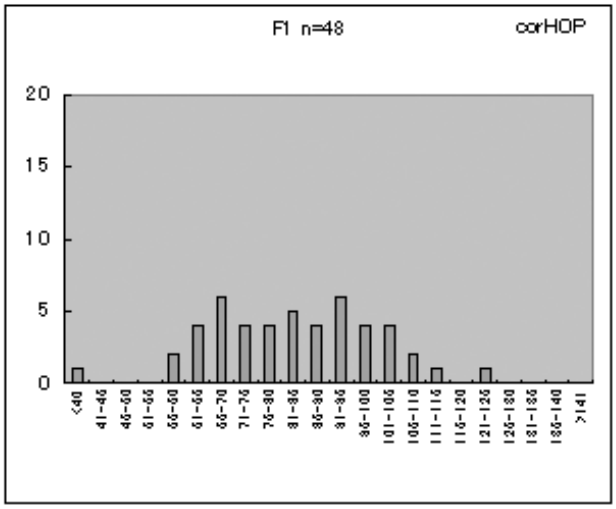


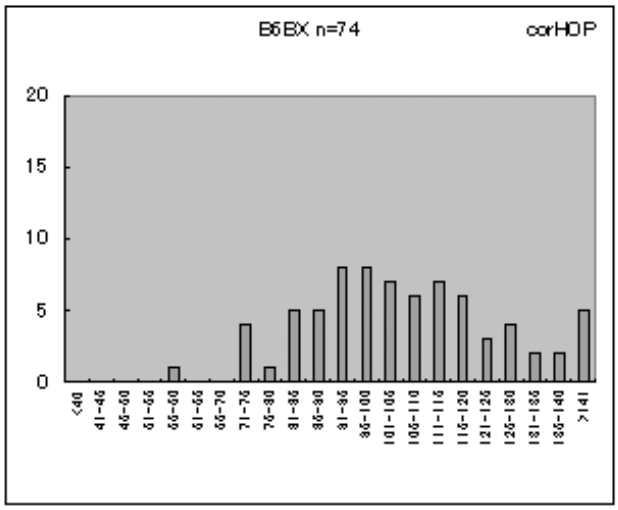
Figure 3. Frequency distribution of the hydroxyproline content per lung (% of control) from C57BL/6J and CBA/J mice and their progeny. Y axis reveals frequency. Male CBA/J mice

were bred with C57BL/6J female to produce B6CBAF1 hybrids. Male B6CBAF1 mice were bred with C57BL/6J female to produce B6:BX. B6CBAF1 were intercrossed to produce B6CBAF2 progeny.


CBA n=10
corHOP
20
15
10
5
0
<40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
>141


B6 n=23
corHOP
20
15
10
5
0
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
>141


F1 n=48
corHOP
20
15
10
5
0
<40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
>141


B6BX n=74
corHOP
20
15
10
5
0
<40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
>141

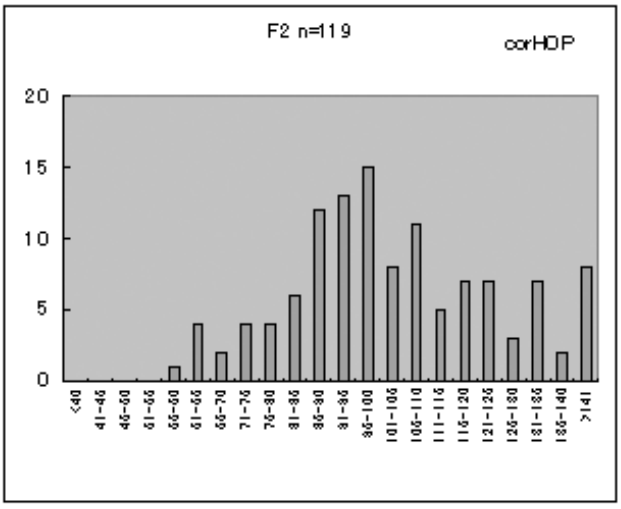

F2 n=119
corHOP
20
15
10
5
0
<40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
>141

Figure 4. A genome-wide search for QTLs by selective genotyping of the 25 F2 cohort (50meiosis), i.e., the most extreme high and low responders to silica intratracheal instillation. The x-axis for each plot is the length of chromosome in centimorgans. The y-axis is the likelihood ratio X2 statistic. The upper and lower horizontal lines in each plot represent significant and suggestive linkage thresholds, respectively. Putative QTLs on chromosomes 3, 4, 6, 10, 11, 14, and 18 that were identified by the genome scan were further analyzed with entire F2 cohort (see Methods).

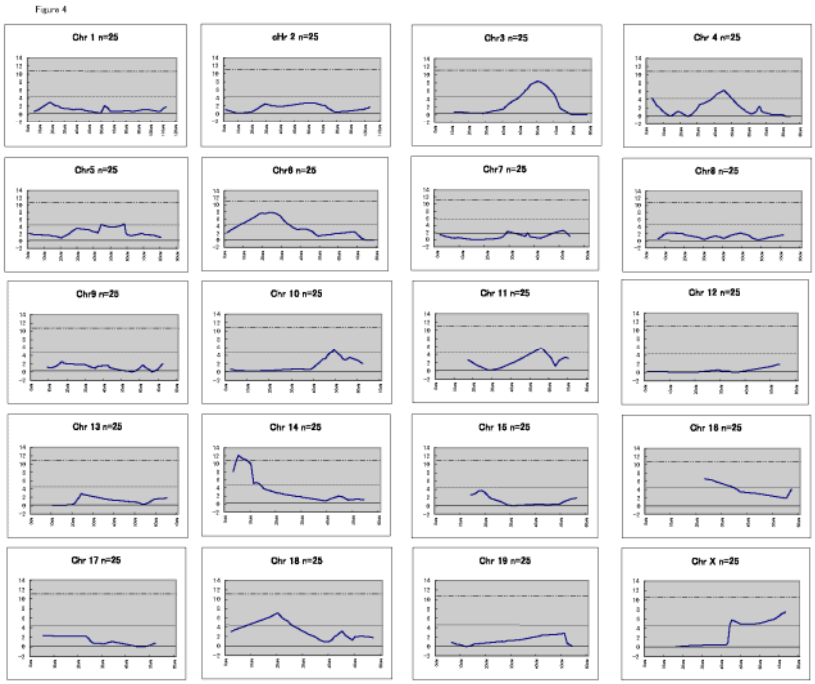


Figure 5. Plot of the likelihood ratio X 2 (khai-square) statistics for the association of silica-instilled hydroxyproline content of the lung (% of controls) phenotype with polymorphic SSLP markers on chromosomes 4, 3 and 18. Internal mappings were done by simple linear regression (see Methods). The upper and lower horizontal lines represent significant and suggestive linkage thresholds, respectively, as determined by permutation test (Map Manager QTX) [15].