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Leptin receptor polymorphisms and lung function decline in COPD

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ABSTRACT: Only a fraction of all smokers develop chronic obstructive pulmonary disease (COPD), suggesting a large role for genetic susceptibility. The leptin receptor (LEPR) is present in human lung tissue and may play a role in COPD pathogenesis. The present study examined the association between genetic variants in the *LEPR* gene and lung function decline in COPD.

In total, 429 European Americans were randomly selected from the National Heart Lung and Blood Institute Lung Health Study. 36 single nucleotide polymorphisms (SNPs) in *LEPR* were genotyped using the Illumina™ GoldenGate platform (Broad Institute, Cambridge, MA, USA). Mean annual decline in forced expiratory volume in 1 s % predicted over the 5-yr period was calculated using linear regression. Linear regression models were also used to adjust for potential confounders. In addition, *in vivo* expression of the receptor gene was assessed with immunohistochemistry on lungs from smoke-exposed inbred mice.

We identified significant associations ($p<0.05$) between lung function decline and 21 SNPs. Haplotype analyses confirmed several of these associations seen with individual markers. Immunohistochemistry results in inbred mice strains support a potential role of LEPR in COPD pathogenesis.

We identified genetic variants in the *LEPR* gene significantly associated with lung function decline in a population of smokers with COPD. Our results support a role for *LEPR* as a novel candidate gene for COPD.

KEYWORDS: Chronic obstructive pulmonary disease, leptin receptor, lung function decline, polymorphisms

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Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the USA and the fifth leading cause of death worldwide, and its prevalence is expected to increase in coming decades [1, 2]. The overwhelming majority of COPD is caused by environmental exposures. In the USA, this exposure is primarily cigarette smoke (CS); however, only 15% of all smokers develop COPD. This suggests a large role for genetic susceptibility.

In addition to its role in obesity (appetite suppression), leptin has been shown to have multiple other functions, including increasing sympathetic nerve activity, maintaining reproductive function, immunity, angiogenesis, preserving normal respiratory function in the presence of obesity and cell proliferation of tracheal epithelial cells and lung growth [3]. After adjustment for obesity, the leptin pathway has been associated with inflammatory markers as well as multiple inflammatory conditions, including cardiovascular disease [4, 5]. Its potential role in the systemic inflammatory response in patients with COPD is evident from the correlation of leptin with other inflammatory markers [6–8]. Leptin has been shown to directly stimulate phagocytic activity of macrophages and enhance endotoxin-induced production of tumour necrosis factor-α, interleukin (IL)-6, and IL-12, cytokines typically involved in COPD pathogenesis [9–12]. Conversely, deficiency in leptin or its receptor may predispose sufferers to both immunodeficiency and infection [10]. Serum leptin levels are elevated in exacerbations of COPD and have also been associated with bacterial pulmonary infections, which are associated with COPD exacerbations and decline in lung function [7, 13–15]. Leptin exerts multiple effects through its leptin receptor (LEPR), located on human chromosome 1p31. The LEPR is produced in several alternatively spliced forms that share extracellular and transmembrane domains but have varying cytoplasmic residues [16], and has a wide tissue distribution, including lung tissue [17].

We tested for association between genetic variants in the *LEPR* gene and lung function decline

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in a subset of the multicentre National Heart, Lung and Blood Institute (NHLBI)-supported Lung Health Study (LHS) cohort. In addition, the potential role of LEPR in COPD pathogenesis was further evaluated in smoke-exposed AKR/J mice, which display marked airspace enlargement by histological and morphometric criteria [18]. To determine whether the LEPR is differentially localised to resident lung cells under conditions that simulate human COPD, we performed immunohistochemistry on murine lung sections obtained from AKR/J mice exposed to 4 months of CS *versus* room air controls.

Identifying pathways and novel molecular targets that modify the clinical course of disease is fundamental to developing preventive strategies and novel therapies.

METHODS

Subjects

Subjects participating in the current study included 429 European Americans (EA) randomly selected from a group of 4,287 participants of the multi-centre NHLBI-supported LHS for whom DNA was available. Lung function was measured annually over 5 yrs and conducted according to American Thoracic Society guidelines using identical spirometers, software, procedures and reading centre personnel [19, 20]. The quality of the spirometry testing conducted by the technicians was monitored centrally throughout the testing and comparison of baseline spirometry measures showed good reproducibility with very small mean short-term intra-individual variations in forced expiratory volume in 1 s (FEV_1) [21]. Lung function data from annual visit 1 to annual visit 5 was used for the current analyses and has been shown to have a good linear fit in previous LHS analyses [19, 22]. Subjects with less than three annual lung function measurements were excluded from the analysis (n=15).

Single nucleotide polymorphism selection and genotyping

Single nucleotide polymorphisms (SNPs) representing the *LEPR* gene were selected from Goldenpath (genome.ucsc.edu) and/or National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov; Bethesda, MD, USA). Criteria included: 1) SNPs approximating inter-SNP distance as close to 5 kb as were available at the time of the dbSNP Build 124 (NCBI); 2) representation of SNPs in the promoter, coding and 3′-untranslated region; and 3) SNPs with acceptable design scores according to the Illumina Assay Design Tool for genotyping on the Illumina™ GoldenGate platform (Broad Institute, Cambridge, MA, USA). Priority for selecting SNPs included: 1) regulatory and coding SNPs; 2) highly polymorphic SNPs, preferably ⩾10% minor allele frequency; 3) validated SNPs; and 4) SNPs at intron/exon boundaries. A total of 36 *LEPR* SNPs spanning 228,294 bp on human chromosome 1p31 with an average inter-SNP distance of 6.53 kb (range 1.9–14 kb) are summarised in table 1.

Statistical analysis

Mean annual decline in lung function (post-bronchodilator FEV_1 % predicted) was calculated as a linear regression slope over the 5-yr study period. Linear regression models were used to adjust for potential confounders, including baseline characteristics, smoking history (pack-yrs), age, sex, FEV_1 % pred and airway reactivity (AR). AR was calculated as a quantitative measure, using the two-point slope [22]. In addition to adjusting for baseline characteristics, 1) change in body mass index (BMI), calculated as a linear regression slope and 2) smoking status at year 5, defined as ‘‘continuous smoker’’, ‘‘intermittent smoker’’, and ‘‘sustained quitter’’, were also analysed.

Forward and backward selection was used to develop a parsimonious model. Residuals from the regression were included in a genetic additive model and the most common homozygote genotype for each SNP served as the reference category. The interaction term for smoking and genetic effect were tested using PLINK (Boston, MA, USA) [23], while testing for quantitative interaction. Each SNP locus was evaluated for Hardy–Weinberg equilibrium. All analyses were performed

TABLE 1 Location, minor allele frequency, and type of selected *LEPR* single nucleotide polymorphisms (SNPs)

ID	SNP	Position	Region	Minor allele	MAF
1	rs7531867	65819567	Downstream	A	0.39
2	rs1805096	65814278	Coding exon (1019 P/P)	T	0.39
3	rs1892535	65809202	Intron	T	0.18
4	rs6588153	65804038	Intron	A	0.38
5	rs1938484	65793303	Intron	A	0.18
6	rs8179183	65787973	Coding exon (656 K/N)	C	0.18
7	rs3790419	65779130	Coding exon (343 S/S)	G	0.22
8	rs3828034	65774346	Intron (boundary)	C	0.19
9	rs12564626	65768563	Intron	A	0.45
10	rs10443259	65763371	Intron	A	0.28
11	rs6691346	65758418	Intron	A	0.28
12	rs4655680	65753490	Intron	T	0.29
13	rs1137100	65748462	Coding exon (109 K/R)	G	0.26
14	rs10889562	65742466	Intron	A	0.22
15	rs6702028	65731912	Intron	C	0.28
16	rs1782763	65719921	Intron	C	0.33
17	rs1171265	65715273	Intron	A	0.36
18	rs1171271	65710811	Intron	C	0.27
19	rs1782754	65705369	Intron	G	0.27
20	rs1171279	65700514	Intron	T	0.27
21	rs1171274	65692859	Intron	C	0.27
22	rs10889558	65688987	Intron	A	0.27
23	rs6694528	65675037	Intron	T	0.13
24	rs1327121	65669358	Intron	C	0.34
25	rs17412682	65664314	Intron	C	0.46
26	rs2025804	65658142	Intron	C	0.34
27	rs17127652	65647164	Intron	G	0.02
28	rs4655811	65635178	Intron	C	0.34
29	rs6657868	65625728	Intron	A	0.39
30	rs9436746	65620494	Intron	A	0.4
31	rs970468	65618511	Downstream	G	0.39
32	rs17097182	65615466	Downstream	T	0.05
33	rs1045895	65610002	3′ UTR	A	0.38
34	rs9436299	65604909	Intron	C	0.33
35	rs1327118	65597590	Promoter	C	0.46
36	rs10493377	65651840	Promoter	G	0.47

MAF: minor allele frequency; UTR: untranslated region.

TABLE 2 Patient characteristics

Subjects n	414
Baseline characteristics	
Mean age yrs	48.6±7.0
Male %	65.0
BMI kg·m^{-2}	25.4±3.6
Smoking pack-yrs	40.5±19.2
Pre-BD FEV_1 %	76.3±6.4
Post-BD FEV_1 %	79.2±6.2
Longitudinal characteristics at 5 yrs	
Smoking history %	
Continuous	50
Intermittent now smoking	12
Intermittent now quit	19
Sustained quitter	19
Post-BD ΔFEV_1 %·yr^{-1}	-0.74±1.63

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; BD: bronchodilator; FEV_1: forced expiratory volume in 1 s.

with StataSE, version 8.0 (Stata Corp, College Station, TX, USA) and PLINK [23].

Individual haplotypes were analysed using PLINK. Pairwise linkage disequilibrium (LD) based on the D′ statistic was measured using Haploview (Broad Institute) [24]. LD blocks were defined using their default algorithm [25]. Sliding windows of two to four adjacent SNPs were used to test for association. Haplotype estimates were computed using PHASE (Seattle, WA, USA) assuming no recombination [26].

Murine model

10-week-old AKR/J mice were exposed to CS 5 h·day^{-1}, 5 days·week^{-1}, for 6 months. The exposure was conducted by burning 2R4F reference cigarettes (University of Kentucky, Tobacco Research Institute, Lexington, KY, USA), using a TE-10 smoking machine (Teague Enterprises, Woodland, CA, USA). Each cigarette was puffed for 2 s, once every minute for a total of eight puffs, at a flow rate of 1.05 L·min^{-1}, to provide a standard puff of 35 cm^3. The smoke machine was adjusted to produce a mixture of sidestream smoke (89%) and mainstream smoke (11%). The smoke chamber was monitored daily for total suspended particles and carbon monoxide, with concentrations of 90 mg·m^{-3} and 350 ppm, respectively. Air-exposed control mice were housed in a filtered air environment.

Immunohistochemistry

5-μm paraformaldehyde-fixed, paraffin-embedded tissue sections from smoke-exposed and room air control AKR/J mice were deparaffinised and rehydrated in an ethanol series. Sections were blocked for nonspecific binding with 3% normal serum from chicken and incubated with the primary antibodies for 1 h at room temperature. Following incubation with the primary antibody overnight at 4°C, slides were washed with PBS Tween-20, incubated for 30 min at room temperature with an appropriate biotinylated secondary antibody and developed by using 3,3′-diaminobenzidine substrate and chromagen from Dako (Stockport, UK). Antigen retrieval was performed using citrate buffer for 30 min. Antibodies were used at the following concentrations: LEPR, ObR (goat polyclonal, I-17, 1:100; Santa Cruz Biotechnology, Santa Cruz, AZ, USA), donkey anti-goat secondary antibody (sc2020, 1:400, Santa Cruz Biotechnology).

TABLE 3 Association of *LEPR* polymorphisms with lung function decline in a subset of the Lung Health Study European American cohort#

LD block	SNP ID	SNP	β	95% CI	p-value
1	1	rs7531867	0.298	-0.111–0.298	**0.006**
	2	rs1805096	0.295	-0.082–0.339	**0.007**
2	3	rs1892535	0.314	-0.008–0.435	**0.018**
	4	rs6588153	0.292	-0.381–0.039	**0.007**
	5	rs1938484	0.351	-0.459–0.469	**0.007**
	6	rs8179183	0.109	-0.014–0.410	0.429
3	7	rs3790419	-0.09	0.013–0.432	0.473
	8	rs3828034	0.026	-0.008–0.419	0.848
	9	rs12564626	0.25	-0.004–0.435	**0.015**
	10	rs10443259	0.334	-1.011–0.509	**0.004**
	11	rs6691346	0.334	0.039–0.477	**0.004**
	12	rs4655680	0.312	-0.436– -0.027	**0.007**
	13	rs1137100	0.33	0.019–0.456	**0.005**
	14	rs10889562	-0.044	-0.350–0.277	0.73
	15	rs6702028	0.32	0.085–0.538	**0.006**
	16	rs1782763	0.318	0.111–0.565	**0.003**
	17	rs1171265	0.321	-0.329–0.138	**0.004**
	18	rs1171271	0.32	0.091–0.543	**0.006**
	19	rs1782754	0.317	0.093–0.547	**0.006**
	20	rs1171279	-0.095	0.105–0.537	0.425
4	21	rs1171274	0.338	0.106–0.529	**0.004**
	22	rs10889558	0.311	0.093–0.547	**0.007**
	23	rs6694528	-0.037	-0.296–0.207	0.818
	24	rs1327121	0.238	0.103–0.557	**0.033**
	25	rs17412682	-0.232	0.087–0.537	**0.027**
	26	rs2025804	0.258	0.106–0.562	**0.022**
	27	rs17127652	-0.251	0.106–0.562	0.518
5	28	rs4655811	0.216	0.049–0.452	0.055
	29	rs6657868	0.206	-0.240–0.292	0.06
	30	rs9436746	0.223	-0.334–0.155	**0.039**
6	31	rs970468	0.198	-0.161–0.378	0.068
	32	rs17097182	0.005	0.096–0.607	0.983
	33	rs1045895	-0.171	0.082–0.502	0.111
	34	rs9436299	0.214	0.056–0.572	0.059
	35	rs1327118	0.129	0.084–0.507	0.231
	36	rs10493377	0.093	0.088–0.508	0.373

Examination of the linkage disequilibrium (LD) structure across these single nucleotide polymorphisms (SNPs) revealed high LD (D′ range 0.80–1.0, with R^2 range 0.65–0.96) for 31 of 35 pairs of contiguous SNPs (fig. 1). In fact, 33 of the SNPs fell into one of six blocks, and most of the 21 SNPs with association signal are noted to have similar effect sizes for the minor allele and fall into one of three LD blocks (2, 3 and 4). CI: confidence interval. Bold p-values are statistically significant. #: n=414.

RESULTS

Clinical characteristics of the subjects are presented in table 2. There were no statistical differences in baseline characteristics between those included in the final analyses (n=414)

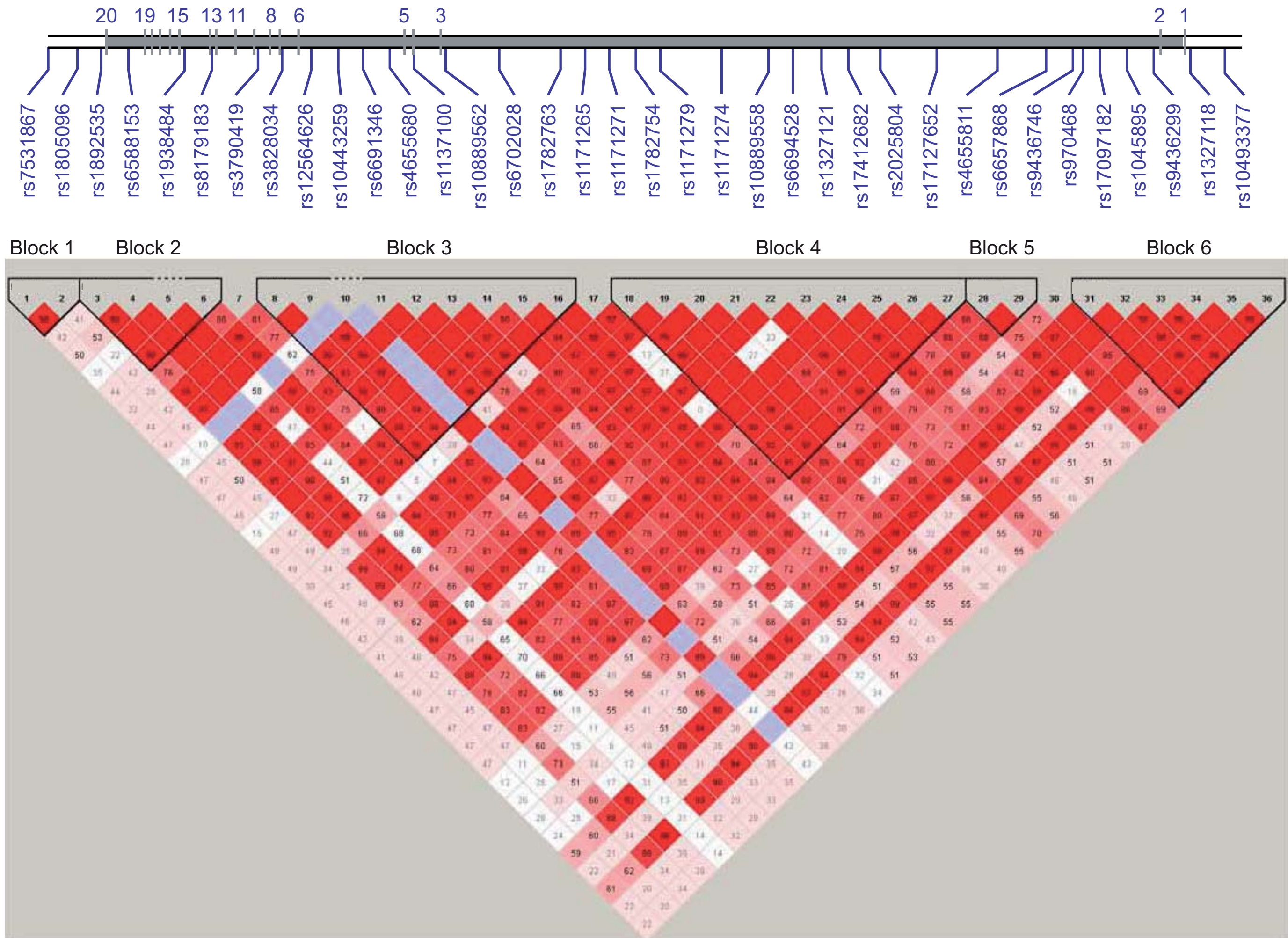

FIGURE 1. Pairwise linkage disequilibrium (LD) in subjects is represented as red squares for strong LD, blue squares for nonsignificant LD, and white squares for little or no LD. LD blocks are identified as noted.

compared with those excluded because more than two data points in lung function were missing (n=15, data not shown). Distributions of baseline characteristics and lung function measurements were also similar to those in the full LHS cohort from which this subset was selected (n=5,887, data not shown). Age, baseline lung function, AR, smoking status at year 5, and change in BMI were independently associated with lung function decline, and were included in the final regression analyses. There was no significant interaction between smoking status at year 5 and LEPR polymorphisms.

Single-marker analyses

All 36 *LEPR* SNPs were in the Hardy–Weinberg equilibrium. All results of the two-point tests for association between *LEPR* markers and FEV1 % pred decline are presented in table 3. We found evidence for significant associations between 21 SNPs in the *LEPR* gene and FEV1 % pred decline, spanning the length of the gene from intron 2 to the 5′ end. The minor alleles of most significant SNPs were associated with attenuation in lung function decline. Of particular interest, each G allele at the functional marker rs1137100, in exon 4, which creates a lysine to arginine amino acid change, was associated with a 0.33%·yr^{-1} attenuation in annual loss of FEV1 % pred.

Haplotype analyses

Considering the clustering of most genotyped SNPs within six LD blocks with high levels of LD also observed between SNPs across different blocks, a systematic sliding window approach was implemented, considering windows of two to four SNPs/window beginning with the first (5′) marker, and working across the gene, one marker at a time. Haplotype tests revealed multiple association signals in three specific regions that overlapped with the single-SNP results described above. 25 haplotypes showed stronger association than single SNP results (fig. 2). One of the most compelling regions was at the 5′ end of *LEPR* (region 1) and the haplotype GCCT (rs7531867, rs1805096, rs1892535 and rs6691346), which revealed a strong association with lung function decline (p=0.003) and includes the coding-synonymous SNP, rs1805096. Furthermore, one 3-SNP and two 4-SNP window haplotypes in region 2 spanning rs10443259 to rs10889562 (introns 2 through 5) were most strongly associated with lung

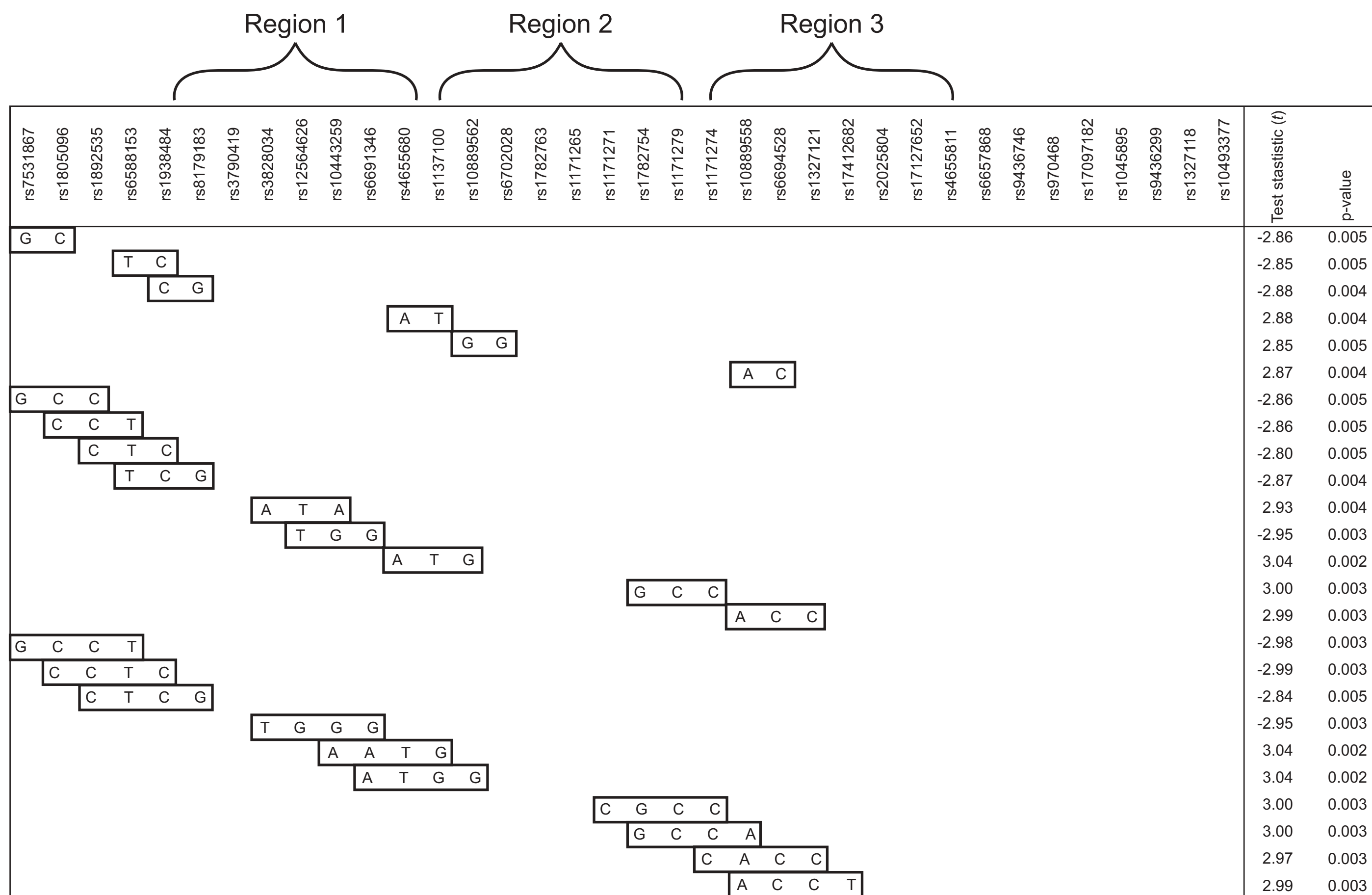

FIGURE 2. Haplotypes (n=25) that show greater association than single nucleotide polymorphism results.

function decline (p=0.002), and included the functional marker rs1137100, as described previously.

Validation of LEPR *as a candidate gene for COPD phenotypes in inbred mice*

AKR/J mice, a well-characterised inbred strain, exposed to 4 months of CS not only exhibit airspace enlargement but also develop airway thickening and inflammation that is highly reminiscent of COPD [18]. We examined whether CS exposure altered LEPR expression in the lungs of this strain. Using immunohistochemistry, we observed that LEPR expression was evident in macrophages, the airways and airspace compartments in room air-exposed AKR/J mice. However, upon smoke exposure, there was a reduction of staining in the airspace and airway wall (fig. 3). These findings are consistent with CS-induced downregulation of LEPR expression in the epithelial compartments of CS-exposed mice.

DISCUSSION

We identified 21 SNPs in the *LEPR* gene that were significantly associated with lung function decline in an EA population with COPD and our haplotype analysis supported results from SNP analysis. The association signals observed across these SNPs likely represent two or three signals in the gene. Furthermore, the potential role of the LEPR in lung architecture and COPD phenotypes is supported by an AKR/J murine model showing decreased *LEPR* expression in airway wall and epithelium after smoke exposure. Our results identify *LEPR* as a novel candidate gene for COPD.

To our knowledge, no previous studies have investigated the role of genetic polymorphisms in the *LEPR* gene and COPD or its associated phenotypes. The phenotype that we studied was rate of decline of lung function rather than COPD *per se*. The rationale for this was that rate of decline in lung function is a more precise phenotype than a single cross-sectional measurement of lung function needed to define COPD, and may serve as a more genetically homogeneous phenotype. We identified 21 SNPs in the *LEPR* gene that were significantly associated with lung function decline in a EA population with COPD. One of the most compelling regions was at the 5′ end of LEPR (region 1), which includes part of the intracellular domain of the receptor [16] and may result in different signalling potency. Most variants identified in *LEPR* were associated with an attenuation of lung function decline, and notably, the G variant at the nonsynonymous SNP rs1137100, which codes for an amino acid substitution in the extracellular domain of the LEPR [27], was associated with a 0.33%$\cdot$yr^{-1} attenuation in loss of FEV_1 % pred. Airflow obstruction that occurs in COPD is caused by a mixture of small airway disease, parenchymal destruction and increased airways responsiveness that develops over decades [28]. Thus, over time, these genetic variants may have a substantial impact on disease progression. Over a 40-yr period, a person homozygous for the G allele at

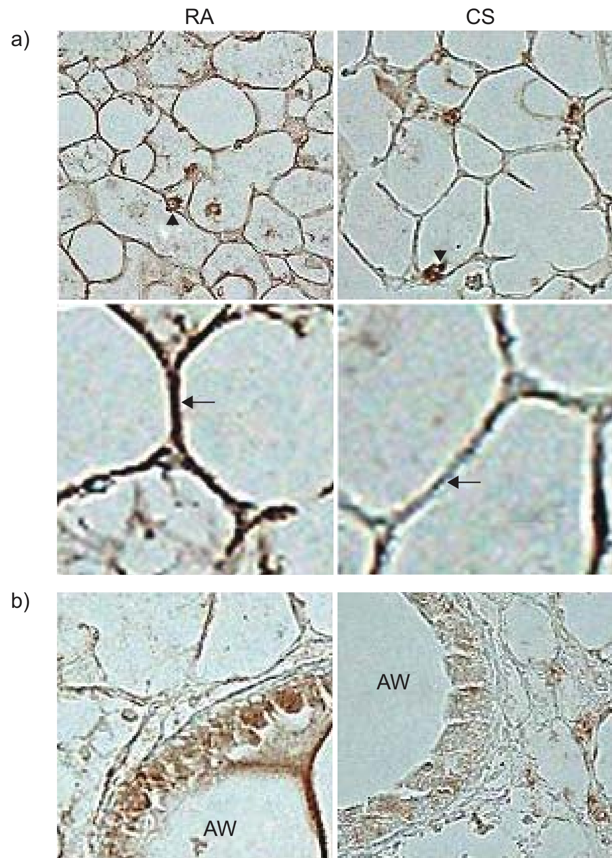

FIGURE 3. Reduced leptin receptor expression in lung parenchyma of AKR/J mice exposed to 4 months of cigarette smoke (CS) or room air. Representative images of a minimum of three mice per condition. a) Decreased immunohistochemical staining of leptin receptor (brown) in alveolar walls of smoke-exposed mice using polyclonal antibody against ObR and avidin-biotin-peroxidase complex method. Arrowheads denote preserved leptin receptor staining of alveolar macrophages after CS exposure. Arrows show reduced airspace wall staining in smoke-exposed mice. b) Reduced leptin receptor expression is also evident in airway epithelial cells of smoke-exposed mice. RA: room air exposed; AW: airway lumen.

rs1137100 will have a FEV1 % pred that is 26.4% higher compared with a homozygote for the major allele. Given a minor allele frequency of 0.26, as seen in our cohort, we might extrapolate 7% of the population to be homozygote for the minor allele (GG), 38% to be heterozygote (AG) and 55% to be homozygote for the major allele (AA). Even after adjusting for smoking status, this genetic variant could explain the difference between mild and moderate or moderate and very severe COPD under current Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [29].

The dearth of well-characterised populations of COPD subjects with longitudinal lung function data comparable to the LHS adds to the uniqueness of our population, but limits the opportunity to replicate these results. However, finding multiple significant SNPs in a single gene reduces the likelihood of spurious results due to multiple testing, though it may also be due in part to the high degree of LD in this gene. Furthermore, when using a false discovery rate of 0.2, six SNPs would remain statistically significantly associated with lung function decline ($p<0.006$) in the present study. Previous studies have shown genetic variants in *LEPR* to be associated with markers of inflammation, including C-reactive protein (CRP) and fibrinogen levels, lending support to the hypothesis that the leptin pathway has a physiological influence on inflammatory traits. Specifically, the minor allele (T) for the rs1805096 locus was associated with lower levels of fibrinogen, CRP and IL-6 levels in a previous study of healthy EAs [30]. Both lower CRP and IL-6 levels have been associated with attenuated lung function decline in the LHS cohort [31]. Interestingly, the minor allele (T) in the coding SNP rs1805096 was also associated with lower rates of lung function decline in our study (0.295 $\%\cdot yr^{-1}$; $p=0.007$). Therefore, we hypothesise one potential mechanism of the disease-modifying effects of the *LEPR* gene may be mediated through inflammatory mechanisms. The notion that leptin may function as an immunomodulatory cytokine has become increasingly accepted. The association between leptin and muscle wasting and cachexia in COPD is well established [32]; and the presence of leptin in induced sputum of patients with moderate COPD and its association with other inflammatory markers [33] suggests leptin may be involved in the local inflammatory response in COPD.

Inbred mice or guinea pigs subjected to chronic CS exposure have been shown to be invaluable models of CS-induced parenchymal lung disease [34, 35]. Furthermore, the use of animal models of complex human diseases to parse candidate genes identified in broad genetic or genomic surveys is a standard approach to initial pathway validation, especially if additional populations are not available for replication and therapeutic targets are an ostensible goal [36, 37]. We noted a reduction in staining in the airspace wall and airway epithelial compartment, but retained expression in macrophages in AKR/J smoke-exposed mice. This is consistent with findings of BRUNO *et al.* [13] who found decreased expression of leptin and its receptor in smokers and subjects with mild to severe COPD as compared with healthy nonsmoking subjects. Accordingly, reduced leptin receptor expression (acquired or genetic) may be a critical predisposing factor to CS-induced lung disease.

In summary, the current study is the first to report an association between *LEPR* polymorphisms and COPD. Our results support the role of the leptin pathway, and particularly the *LEPR* in COPD and lung function decline. Specifically, individuals with the minor allele at the selected polymorphisms were less susceptible to loss of lung function and COPD progression. The SNP and haplotype tests point to three clusters of signal highlighting two potential loci considering the linkage disequilibrium between these signals: the functional SNP rs137100 and at the synonymous coding SNP rs1805096. While these two SNPs are not in LD with each other ($D'=0.45$, $R^2=0.125$), haplotypes of significance that include each SNP overlap neighbouring LD blocks, making it somewhat difficult to tease apart these two signals. It is necessary to replicate our findings in other populations; however, our results identify *LEPR* as a novel target in COPD and lung function loss.

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STATEMENT OF INTEREST
None declared.

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