Superoxide dismutase 3, extracellular (SOD3) variants and lung function

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1Institute of Lung Biology and Disease, Helmholtz Zentrum München, German Research Center for Environmental Health and 2University Children’s Hospital, Ludwig Maximilian University, Munich, Germany; 3Department of Environmental and Occupational Health and 4Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania; 5Centers for Environmental Genetics, University of Cincinnati, Cincinnati, Ohio; and 6Center for Pediatrics, Clinic for Pediatric Pneumology and Neonatology, Hannover Medical School, Hannover, Germany

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Ganguly K, Depner M, Fattman C, Bein K, Oury TD, Wesselkamper SC, Borchers MT, Schreiber M, Gao F, von Mutius E, Kabesch M, Leikauf GD, Schulz H. Superoxide dismutase 3, extracellular (SOD3) variants and lung function. Physiol Genomics 37: 260–267, 2009. First published March 24, 2009; doi:10.1152/physiolgenomics.90363.2008.—Polymorphisms in Superoxide dismutase 3, extracellular (SOD3) have been associated with reduced lung function and susceptibility to chronic obstructive pulmonary disease (COPD) in adults. Previously, we identified SOD3 as a contributing factor to altered ventilation efficiency (dead space volume/total lung capacity) in mice. Because SOD3 protects the extracellular matrix of the lung, we hypothesized that SOD3 variants also may influence postnatal lung function development. In this study, SOD3 transcript and protein localization were examined in mouse strains with differing ventilation efficiency [C3H/HeJ (high), JF1/Msf (low)] during postnatal development. Compared with C3H/HeJ mice, JF1/Msf mice had Sod3 promoter single nucleotide polymorphisms (SNPs) that could affect transcription factor binding sites and a decline in total lung SOD3 mRNA during postnatal development. In adult JF1/Msf mice, total lung SOD3 activity as well as SOD3 transcript and protein in airway epithelial and alveolar type II cells and the associated matrix decreased. In children (n = 1,555; age 9–11 yr), two common SOD3 SNPs, one located in the promoter region [C/T affecting a predicted aryl hydrocarbon receptor-xenobiotic response element (AhR-XRE) binding motif] and the other in exon 2 (Thr/Ala missense mutation), were associated with decreased forced expiratory volume in 1 s (FEV1), and the promoter SNP was associated with decreased maximal expiratory flow at 25% volume (MEF25). In vitro, a SOD3 promoter region-derived oligonucleotide containing the C variant was more effective in competing with the nuclear protein-binding capacity of a labeled probe than that containing the T variant. Along with the previous associated risk of lung function decline in COPD, these findings support a possible role of SOD3 variants in determining lung function in children.

complex trait; asthma; chronic obstructive pulmonary disease; lung development

Superoxide dismutase 3, extracellular (SOD3) is the primary extracellular antioxidant enzyme in the lung (14, 16) and protects the extracellular matrix during lung injury (2, 4, 20, 40). Recently, genetic variants in SOD3 have been associated with reduced lung function in adults (10) and lung function decline in chronic obstructive pulmonary disease (COPD) (25, 44, 45). Individual genetic differences influencing the development of lung structure and function may contribute to subsequent vulnerability to environmental stress and possibly the progression of chronic pulmonary diseases (6, 35, 37). Despite this apparent connection, little is known about the genetic factors linking unfavorable lung function development with increased susceptibility to lung diseases. We previously identified (34) quantitative trait loci (QTL) for dead space volume (Vd), total lung capacity (TLC), compliance, and diffusion capacity in C3H/HeJ and JF1/Msf mouse strains. Compared with C3H/HeJ mice, JF1/Msf mice had a greater percentage of TLC occupied by Vd (specific dead space volume: Vd/TLC) and thus reduced ventilation efficiency. Sod3 was identified as a candidate gene for Vd contained within the QTL on mouse chromosome (mCh) 5 (34). We also detected lower lung SOD3 transcript and protein levels in JF1/Msf mice compared with C3H/HeJ mice at the age of 4 wk (19). Previous studies had found that gene-targeted Sod3+/− mice are susceptible to lung injury (7, 15). In adult Sod3−/− mice, we found Vd/TLC to be similar to that of the JF1/Msf strain, which provided experimental evidence for the role of SOD3 in determining ventilation efficiency (19).

As in humans, mouse lung function continues to develop postnatally, and transgenic mice expressing increased SOD3 in the lung are protected from hyperoxia-induced alveolar damage during postnatal lung development (2, 4, 21, 30). Plasma SOD3 levels change in an age-dependent manner, peaking in early childhood as the lung develops (1). Diminished SOD3 levels during lung growth could provide a plausible link between unfavorable lung function development and subsequent vulnerability to disease. Hence, to understand the possible role of SOD3 in lung function development, we compared transcript levels of JF1/Msf and C3H/HeJ mice at various postnatal stages of lung development. To further assess the possible role of SOD3 in lung function development, we performed a single nucleotide polymorphism (SNP) association study examining lung function in children (9–11 yr). SOD3 consists of two exons (exon 2 being the entire coding region) and is expressed primarily in the lung, kidney, blood vessels, and cartilage (14, 16). The 5′-untranslated region of SOD3 contains various regulatory elements including aryl hydrocarbon receptor-xenobiotic response elements (AhR-XRE), antioxidant response elements (ARE), AP-1, and NF-κB motifs (16). The SOD3 protein (251 amino acids) exists as multimeric active and inactive folding variants (32). Therefore, we examined two common (heterozygosity ≥40%) tag SNPs located in the promoter region (C/T eliminating a pre-
dicted AhR-XRE binding motif) and exon 2 (Thr/Ala missense mutation) of SOD3.

METHODS

Mouse Procedures

Animals. Mice (C3H/HeJ and JF1/Msf strains, Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free conditions, and the procedures used were approved by the Bavarian Animal Research Authority.

Polymerase chain reaction. PCR amplification of the Sod3 promoter region using genomic DNA from C3H/HeJ and JF1/Msf mice (Jackson Laboratory) was performed and sequenced in forward and reverse directions (Sequiserve, Vaterstetten, Germany). The sequences of Sod3 promoter (913 bp starting −593 bp proximal to and spanning exon 1) were compared to identify SNPs that could affect transcription factor binding domains (Genomatix); pair 1, CCAGACCTGTGTTTATCC (FORWARD) and CTGCTACTCCGGTCTCCTTCTC (REV), pair 2, GAACGGCAGAGTTAGGAAAG (forward) and CCAACCCAGTTTCCAGAAC (reverse).

To determine SOD3 mRNA levels, lungs were removed from C3H/HeJ and JF1/Msf mice [n = 4 mice per strain per age; postnatal day (P)-1-P70] and immediately frozen in liquid nitrogen. Total RNA was isolated (Qiagen, Germany), quality confirmed by denaturing formaldehyde-agarose-SYBR Gold gel, and quantified (PeqLab NanoDrop Bioanalyzer). Quantitative real-time PCR (qRT-PCR) was used to determine SOD3 mRNA levels. Two micrograms of RNA from mouse lung (n = 4 mice per strain per age) was reverse transcribed into cDNA (Ready-To-Go T-primed First-Strand Kit, Amersham). PCR amplification of the SOD3 promoter region using genomic DNA from C3H/HeJ and JF1/Msf mice (Jackson Laboratory) was performed and sequenced in forward and reverse directions (Sequiserve, Vaterstetten, Germany). The sequences of Sod3 promoter (913 bp starting −593 bp proximal to and spanning exon 1) were compared to identify SNPs that could affect transcription factor binding domains (Genomatix); pair 1, CCAGACCTGTGTTTATCC (FORWARD) and CTGCTACTCCGGTCTCCTTCTC (REV), pair 2, GAACGGCAGAGTTAGGAAAG (forward) and CCAACCCAGTTTCCAGAAC (reverse).

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In situ hybridization and immunohistochemistry. To localize SOD3 mRNA and protein, mouse adult lungs (P70) were fixed (10% formaldehyde-agarose-SYBR Gold gel, and quantified (PeqLab NanoDrop Bioanalyzer). Quantitative real-time PCR (qRT-PCR) was used to determine SOD3 mRNA levels. Two micrograms of RNA from mouse lung (n = 4 mice per strain per age) was reverse transcribed into cDNA (Ready-To-Go T-primed First-Strand Kit, Amersham Biosciences, Freiburg, Germany), and 1 μl of cDNA was used in the PCR reaction [11.5 μl of Absolute qPCR SYBR Green ROX mix (ABgene, Hamburg, Germany), 1 μl of each primer, and 10.5 μl of RNase-free water]. qRT-PCR was performed (Applied Biosystems 7900HT System; 95°C for 15 min and 40 cycles of 95°C for 15 s, 60°C for 1 min) with the following primer pairs: SOD3 (39): forward GTGTCCCAAAGACAATC, reverse GTGCTATGGGGACAGG and ACTB: forward TCCATCATAGGTTGAGCCT, reverse GAGCAATGATCTG ATCTTCTTCA (Sigma-Genosys, Steinheim, Germany). SOD3 transcript levels determined with the 2−ΔΔCt method (where Ct is threshold cycle) were normalized to ACTB (27).

In situ hybridization and immunohistochemistry. To localize SOD3 mRNA and protein, mouse adult lungs (P70) were fixed (10% formalin, 4 h; 70% EtOH, 20 h), paraffin embedded, and sectioned (5 μm). We used in situ hybridization as described previously (22) with the above Sod3 qRT-PCR primer pair to generate digoxigenin-rUTP-labeled (Roche, Mannheim, Germany) RNA probes from linearized cDNA templates. The immunohistochemical method (described in Ref. 13) used deparaffinized sections incubated with 1% H2O2 in methanol to inactivate endogenous peroxidases, followed by antigen retrieval (DEKO, Hamburg, Germany). Sections were incubated (1 h) with protein-blocking solution to reduce nonspecific binding, polyclonal mouse SOD3 antibody (20 h, 4°C), and biotinylated goat anti-rabbit IgG and streptavidin-horseradish peroxidase (Jackson ImmunoResearch Europe). Primary and secondary antibody dilutions were determined empirically (1:500). The negative controls lacking primary antiserum showed no staining. Sections were incubated in ABC reagent (Vector Laboratories) to enhance staining and developed with diaminobenzidine (DAB, Vector Laboratories).

Analysis of SOD3 activity. Before removal, lungs from C3H/HeJ or JF1/Msf mice [n = 10 mice/strain] were perfused with 5 ml of phosphate-buffered saline through the right ventricle of the heart. The lungs were homogenized in 3 ml of a solution containing 50 mM KH2PO4, 0.3 M KBr (pH 7.4). After separation from SOD1 (CuZnSOD) and SOD2 (Mn-SOD) by concanavalin A-Sepharose chromatography (28), SOD3 activity was measured by inhibition of partially acetylated cytochrome c reduction at pH 10.0 as previously described (9). The total protein concentration in the homogenates was determined with the Coomassie Plus protein assay (Pierce, Rockford, IL).

Human Study

Population. This study was approved by the local ethics committees; the children who participated provided oral assent, and their parents provided written informed consent. The study was performed in Munich and Dresden as part of the International Study of Asthma and Allergy in Childhood phase II (ISAAC II) (43) (n = 5,629). DNA was available for 3,099 school children age 9–11 yr (European decent; all German). The characteristics of this population are presented in Supplemental Table S1.1

Phenotyping. Lung function was measured in a 50% subsample (MasterScope, Jäger, Würzburg, Germany) (n = 1,555). With a minimum of two spirometries, the greater of two reproducible (within 5%) measurements of forced expiratory volume in 1 s (FEV1) was recorded. In addition to FEV1, forced vital capacity (FVC), maximal expiratory flow at 75% (MEF75), maximal expiratory flow at 50% (MEF50), and maximal expiratory flow at 25% (MEF25) were determined, and all values were expressed as percentage predicted by internal reference values. The ratio FEV1/FVC was also calculated.

SNP selection and genotyping. SNPs were selected to tag common haplotypes in SOD3 and within the 2 kb upstream of its transcriptional start site. Ten SNPs genotyped were identified with HapMaps (18), and linkage disequilibrium (LD) was assessed (Haploview) (5). Four SNPs exceeded a minor allele frequency >0.10, with three SNPs being in tight LD (r2 = 1). Therefore, we included in the analysis the validated nonsynonymous SNP (rs2536512) and the promoter SNP (rs699473) as haplotype tagging SNPs for genotyping. Genomic DNA had been extracted from whole blood (29) and amplified (49). DNA was genotyped with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA) (11). SNPs were tested for deviation from Hardy-Weinberg equilibrium with the χ2-test, with expected frequencies derived from allele frequencies. Levels of lung function were analyzed with percentages of reference values. To test for differences between genotypes in lung function parameters, univariate variance analyses were performed with SAS (version 9.1.3). Associations between SNPs and dichotomous outcomes were assessed with a logistic regression model. Regression analysis for a linear model was used in which genotypes are coded according to the number of rare alleles (0-1-2) and as well as for a dummy coding, which is the same as using variance analyses. Bonferroni correction was performed for each lung function measurement. For the significant results, an adjusted model (adjusted for sex, asthma, and environmental tobacco smoke) was performed. Additionally, we stratified the two cities and repeated the analyses in each city to examine the homogeneity of the result. All tests were two sided, and differences were considered significant at p < 0.05.

Electrophoretic mobility shift assay. Nuclear protein extracts from NCI-H441 human lung cells (American Type Culture Collection (ATCC); HTB-174 grown in ATCC-formulated RPMI-1640 medium 30-2001 supplemented with 10% fetal bovine serum), prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Bio-technology, Rockford, IL), were subjected to electrophoretic mobility shift assay (EMSA). The binding reaction mixture containing 3.5 μg of nuclear extract, 2.25 ng of biotin-labeled oligonucleotide probe(s) (C-polymorphic), and 1× binding buffer [in mM: 12 HEPES, pH 7.9, 4 Tris-HCl, pH 7.9, 25 KCl, 50 MgCl2, 1 EDTA, 4 diithiothreitol, 0.2 phenylmethylsulfonyl fluoride, with 100 ng/μl poly(dI-dC) and 0.05% IGEPAL CA-630] was incubated at 15°C for 30 min in a final volume of 10 μl. Double-stranded oligonucleotides were prepared by annealing complementary synthetic oligonucleotides corresponding to the SOD3 promoter region −441 to −417 (47). The nucleotide sequence of the sense strands was C-polymorphic: forward AGCAGAATGC-

1 The outline version of this article contains supplemental material.
CACGCACATTGCAA; T-polymorphic: forward AGCAAATGC-
CATGCACATTGCAA.

For competition assays, 100-fold excess unlabeled double-stranded
variant oligonucleotides were incubated with the extracts (23°C, 30
min) before probe addition. Bound complexes were separated on 6%
polyacrylamide gels and blotted onto membrane. The blot was pro-
cessed with a streptavidin-horseradish peroxidase conjugate-based
detection method (LightShift Chemiluminescent Kit, Pierce Biotech-
nology). Chemiluminescence signal was detected by exposing the blot
to film.

RESULTS

JF1/Msf Mice Have Three Sod3 Promoter SNPs and
Decreased SOD3 mRNA Levels and Enzyme Activity

Previously, we identified (19, 34) a region on mCh 5
(containing Sod3) with linkage to increased VDS. Subsequent
exonwise Sod3 sequencing of these strains uncovered polymor-
phisms that could lead to nonsynonymous amino acid changes
and altered predicted protein structure (19). The promoter
region of Sod3 is complex, with no obvious TATA box but
with a highly purine-rich region from −208 to +104 containing
several overlapping putative Myeloid Zinc Finger-1 (MZF-
1), Kruppel-like factor (KLF), and Ets-family transcription
factor binding domains (46). Sequencing of the Sod3
promoter of JF1/Msf mice revealed three G → A SNPs within 100 bp
from the start codon (Supplemental Fig. S1). These SNPs
would eliminate cognition sites in the core sequences of
MZF-1, KLF, and Ets-family transcription factor binding do-
mains (8) previously determined to be essential for basal Sod3
transcription (46).

As in humans (48), lung function continues to develop as a
postnatal event in mice (3). We therefore compared at selected
times the postnatal lung SOD3 transcript levels of JF1/Msf
mice to those of C3H/HeJ mice (Fig. 1A) and determined that
the JF1/Msf strain had decreased SOD3 mRNA. This differ-
ence became greater with time and reached approximately
twofold during the second week (P14) onward, which is the
peak alveologenesis stage in mice (3). The difference became
greatest at P28 (~4-fold), when the development of the terminal
sacs into mature alveolar ducts and alveoli is completed.
SOD3 enzyme activity was approximately fourfold greater in
the lung of adult C3H/HeJ compared with JF1/Msf mice, consistent with the transcript expression levels (Fig. 1B).

SOD3 Transcript and Protein Localization

As has been noted previously, the expression pattern of
SOD3 transcript and protein in human lung is similar to that in
mouse lung (13, 17, 31, 38). To determine the location of the
decreased SOD3 transcripts within the adult C3H/HeJ and
JF1/Msf lungs (P70), we performed in situ hybridization using
tissue sections stained simultaneously. SOD3 transcripts in
airway epithelial and alveolar type II cells were significantly
decreased in the JF1/Msf lung compared with the C3H/HeJ
lung (Fig. 2). In addition, significant reduction of SOD3
immunoreactive staining was detected in the airway extracel-
lar matrix of JF1/Msf compared with that of C3H/HeJ mice
(Fig. 3). Staining typically occurred in discrete patches in
JF1/Msf lung compared with a continuous pattern in C3H/HeJ
lung. A substantial depletion of SOD3 in the alveolar paren-
chyma was also noted in JF1/Msf lung, which was particularly
evident in the alveolar septal tips and regions associated with
type II cells.

Fig. 1. A: temporal expression pattern of superoxide dismutase 3 extracellular (SOD3) transcript levels in C3H/HeJ and JF1/Msf mice. Expression of SOD3 mRNA in JF1/Msf decreased with time compared with C3H/HeJ mice. The difference was ~2-fold at postnatal day (P)14 and peaked at P28 (~4-fold), when the development of the terminal sacs into mature alveolar ducts and alveoli is completed. Quantitative real-time polymerase chain reaction was performed, and the comparative cycle number threshold (CT) method (ΔΔCT) was used [ΔΔCT = CT (gene) – CT (Actb)]. Data are presented as expression relative to time-matched C3H/HeJ level (means ± SE; n = 4 mice·strain ·day −1). *Significantly different from C3H/HeJ (1-way ANOVA followed by all pairwise multiple-comparison procedures with Holm-Sidak method, P = 0.05).

B: SOD3 activity in adult C3H/HeJ and JF1/Msf mouse lung. SOD3 activity in JF1/Msf mice was decreased (~4-fold) compared with C3H/HeJ mice (n = 10 mice·strain, 6–8 wk old). *Significantly different from C3H/HeJ (1-way ANOVA followed by all pairwise multiple-comparison procedures Tukey test, P ≤ 0.001).
SOD3 Gene Variants Are Associated with Decreased Lung Function in Children

Four SNPs exceeding a minor allele frequency of 0.1 exist within SOD3 and the associated 2-kb 5′-untranslated region. Two tag SNPs (rs699473 and rs2536512) capturing the genetic information of the SOD3 gene locus were genotyped in a cross-sectional study population of children [European (German) decent] from Munich and Dresden (n = 3,099). No significant deviations from Hardy-Weinberg equilibrium were observed, and observed allele frequencies were similar to those previously reported. (For example, the level of heterozygosity for rs699473 was 0.42 compared with the reported 0.49.)
Variants in both SNPs were statistically associated with decreased FEV₁, and rs699473 was associated with MEF₂₅ (Table 1). Homogeneity analysis by the pooled sample of \( n = 1,555 \) into two subgroups of Munich and Dresden children also revealed a consistent trend in both cities (Table 2). Interestingly, the association with decrease in FEV₁ was somewhat more pronounced in girls by \( \sim 1\% \) (Supplemental Table S2). Analysis of the predicted consequences of the promoter polymorphism (C/T in rs699473) on transcriptional binding partners was performed with the Transcription Element Search System (TESS) (36), and an AhR-XRE was identified. The binding motif TGGCAC/TGCAC requires a C nucleotide, and cognition would be lost with a T conversion (12). The non-synonymous SNP (rs2536512) would result in a threonine/alanine conversion that would replace a polar, hydrophilic with an aliphatic, hydrophobic amino acid at position 58 of the SOD₃ protein, which would eliminate a PKC delta motif. To examine whether the C/T variation could alter DNA-protein binding, we performed competitive electrophoretic mobility shift binding assay, using nuclear extract from NCI-H441 cells. Three shifted bands (Fig. 4, arrows) were detected. In the presence of 100-fold molar excess competitor oligonucleotide (C or T) the intensity of the two slower-migrating bands decreased. Interestingly, the intensity of the slowest-migrating shifted band (Fig. 4, top arrow) was stronger when the competitor had a T at nucleotide position \(-430\) compared with the oligonucleotide competitor containing a C. These observations suggest that the presence of a T at this specific site reduces the presence of 100-fold molar excess competitor oligonucleotide.

**DISCUSSION**

To begin to identify novel genetic loci for lung function in an experimental approach we previously surveyed several mouse strains and found that JF1/Msf and C3H/HeJ had the greatest difference in respiratory function. Using genomewide mouse strains and found that JF1/Msf and C3H/HeJ had the experimental approach we previously surveyed several cities (Table 2). Interestingly, the intensity of the two slower-migrating bands was decreased. Interestingly, the intensity of the slowest-migrating shifted band (Fig. 4, top arrow) was stronger when the competitor had a T at nucleotide position \(-430\) compared with the oligonucleotide competitor containing a C. These observations suggest that the presence of a T at this specific site reduces the nuclear protein binding capacity of the SOD₃ promoter region.

**Table 1. Regression analyses for SNPs in SOD₃ and lung function parameters in children aged 9–11 yr**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lung function parameters</th>
<th>Crude Model (lung function parameter = SNP)</th>
<th>Adjusted Model</th>
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<tbody>
<tr>
<td></td>
<td>Parameter Estimate</td>
<td>SE</td>
<td>P value additive model</td>
</tr>
<tr>
<td>SOD3_rs699473</td>
<td>FEV₁</td>
<td>-1.16</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>FVC</td>
<td>-0.26</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>MEF₂₅</td>
<td>-1.72</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>MEF₅₀</td>
<td>-0.82</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>MEF₇₅</td>
<td>-0.37</td>
<td>0.11</td>
</tr>
<tr>
<td>SOD3_rs2536512</td>
<td>FEV₁</td>
<td>-1.05</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>FVC</td>
<td>-0.61</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>MEF₂₅</td>
<td>-2.40</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>MEF₅₀</td>
<td>-1.72</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>MEF₇₅</td>
<td>-1.34</td>
<td>0.71</td>
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Adjusted model was adjusted for sex, asthma and environmental tobacco smoke; adjusted models were calculated for the additive model if the crude model was significant after Bonferroni correction. Indexes of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV₁), and maximal expiratory flow at 75% (MEF₅₀), 50% (MEF₇₅), and 25% (MEF₂₅) were expressed as % predicted by internal reference values. We also report the FEV₁-to-FVC ratio. SOD₃, superoxide dismutase 3, extracellular; SNP, single nucleotide polymorphism.
Table 2. Association of SNPs in SOD3 and decrease in % FEV1 and MEF25 in children aged 9–11 yr for pooled population or Dresden or Munich separately

<table>
<thead>
<tr>
<th>SNP Position</th>
<th>Genotype</th>
<th>n</th>
<th>P</th>
<th>D</th>
<th>M</th>
<th>Value</th>
<th>P value</th>
<th>Value</th>
<th>P value</th>
<th>Value</th>
<th>P value</th>
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<tbody>
<tr>
<td>rs699473-15472161 (−4341)</td>
<td>Wild type</td>
<td>705</td>
<td>447</td>
<td>258</td>
<td>100.54±0.38</td>
<td>0.0025</td>
<td>0.0015</td>
<td>100.36±0.51</td>
<td>0.1334/0.3218</td>
<td>100.84±0.59</td>
<td>0.0026/0.0091</td>
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<tr>
<td></td>
<td>Heterozygote</td>
<td>659</td>
<td>400</td>
<td>259</td>
<td>99.57±0.41</td>
<td>0.6379</td>
<td></td>
<td>99.67±0.53</td>
<td>0.9723</td>
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<td></td>
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<tr>
<td></td>
<td>Variant</td>
<td>191</td>
<td>111</td>
<td>80</td>
<td>98.04±0.75</td>
<td></td>
<td>0.3978</td>
<td>98.77±0.96</td>
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<td>0.9723</td>
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<tr>
<td>rs2536512-15476673 (377)</td>
<td>Wild type</td>
<td>667</td>
<td>426</td>
<td>241</td>
<td>100.50±0.40</td>
<td>0.0206/0.0064</td>
<td>100.29±0.52</td>
<td>0.3037/0.5894</td>
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<tr>
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<td>Heterozygote</td>
<td>676</td>
<td>406</td>
<td>270</td>
<td>99.68±0.40</td>
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<td>0.4171</td>
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<td>117</td>
<td>81</td>
<td>98.20±0.73</td>
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<td>99.25±0.94</td>
<td>0.9723</td>
<td>96.69±1.15</td>
<td>0.9723</td>
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</table>

Values for FEV1 and MEF25 are expressed as mean ± SE percentages predicted by internal reference values. Slight differences in numbers are explained by different numbers of missing values. P, pooled; D, Dresden; M, Munich. First P value under assumption of linear model, second under a dummy-coded model.

We then assessed the association of lung function in children to SNPs carrying essential genetic information of SOD3. We found two common SNPs (occurring ≥40% heterozygosity) in SOD3 to be associated with a diminished lung function, as determined by FEV1, and one SNP to be associated with diminished MEF25. FEV1 and MEF25 are measures of the performance of large and small conducting airways, which is consistent with the observed alteration in VDS in mice. Interestingly, SOD3 SNPs in children were not associated with FVC, a measure of lung size, a finding that is reflected in SOD3−/− and wild-type mice exhibiting similar TLCs (19). VDS is difficult to measure in children (especially in the sample size needed for this study), and FEV1 is of greater clinical significance in determining lung development. For example, Stern et al. (37) determined that poor airway function in infants (measured within the first 6 mo) led to diminished FEV1 at ages 11, 16, and 22 yr, indicating that diminished airway function in early infancy is a risk factor for airflow obstruction in young adults. Similarly, Burrows et al. (6) and Samet et al. (35) proposed that respiratory illness in childhood can lead to chronic airflow obstruction in adulthood.

The associations we detected involve two common SNPs, one in the promoter (rs699473) and the other in exonic (rs2536512) portions of SOD3. The promoter SNP (rs699473) in the human SOD3 could theoretically eliminate an AhR-XRE DNA binding site. Importantly, we established a functional role for the promoter SNP and found that an oligonucleotide containing the C variant was more effective at competing DNA-protein binding. Thus the allele associated with decreased lung function (the T variant) was less effective and thereby is expected to have diminished capacity to transactivate the gene. Although this is in need of further functional assessment, Hanlon et al. (23) predicted a role for AhR-XRE in SOD3 gene regulation in mouse cells. Jain et al. (24) reported AhR transcript expression in mouse airway epithelium and parenchyma commencing from embryonic day 13.5 (late pseudoglandular stage) onward, which is consistent with a possible role in development. In rats, high doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a high-affinity AHR ligand, arrested the transition from the canalicular to the saccular stage of lung development.
tions, rs10489030, heterozygosity = 0.14) that lies near SOD3 was linked to FEV1 by Wilk et al. (44) in a genomewide association analysis of a large cohort (Framingham Heart Study). This SNP was thought to be within the 3’ region of the SOD3 gene. However, it has since been localized to another gene, coiled-coil domain containing 149 (CCCD149). Nonetheless, rs10489030 is in very low LD with our association of separated by 43.5 kb, does not suggest a clear replication of the investigators identified an association between reduced FEV1 obtained by Dahl et al. (10). In two separate large cohorts, these investigation of this region. Supportive evidence for an association of SOD3 variants with FEV1 in adults has been obtained by Dahl et al. (10). In two separate large cohorts, these investigators identified an association between reduced FEV1 and two SNPs in noncoding exon 1 (rs8192287; heterozygosity: 0.11) and in intron 1 (rs8192288; heterozygosity: 0.09) (10). Homozygotes also had an adjusted hazard ratio for COPD hospitalization of 2.5 (1.0–5.9), independent of the other exon SOD3 SNP (rs1799895) that had been associated with lung function decline in smokers with COPD (25, 45). Interestingly, Dahl et al. (10) also reported an association of decreased FEV1 with the rs192287/rs8192288 genotype in nonsmokers, which is consistent with our findings in nonsmoking children. A noteworthy difference between our study and the previous studies is that the tag SNPs (rs699473 and rs2536512) we selected have a greater allelic frequency (~45% vs. ~10%) and thus could be relevant to more people. The selected promoter SNPs also have functional significance in that rs699473 altered DNA-protein binding in a predicted AhR-XRE binding domain (as noted above) and rs2536512 could lead to a disruptive amino acid substitution. The other exon SNP (rs1799895) in SOD3 previously associated with FEV1 decline in smokers with COPD is also relatively uncommon (heterozygosity = 0.02). Nonetheless, the rs1799895 association was found, while none was found with other antioxidant genes (i.e., SOD1:CuZn-SOD, SOD2:Mn-SOD, or catalase), and this association was reproduced in two studies (25, 45).

In summary, the protective role of SOD3 in the lung extracellular matrix has been demonstrated extensively in transgenic mice expressing human SOD3 (2, 4, 20). Similarly, gene-targeted Sod3−/− mice are sensitive to various forms of lung injury (7, 15) and have diminished ventilation efficiency similar to JFI/Msf mice (19). Thus genetic variants leading to loss of function or diminished expression appear critical to these phenotypes, and we propose that if such differences occurred early they could alter the course of lung development. Consistent with our theory, we detected lowered lung SOD3 mRNA expression levels during JFI/Msf mouse lung development. We localized the difference in SOD3 expression in adult mouse lungs to specific regions of the lung extracellular matrix and measured a decrease in lung SOD3 activity. Moreover, we also detected significant association of two tag SNPs (rs699473 and rs2536512) capturing essential genetic information of the SOD3 gene locus to be associated with a diminished lung function in children. The C to T conversion rs699473 SNP was associated with altered DNA-protein binding capacity. Although we cannot rule out whether other genetic variants in LD with our SNPs are functionally important, SOD3 has a protective role in the lung and several SNPs have been linked to decreased FEV1 in children and nonsmoking adults. Together these findings support a role of SOD3 in susceptibility for reduced lung function development.

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