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ORIGINAL ARTICLE

Association of *IREB2* and *CHRNA3/5* polymorphisms with COPD and COPD-related phenotypes in a Chinese Han population

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Genome-wide association studies and integrative genomics approaches have demonstrated significant associations between chronic obstructive pulmonary disease (COPD) and single-nucleotide polymorphisms (SNPs) in the chromosome 15q25 region that includes iron-responsive element binding protein 2 gene (*IREB2*) and *CHRNA3/5* in non-Asian populations. We investigated whether *IREB2* and *CHRNA3/5* polymorphisms would be associated with COPD susceptibility and COPD-related phenotypes in a Chinese Han population. Eight SNPs (rs2568494, rs2656069, rs10851906, rs1964678, rs12593229, rs965604, rs13180, rs17483929) in *IREB2* gene and four SNPs (rs16969968, rs1051730, rs938682, rs8034191) in or near *CHRNA3/5* locus were genotyped in a case–control study (680 COPD patients and 687 controls). No significant associations were found between any of the SNPs and COPD in either former-smokers or current-smokers. Two SNPs (rs2656069 and rs10851906) in *IREB2* were associated with COPD ($P = 0.045$ and 0.032, respectively) in non-smoker. Four SNPs (rs1964678, rs12593229, rs965604 and rs13180) in *IREB2* were associated with forced expiratory volume in 1 s (FEV_1)% predicted and three SNPs (rs16969968, rs8034191 and rs1051730) in *CHRNA3/5* were both associated with FEV_1% predicted and FEV_1/FVC in COPD cases (P range 0.007–0.050). The SNP rs8034191 near *CHRNA3/5* locus was significantly associated with pack-years of smoking in COPD patients ($P = 0.033$). We demonstrated *IREB2* polymorphisms were associated with COPD in non-smoking subjects, and the effect of *IREB2* gene on COPD may be independent from smoking and independent from *CHRNA3/5* gene cluster. Besides, we confirmed that SNPs in these two gene loci were associated with pulmonary function and *CHRNA3/5* polymorphism was associated with pack-year of smoking in COPD patients in the Chinese Han population.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide and results in an economic and social burden, which is both substantial and still increasing.[1] COPD is a complex human disease, associated with persistent airway inflammation, protease-anti-protease imbalance, oxidative stress, chronic obstructive bronchitis and emphysema, resulting in progressive airflow limitation that is not fully reversed by bronchodilators.[2] Although smoking is a significant environmental cause of COPD, there is considerable variability in the susceptibility of smokers to develop COPD, and non-smokers can also get the disease even after eliminating the influence of passive smoking. These indicate that the genetic factors might contribute to the individual susceptibility. To date, the only proven genetic risk factor for COPD is severe deficiency of α1-antitrypsin, which is present in only 1–2% of individuals with COPD.[3,4]

Genome-wide association studies (GWAS) have revolutionized the identification of susceptibility genes for complex diseases. Recently, GWAS and integrative genomic approaches, which combine gene expression data with association studies, demonstrated that single-nucleotide polymorphisms (SNPs) in a region of chromosome 15q25 were significantly associated with COPD;[5,6] this region contains several genes, including the nicotinic acetylcholine receptor genes (*CHRNA3* and *CHRNA5*) and the iron-responsive element binding protein 2 gene (*IREB2*). The polymorphisms at the two loci have also been reported to be associated with COPD-related phenotypes, such as pulmonary function, smoking behavior and emphysema.[7–10] However, all of the GWAS were conducted in non-Asian

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populations. Given the large differences in genetic background of different ethnic populations, replication studies in other populations are warranted to evaluate the association between these polymorphisms and COPD. Besides, the high levels of linkage disequilibrium (LD) in the chromosome 15q25 region make it difficult to identify the specific functional variant or gene, which is underlying the observed association.

The primary aim of the present study was to investigate the associations between SNPs at the *IREB2* gene and *CHRNA3/5* locus and COPD and COPD-related phenotypes in a Chinese Han population. In addition, by stratified analysis and analyses of COPD-related phenotypes, we also hoped to better differentiate which was the real functional polymorphism or gene in the region.

MATERIALS AND METHODS

Subjects

A total of 680 COPD patients and 687 control subjects were included in this case–control association study. All cases and controls were unrelated individuals and from a southwestern Chinese Han population. Approval for the study was obtained from China's Ministry of Health and the Institutional Review Board of the West China Hospital of Sichuan University. Written informed consent was obtained from all subjects.

Inclusion criteria for COPD subjects were as follows: age $\geqslant$40, physician-diagnosed COPD, pulmonary function test showing post-bronchodilator forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) of $<$70% and FEV_1 of $<$80% predicted.[1] Patients were excluded from the study if they had an established diagnosis of asthma, lung cancer, a history of atopy and known AAT deficiency. Patients with acute exacerbations 4 weeks preceding study assessment were also excluded. Disease severity was classified according to the criteria of Global Initiative for Chronic Obstructive Lung Disease (GOLD).[1] Inclusion criteria for control patients were age $\geqslant$40 and normal pulmonary function, FEV_1 predicted $\geqslant$80% and FEV_1/FVC% $\geqslant$70%. Exclusion criteria for controls were as described for cases and also included a family history of COPD. Efforts were made to match cases by age, gender and smoking history.

SNPs selection and Genotyping

Eight SNPs (rs2568494, rs2656069, rs10851906, rs1964678, rs12593229, rs965604, rs13180, rs17483929) in *IREB2* gene and four SNPs in (rs16969968, rs1051730, rs938682) or near (rs8034191) *CHRNA3/5* locus were chosen, which were all in the region of chromosome 15q25 and were found to be significantly associated with COPD and COPD-related phenotypes in non-Asian populations by recent GWAS and integrative genomic approaches.[5–7,11]

Genomic DNA was extracted from blood using the commercially extraction kit (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. Genotyping was carried out commercially by BGI (Shenzhen, China) using Sequenom's iPLEX SNP genotyping protocol developed for measurement with the MassARRAY mass spectrometer (Sequenom, San Diego, CA, USA).[12] Genotyping was blind to case or control status of samples. As a quality control measure, $\sim$5% of samples were genotyped in duplicate to check for concordance. In addition, a selection of samples were also genotyped using restriction enzyme digestion or direct sequencing to confirm the genotyping results from BGI.

Statistical analysis

Hardy–Weinberg equilibrium for all SNPs was assessed in control subjects by using a goodness-of-fit χ^2-test. The differences of allele frequencies between cases and controls were tested by χ^2-test. Logistic regression analyses were performed to test the association between each SNP with COPD case/control status, adjusting for age, gender, body mass index (BMI), pack-years of smoking and current smoking status. In addition to the overall analysis, we also carried out a smoking status-stratified analysis. Linear regression analyses were performed to assess the relationship between SNPs and quantitative phenotypes, such as pulmonary function, pack-years of smoking and BMI among COPD cases only and the entire cohort. SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) was used in statistical evaluation of the above data.

LD structure in the *CHRNA3/5* and *IREB2* region was examined with the program Haploview 4.2 (Broad Institute of MIT and Harvard, Boston, MA, USA) and haplotype analysis was also conducted using the same software.[13]

In every case, a two-sided *P*-value of $<$0.05 was considered statistically significant.

RESULTS

Demographic characteristics and results of quality control

Table 1 summarizes the demographic data and baseline characteristics of the study groups. Despite attempts to match cases and controls there were significant differences observed for age, current smoking status and pack-years for ever-smokers and adjustments were made by logistic regression and linear regression to take this into account in the statistical analysis.

The locations and the characteristics of the 12 SNPs genotyped are summarized in Table 2. The overall genotype call rate was 99.93% (range, 99.56–100%) and the accuracy was 100% according to duplicate genotyping of 5% of samples (69 samples and 828 duplicate genotyping reactions). The samples genotyped by alternative methods were 100% concordant, satisfying criteria for the assays to be accepted for further analysis. There were no deviations from Hardy–Weinberg equilibrium for any of the genotyped SNPs in control subjects (Table 2).

Association analysis of COPD susceptibility

Individual SNP association analysis. The allele frequencies for SNPs in cases and controls are shown in Table 2. None of these SNPs showed significant associations with COPD in the crude analysis. Table 3 compares the genotype frequencies between cases and controls for the 12 SNPs analyzed. There were no significant differences in

Table 1 Characteristics of COPD patients and control subjects

	COPD patients (n = *680*)	*Controls* (n = *678*)	P-*value*
Male (%)	483 (71.0)	476 (69.3)	0.481[a]
Age (±s.d.)	62.74 (±9.08)	60.90 (±10.21)	0.002[b]
BMI (±s.d.)	22.45 (±3.49)	22.63 (±3.32)	0.085[b]
Current smoking status			
Non-smoker (%)	225 (33.1)	231 (33.6)	0.028[a]
Former smoker (%)	204 (30.0)	165 (24.0)	
Current smoker (%)	251 (36.9)	291 (42.4)	
Pack-years[c] for ever-smokers (±s.d.)	33.76 (±20.70)	29.17 (±21.32)	0.001[b]
Post- FEV_1% predicted (±s.d.)	64.22 (±24.91)	102.35 (±15.55)	$<$0.0001[b]
Post- FEV_1/FVC ratio (±s.d.)	53.58 (±12.49)	79.26 (±6.14)	$<$0.0001[b]
GOLD status			
Stage I (mild)	192	—	
Stage II (moderate)	262	—	
Stage III (severe)	187	—	
Stage IV (very severe)	39	—	

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; Post-, post-bronchodilator.
[a]Pearson's χ^2-test.
[b]Student's *t*-test.
[c]Pack years = (number of cigarettes smoked per day × number of years smoked)/20.

Table 2 Characteristics of the *IREB2* and *CHRNA3/5* SNPs genotyped and allele frequencies of these SNPs in COPD patients and controls

							MAF		
Gene	*SNP no.*	*SNP*	*Chromosome position (NCBI)*	*Location in gene*	*Major/minor alleles*	*HWE P-value in controls*	*COPD (n = 680)*	*Control (n = 687)*	*P-value*[a]
IREB2	1	rs2568494	76528019	Intron	G/A	0.522	0.132	0.133	0.982
	2	rs17483929	76529431	Intron	G/A	0.933	0.117	0.116	0.948
	3	rs2656069	76532762	Intron	A/G	0.449	0.268	0.262	0.706
	4	rs1964678	76541055	Intron	T/C	0.769	0.482	0.489	0.726
	5	rs12593229	76552345	Intron	T/G	0.961	0.477	0.484	0.724
	6	rs10851906	76561731	Intron	A/G	0.427	0.273	0.264	0.612
	7	rs965604	76576278	Intron	C/T	0.941	0.472	0.480	0.664
	8	rs13180	76576543	Exon	C/T	0.911	0.472	0.480	0.666
CHRNA3/5	9	rs8034191	76593078	Near *CHRNA5*	T/C	0.456	0.028	0.028	0.964
	10	rs16969968	76669980	Exon	G/A	0.493	0.030	0.025	0.457
	11	rs1051730	76681394	Exon	C/T	0.493	0.030	0.025	0.457
	12	rs938682	76683602	Intron	T/C	0.374	0.459	0.431	0.140

Abbreviations: COPD, chronic obstructive pulmonary disease; HWE, Hardy–Weinberg equilibrium; IREB2, iron-responsive element binding protein 2 gene; MAF, minor allele frequency; NCBI, National Center for Biotechnology Information; SNP, single-nucleotide polymorphism.
[a]χ^2-test for allele frequency difference between COPD and control.

either the crude analysis or the analysis adjusting for age, gender, BMI, pack-years and current smoking status among all COPD cases and controls (Supplementary file: Table S1). To evaluate potential genetic contribution to more severe manifestations of the disease, those with mild level of disease were excluded from the cases and patients with moderate to very severe disease (GOLD groups II, III and IV) were compared with controls. Among these subjects, unadjusted analysis indicated a borderline association between genotype frequencies of SNP (rs10851906) in *IREB2* and COPD. However, the level of association was not changed when the model was adjusted for age, gender, BMI, pack-years and current smoking status (Table 3).

As former-smokers, current-smokers as well as non-smokers were all included in this study and the distributions of these subjects in cases and controls were significantly different, we carried out a smoking status stratification analysis to eliminate the potential confounding which may be caused by the difference in smoking history. None of the SNPs was significantly associated with COPD in former-smokers ($n = 369$) and current-smokers ($n = 542$) even after adjusting the model for age, gender, BMI and pack-years in logistic regression. When the analysis was conducted in non-smokers ($n = 456$), two SNPs (rs2656069 and rs10851906) in *IREB2* were associated with COPD ($P = 0.045$ and 0.032, respectively, Table 4). Under the assumption of a recessive mode of inheritance (GG vs AG + AA for both SNPs), the GG genotypes in both SNPs were associated with decreased risk of COPD (OR = 0.459, 95% CI = 0.223–0.945 for rs2656069; OR = 0.441, 95% CI = 0.215–0.902 for rs10851906).

LD and haplotype association analysis. We calculated the pairwise LD (r^2) values for the 12 SNPs in the region of chromosome 15q25 (Figure 1). This revealed very strong levels of LD between groups of SNPs in both genes. For example, there was strong LD between SNPs rs2656069 and rs10851906 ($r^2 = 0.983$), which was reflected in the very similar odds ratios calculated within the non-smokers in the smoking status-stratified analysis. Global tests for haplotype were predicted from genotypic data. These analyses revealed no differences in frequency distribution of haplotypes either between all COPD cases and controls (Supplementary file: Table S2) or between COPD severity II, III and IV cases and controls (data not shown). Haplotype association analysis in non-smokers also failed to demonstrate any significant association with COPD, although the haplotype with G allele of rs2656069 and G allele of rs10851906 in it was less frequent among cases (26.7% vs the 29.0% in controls, $P = 0.431$, data not shown).

Association analyses of COPD-related phenotypes

SNP and pulmonary function. As association analysis of quantitative traits has increased power in comparison with qualitative phenotypes and because FEV_1% predicted is the primary measure of COPD severity, quantitative genetic association analysis was carried out for FEV_1% predicted and FEV_1/FVC using general linear models under the assumption of an additive mode of inheritance, adjusting for age, gender, BMI, pack-years and current smoking status. When the analysis was conducted among cases only, the results showed that four SNPs at *IREB2* (rs1964678, rs12593229, rs965604, rs13180) and three SNPs at *CHRNA3/5* locus (rs16969968, rs8034191, rs1051730) were associated with FEV_1% predicted. The same three SNPs at *CHRNA3/5* locus were also associated with FEV_1/FVC, while no significant association between SNPs in *IREB2* and FEV_1/FVC was found (Table 5). Among these SNPs, rs1964678 and rs8034191 were the most significant SNPs associated with FEV_1% predicted in case group (Figure 2). The presence of the minor allele (C) for rs1964678 was associated with a 2.65% increase in FEV_1% predicted ($\beta = 2.647$, s.e. = 1.283, $P = 0.039$), and the presence of the minor allele (C) for rs8034191 was associated with a 8.59% decrease in FEV_1% predicted ($\beta = -8.593$, s.e. = 3.997, $P = 0.032$) as well as 4.95% decrease in FEV_1/FVC ($\beta = -4.946$, s.e. = 1.971, $P = 0.012$). The same four significant pulmonary function associated SNPs of *IREB2* in cases were still associated with FEV_1% predicted when investigating all subjects, while no significant association between these SNPs for *CHRNA3/5* and FEV_1% predicted in the entire cohort.

Table 3 Genotype frequencies of SNPs analyzed and odds ratios in COPD patients and controls

Gene	SNP no.	SNP	Genotypes	COPD (severity II, III and IV) (n = 488)	Controls (n = 687)	P-value		Adjusted odds ratio (95% CI)[a]
						Unadjusted	Adjusted[b]	
IREB2	1	rs2568494	GG	368 (75.4)	518 (75.5)	0.554	0.654	1
			GA	114 (23.4)	154 (22.4)			1.052 (0.795–1.392)
			AA	6 (1.2)	14 (2.0)			0.664 (0.251–1.756)
	2	rs17483929	GG	379 (78.1)	535 (78.1)	0.906	0.933	1
			GA	101 (20.8)	141 (20.6)			1.020 (0.762–1.366)
			AA	5 (1.0)	9 (1.3)			0.826 (0.273–2.499)
	3	rs2656069	AA	246 (50.4)	378 (55.0)	0.118	0.138	1
			AG	212 (43.4)	258 (37.6)			1.265 (0.989–1.617)
			GG	30 (6.1)	51 (7.4)			0.942 (0.579–1.532)
	4	rs1964678	TT	140 (28.7)	181 (26.4)	0.553	0.470	1
			TC	241 (49.4)	339 (49.4)			0.883 (0.667–1.167)
			CC	107 (21.9)	166 (24.2)			0.815 (0.584–1.139)
	5	rs12593229	TT	142 (29.1)	183 (26.7)	0.578	0.494	1
			TG	241 (49.4)	342 (49.9)			0.878 (0.665–1.159)
			GG	105 (21.5)	161 (23.5)			0.825 (0.590–1.153)
	6	rs10851906	AA	242 (49.6)	376 (54.7)	0.071	0.086	1
			AG	216 (44.3)	259 (37.7)			1.297 (1.015–1.658)
			GG	30 (6.1)	52 (7.6)			0.934 (0.575–1.517)
	7	rs965604	CC	145 (29.7)	186 (27.1)	0.507	0.446	1
			CT	241 (49.4)	342 (49.8)			0.879 (0.667–1.159)
			TT	102 (20.9)	159 (23.1)			0.810 (0.579–1.133)
	8	rs13180	CC	145 (29.7)	186 (27.1)	0.508	0.449	1
			CT	241 (49.4)	341 (49.7)			0.882 (0.669–1.163)
			TT	102 (20.9)	159 (23.2)			0.810 (0.579–1.133)
CHRNA3/5	9	rs8034191	TT	456 (93.4)	649 (94.5)	0.465	0.445	1
			TC	32 (6.6)	38 (5.5)			1.211 (0.740–1.981)
			CC	0	0			—
	10	rs16969968	GG	454 (93.0)	652 (94.9)	0.180	0.178	1
			GA	34 (7.0)	35 (5.1)			1.403 (0.857–2.298)
			AA	0	0			—
	11	rs1051730	CC	454 (93.0)	652 (94.9)	0.180	0.178	1
			CT	34 (7.0)	35 (5.1)			1.403 (0.857–2.298)
			TT	0	0			—
	12	rs938682	TT	152 (31.1)	228 (33.2)	0.635	0.565	1
			TC	232 (47.5)	325 (47.4)			1.049 (0.801–1.373)
			CC	104 (21.3)	133 (19.4)			1.196 (0.857–1.669)

Abbreviations: COPD, chronic obstructive pulmonary disease; IREB2, iron-responsive element binding protein 2 gene; SNP, single-nucleotide polymorphism.
[a]Odds ratios are relative to the major homozygous genotype.
[b]Adjusted by logistic regression for age, gender, BMI, pack-years and current smoking status.

SNP and Smoking behavior of ever-smokers. We investigated the relationship between these SNPs and smoking behavior (pack-years of smoking) in ever-smoking subjects including current-smokers and former-smokers. Among all ever-smoking subjects investigated together ($n = 911$), no SNP was associated with pack-years (Table 5). When investigating cases only ($n = 455$), one SNP at *CHRNA3/5* locus (rs8034191) was associated with this quantitative phenotype ($P = 0.033$) using linear regression analysis after adjusting

Table 4 Genetic association results between SNPs in *IREB2* and *CHRNA3/5* and COPD by smoking status-stratified analysis

			P-*values by different smoking status*		
Gene	*SNP no.*	*SNP*	*Non-smokers (n = 456)*[a]	*Former-smokers (n = 369)*[b]	*Current-smokers (n = 542)*[b]
IREB2	1	rs2568494	0.730	0.403	0.585
	2	rs17483929	0.863	0.329	0.962
	3	rs2656069	0.045[c]	0.591	0.120
	4	rs1964678	0.248	0.078	0.317
	5	rs12593229	0.270	0.102	0.299
	6	rs10851906	0.032[c]	0.528	0.086
	7	rs965604	0.231	0.115	0.277
	8	rs13180	0.231	0.115	0.291
CHRNA3/5	9	rs8034191	0.346	0.789	0.363
	10	rs16969968	0.470	0.290	0.276
	11	rs1051730	0.470	0.290	0.276
	12	rs938682	0.622	0.430	0.237

Abbreviations: COPD, chronic obstructive pulmonary disease; IREB2, iron-responsive element binding protein 2 gene; SNP, single-nucleotide polymorphism.
[a]*P*-values adjusted by logistic regression for age, gender, BMI in non-smokers.
[b]*P*-values adjusted by logistic regression for age, gender, BMI and pack-years in former-smokers and current-smokers.
[c]$P \leq 0.05$.

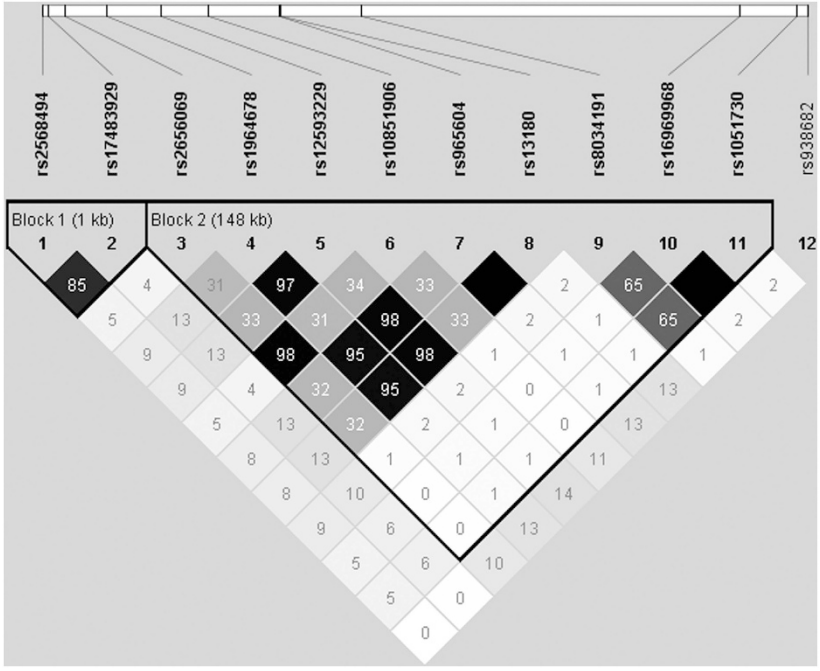

Figure 1 Linkage disequilibrium (LD) among single-nucleotide polymorphisms (SNPs) analyzed in chromosome 15q25. LD values are presented as r^2 and LD block was defined according to the Four Gamete Rule in the Haploview software.

for age, gender and current smoking status (Table 5, Figure 3). The minor allele (C) of this SNP was associated with 8.49% increase in pack-years of smoking ($\beta = 8.487$, s.e. = 3.965).

SNP and BMI. *IREB2* SNP rs10851906 was found to be associated with BMI in cases after adjusting for age, gender, pack-years and current smoking status ($P = 0.048$, data not shown). There was no significant relationship between any of these SNPs and BMI in all subjects.

DISCUSSION

Recently, SNPs at *CHRNA3/5* and *IREB2* region have been shown to have significant associations with COPD and COPD-related phenotypes by GWAS and integrative genomic approaches in non-Asian populations. In this study, we chose eight SNPs in the *IREB2* gene and four SNPs in or near the *CHRNA3/5* locus, which were significant reported SNPs for each gene and investigated the association of these SNPs with COPD susceptibility and COPD-related phenotypes in a Chinese Han population.

Table 5 Genetic association results between SNPs in *IREB2* and *CHRNA3/5* and pulmonary function and smoking behavior

		P-*values for pulmonary function phenotypes*				P-*values for smoking behavior*	
Gene	*SNP*	*FEV_1% predicted (case only)*	*FEV_1/FVC (case only)*	*FEV_1% predicted (all subjects)*	*FEV_1/FVC (all subjects)*	*Pack-years for ever-smoker (case only)*	*Pack-years for ever-smoker (all subjects)*
IREB2	rs2568494	0.649	0.287	0.949	0.336	0.252	0.716
	rs17483929	0.612	0.197	0.998	0.183	0.498	0.750
	rs2656069	0.425	0.984	0.914	0.748	0.851	0.332
	rs1964678	0.039*	0.645	0.022*	0.706	0.968	0.092
	rs12593229	0.045*	0.677	0.031*	0.658	0.896	0.113
	rs10851906	0.437	0.910	0.958	0.810	0.847	0.339
	rs965604	0.042*	0.708	0.029*	0.574	0.852	0.128
	rs13180	0.042*	0.708	0.029*	0.573	0.852	0.128
CHRNA3/5	rs8034191	0.032*	0.012*	0.106	0.093	0.033*	0.116
	rs16969968	0.050*	0.007*	0.217	0.033*	0.159	0.297
	rs1051730	0.050*	0.007*	0.217	0.033*	0.159	0.297
	rs938682	0.797	0.169	0.627	0.112	0.454	0.091

Abbreviations: FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IREB2, iron-responsive element binding protein 2 gene; SNP, single-nucleotide polymorphism.
Analyses of pulmonary function phenotypes were adjusted for age, gender, BMI, current smoking status and pack-years by linear regression; analyses of pack-years for ever-smoker were adjusted for age, gender and current smoking status by linear regression.
Analyses including all subjects were additionally adjusted for COPD case/control status.
*$P \leqslant 0.05$.

None of these SNPs were significantly associated with COPD either before or after adjusting the model for age, gender, BMI, pack-years and current smoking status among all subjects investigated together. We noticed that the subjects included by the GWAS[5,6] and related replication studies[14,15] were only ever-smokers (current-smokers and/or former-smokers), while we additionally included non-smokers in this study. In order to make it comparable between our results and that of previous studies and eliminate the effect of smoking on results, we conducted a smoking status-stratified analysis, in which the study population was stratified into groups of non-smokers ($n = 456$), former-smokers ($n = 369$) and current-smokers ($n = 542$). None of the SNPs was significantly associated with COPD in former-smokers or current-smokers, which was inconsistent with the findings of previous studies. The first GWAS for COPD demonstrated and replicated genetic associations between SNPs at the *CHRNA3/5* locus and COPD in four study populations and rs8034191 and rs1051730 were the most significant SNPs.[6] DeMeo *et al.*[5] found that seven SNPs at *IREB2* that we also investigated showed associations in both a COPD case–control study and family-based study. In two recent replication case–control studies, the same seven SNPs at *IREB2* and one SNP rs8034191 at *CHRNA3/5* were associated with COPD in a European population (cases = 900, controls = 1002)[14] and in a Polish population (cases = 315, controls = 330).[15] The subjects included in the above studies were all ever-smokers. However, in agreement with our study, one recent case–control study, which was also conducted in a Chinese Han population (cases = 275, controls = 434), found no association between *IREB2* rs2568494 and COPD[16] in ever-smokers. The limit of the study is that only one SNP of *IREB2* was investigated, so we can not get the information about other SNPs in *IREB2* gene in that Chinese population. When the analysis was conducted in non-smokers, two SNPs (rs2656069 and rs10851906) in *IREB2* were associated with COPD after adjusting for age, gender and BMI. While no such investigation has been conducted in the non-smokers before, little information was get from the literature. However, the minor alleles (G) for both SNPs were associated with decreased risk of COPD, which was in the same direction as previously reported among the ever-smokers of the non-Asian populations.[5,11,14]

Previous GWAS also revealed significant associations between SNPs in this region and COPD-related phenotypes, such as lung function, smoking behavior and emphysema.[7–10] It was reported that association analysis of quantitative traits can increase power in comparison with qualitative phenotypes and FEV_1 is an important COPD intermediate phenotype that provides a quantitative assessment of COPD severity, we analyzed FEV_1% predicted and FEV_1/FVC in our COPD patients who have a wide range of pulmonary function. Using an additive genetic model, four SNPs in *IREB2* (rs1964678, rs12593229, rs965604, rs13180) were associated with FEV_1% predicted and three SNPs in *CHRNA3/5* locus (rs16969968, rs8034191, rs1051730) were both associated with FEV_1% predicted and FEV_1/FVC after adjusting for age, gender, BMI, pack-years and current smoking status. These findings were consistent with the results reported in the GWAS.[5–8] We found that the SNP rs8034191 near *CHRNA3/5* locus was significantly associated with pack-years of smoking in the same direction as reported by the GWAS conducted by Pillai *et al.*[7] in COPD cases. The same SNP rs8034191 has also been found to be associated with heavier smoking behaviors by Stevens *et al.*[17] However, in a recent replication case–control study in polish population, the same SNP was not associated with pack-years of smoking in COPD cases.[15]

Previous GWAS and replication studies have found associations between *IREB2* polymorphisms and COPD and COPD-related phenotypes.[5,11,14,15] However, because the region of chromosome 15q25 where *IREB2* gene is located was found to be linked to nicotine addiction,[18–21] and there is high levels of LD between SNPs in *IREB2* and SNPs in *CHRNA3/5* in this region,[5] association findings in *IREB2* may be the result of independent association with smoking behavior or the result of high LD with SNPs in the *CHRNA3/5* gene cluster, and key functional variants may actually be in the *CHRNA3/5* gene cluster. We identified two SNPs in *IREB2* associated with COPD in non-smoking subjects, in the same direction as previously reported, while the association was not observed in current-smokers and

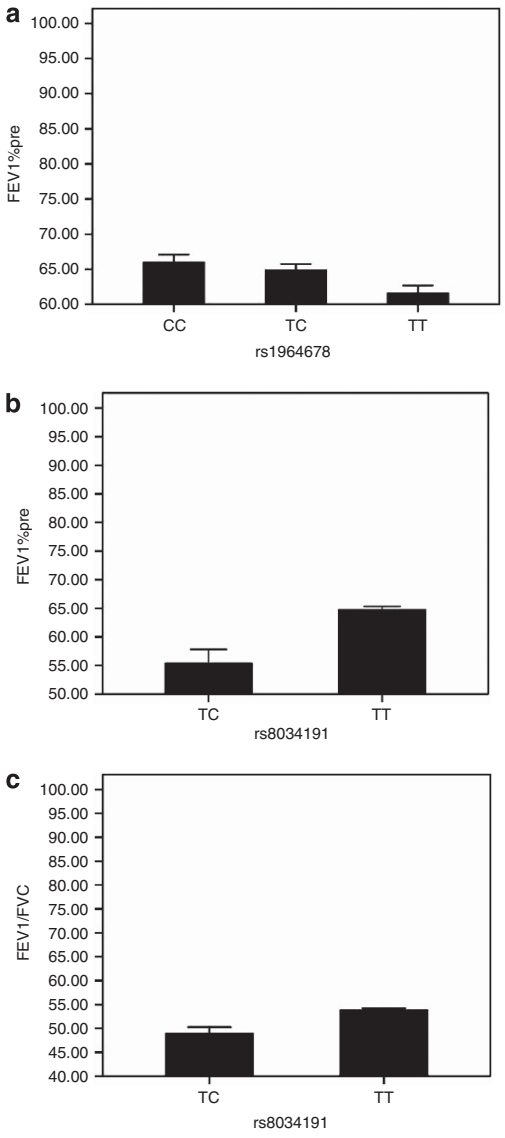

Figure 2 The association between iron-responsive element binding protein 2 gene (*IREB2*; rs1964678, **a**) and *CHRNA3/5* (rs8034191, **b** and **c**) genotypes and forced expiratory volume in 1 s (FEV_1)% predicted and FEV_1/forced vital capacity (FVC) in cases. Data represent mean + s.e. Assuming additive model of inheritance and adjusting for age, gender, body mass index (BMI), pack-years and current smoking status.

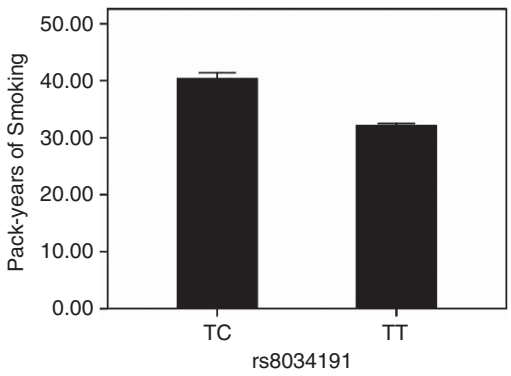

Figure 3 Pack-years of smoking by genotype of *CHRNA3/5* single-nucleotide polymorphism (SNP) rs8034191 in the ever-smoking cases. Data represent mean + s.e. and adjusted for age, gender and current smoking status.

former-smokers. Furthermore, in consist with the findings of DeMeo *et al.*,[5] we did not detect any association for these SNPs at *IREB2* with pack-years of smoking. So the role of *IREB2* polymorphisms in the development of COPD appears to be independent of an effect on smoking. Additionally, we demonstrated that *IREB2* SNPs were associated with FEV_1% predicted after adjusting for pack-years of smoking and current smoking status, which also indicates that *IREB2* may have an independent effect on airflow obstruction. Besides, we failed to demonstrate significant association between any of the SNPs at *CHRNA3/5* locus and COPD in all the analyses, which suggests the association observed for *IREB2* polymorphisms is less likely due to the LD with *CHRNA3/5* SNPs.

In support of *IREB2* involvement, DeMeo *et al.*[5] found increased IREB2 protein and mRNA expression in human lung tissues from COPD patients vs controls. The *IREB2* gene codes for an iron-binding protein, which is involved in maintaining human cellular iron metabolism. It was known that iron homeostasis and free iron concentration are likely to be important mediators of oxidative stress and iron could therefore contribute to local damage by this mechanism. The *IREB2* knockout mouse has been observed to develop neurodegenerative disease, which is probably because of aberrant iron homeostasis in the brain,[22] while the lungs of these animals were not examined in any detail. Another important feature of IREB2 is that it is suggested to be active at lower oxygen tensions[23] and has been observed to be post-translationally regulated by hypoxia.[24,25] Thus, the aberrant iron homeostasis in the presence of hypoxia could lead to increased oxidative stress resulting in tissue damage and reduced FEV_1. Functional studies will be required to clarify the role of iron regulation in COPD pathogenesis in the future.

CHRNA3/5 have been demonstrated to be associated with nicotine-dependence, COPD and other smoking related diseases such as lung cancer and peripheral arterial disease through GWAS,[6,9,19] as well as candidate gene analyses,[26] integrative genomics approaches,[11] meta-analysis[18,27,28] and replication study[15] in diverse populations, including European, Polish and African–American populations. The CHRNA3–CHRNB4–CHRNA5 region encodes the subunits of alpha-nicotinic acetylcholine receptor (nAChR) expressed in neurons as well as bronchial and alveolar epithelium.[29–31] These receptors have been implicated in nicotine addiction and appear to be upregulated with chronic tobacco use.[32] Although many studies demonstrated and replicated genetic associations between SNPs at the *CHRNA3/5* locus and COPD, it was controversial that the observed association was attributed to the differences in smoking behavior between COPD cases and controls or there could be a direct effect of *CHRNA3/5* locus on the development of COPD, independent of smoking behavior.

In the first GWAS of COPD,[6] they did not identify an association between pack-years and this locus, and the association between *CHRNA3/5* and COPD remained robust even after adjusting for current smoking status and pack-years by logistic regression. However, they also observe a genotype-by-environment interaction between the risk of the rs8034191 genotype and current smoking status on COPD, which suggests that some of the increased risk for COPD associated with this gene locus could be mediated by smoking behavior. A recent case–control study in a polish population came to similar conclusions: the effect of CHRAN3/5 SNPs may be

independent from smoking, while some evidence suggests a gene-by-environment interaction also exists.[15] In this study, we failed to demonstrate an association between any *CHRNA3/5* SNPs and COPD susceptibility, either before or after adjusting for age, gender, BMI, pack-years and current smoking status, and either analyzed in all subjects or in subgroup with different smoking status. However, we observed association between *CHRNA3/5* SNP rs8034191 and pack-year of smoking in COPD cases. It is possible that *CHRNA3/5* may be not associated with COPD susceptibility, at least in this Chinese population. Our findings also tend to support that the observed association in previous study may be mediated by smoking behavior, with no independent effect of this locus on the development of COPD. However, as the direction of effect remained the same with previous study despite loss of significance and three out of four SNPs investigated for *CHRNA3/5* locus were found to be associated with deceased FEV_1% predicted and FEV_1/FVC, it is also likely that our study was underpowered to demonstrate a persistent impact on COPD susceptibility from *CHRNA3/5*, especially in the smoking status-stratified analysis with reduced sample size in each subgroup. Further investigation including fine mapping of this region and more homogeneous population with COPD with larger simple size may help to tease apart these issues.

The present study has several limitations. First, we included COPD patients with a wide range of disease severity, which may reduce the homogeneity of affected individuals. To lessen the impact, we additionally conducted analysis excluding the patients with mild levels of disease from the cases and compared the rest cases with controls. Second, although the smoking status-stratified analysis can decrease the impact of smoking, which is an important confounder on association analysis, it also decreases the power to detect true associations because of reduced sample size in each subgroup. However, we were still able to demonstrate two significant associations with COPD in the non-smoking group. Third, we did not perform some correction for multiple testing, which may lead to false-positive results. However, the adjustments for potential confounding variables were performed in all the association analyses and all the significant effects in our study were in the same direction as previously reported, which reduced the possibility for false-positive results.

To our knowledge, this is the first study to investigate the association of the SNPs (rs16969968, rs8034191, rs1051730, rs938682) in *CHRNA3/5* locus and SNPs (rs2656069, rs10851906, rs1964678, rs12593229, rs965604, rs13180, rs17483929) in *IREB2* gene with COPD and COPD-related phenotypes in a large cohort of Chinese Han patients (rs2568494 in *IREB2* has recently been investigated in another Chinese Han population), and also the first study to investigate the above association in non-smokers. In summary, we failed to replicate previous reports of associations between SNPs in *IREB2* and *CHRNA3/5* and susceptibility to COPD in ever-smokers. However, we demonstrated associations between two SNPs in IERB2 and COPD susceptibility in non-smokers, with negative findings for *CHRNA3/5* SNPs. This suggests the effect of *IREB2* polymorphisms on COPD may be independent from smoking and independent from *CHRNA3/5* gene cluster. At the same time, we confirmed that SNPs in these two gene loci were associated with pulmonary function and *CHRNA3/5* was associated with pack-year of smoking in COPD cases. However, further research is warranted to confirm our observation in Chinese Han population and other ethnic populations and identify the exact functional variants in this region involved in the pathogenesis of COPD and COPD-related phenotypes.

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