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An interaction between *Nrf2* polymorphisms and smoking status affects annual decline in FEV_1: a longitudinal retrospective cohort study

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Abstract

Background: An Nrf2-dependent response is a central protective mechanism against oxidative stress. We propose that particular genetic variants of the *Nrf2* gene may be associated with a rapid forced expiratory volume in one second (FEV_1) decline induced by cigarette smoking.

Methods: We conducted a retrospective cohort study of 915 Japanese from a general population. Values of annual decline in FEV_1 were computed for each individual using a linear mixed-effect model. Multiple clinical characteristics were assessed to identify associations with annual FEV_1 decline. Tag single-nucleotide polymorphisms (SNPs) in the *Nrf2* gene (rs2001350, rs6726395, rs1962142, rs2364722) and one functional SNP (rs6721961) in the *Nrf2* promoter region were genotyped to assess interactions between the *Nrf2* polymorphisms and smoking status on annual FEV_1 decline.

Results: Annual FEV_1 decline was associated with smoking behavior and inversely correlated with FEV_1/FVC and FEV_1 % predicted. The mean annual FEV_1 declines in individuals with rs6726395 G/G, G/A, or A/A were 26.2, 22.3, and 20.8 mL/year, respectively, and differences in these means were statistically significant (p_{corr} = 0.016). We also found a significant interaction between rs6726395 genotype and smoking status on the FEV_1 decline (p for interaction = 0.011). The haplotype rs2001350T/rs6726395A/rs1962142A/rs2364722A/rs6721961T was associated with lower annual decline in FEV_1 (p = 0.004).

Conclusions: This study indicated that an Nrf2-dependent response to exogenous stimuli may affect annual FEV_1 decline in the general population. It appears that the genetic influence of *Nrf2* is modified by smoking status, suggesting the presence of a gene-environment interaction in accelerated decline in FEV_1.

Background

Among pulmonary function test (PFT) measurements, forced expiratory volume in one second (FEV_1) is the most reproducible [1]. Therefore, it is suitable for analyzing changes in pulmonary function over time. Accelerated decline in FEV_1 is considered as an important predictor for the development of inflammatory obstructive lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [2,3]. A rapid decline in FEV_1 may be affected by multiple factors, including environmental and genetic factors.

The most important environmental factor for FEV_1 decline is cigarette smoking. In their landmark study, Fletcher et al. [4] demonstrated that smokers had a steeper decline in FEV_1 than non-smokers. Subsequent studies have revealed that the rate of decline in FEV_1 depends on pack-years smoked and that the accelerated decline in FEV_1 in smokers slows to normal rates of decline upon smoking cessation [4-6]. Cigarette smoke contains high concentrations of oxidants, including reactive oxygen species and reactive nitrogen species [7]. Oxidative stress due to cigarette smoking promotes direct injury to airway epithelium, expression of genes encoding proinflammatory

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mediators, and protease/antiprotease imbalance [8], all of which induce chronic inflammation in the lung of smokers that results in deterioration of lung function.

However, only 10-15% of smokers develop a severe impairment of lung function [4]. In addition to environmental factors, genetic determinants play an important role in rapid decline in lung function. A pedigree-based study has shown that FEV_1 levels have a heritability that is independent of cigarette smoking and disease status such as asthma [9]. Furthermore, recent large-scale genome-wide association studies have identified several loci associated with FEV_1 and the FEV_1/forced vital capacity (FVC) ratio [10,11].

It is possible that oxidant/antioxidant imbalance in the lungs of smokers results in an accelerated loss of lung function. Nrf2 is a major regulator of the antioxidant response [12]. Nrf2 regulates the expression of several genes encoding antioxidant and detoxification proteins [13]. In animal models, Nrf2 plays an important role in reducing inflammation associated with elastase-induced emphysema [14]. In human studies, attenuation of Nrf2 due to the down-regulation of the *Nrf2* mRNA has been detected in alveolar macrophages of COPD patients [15]. Moreover, 3 single-nucleotide polymorphisms (SNPs) in the promoter region of the *Nrf2* gene have an influence on the gene's transcriptional activity, and one of these SNPs is associated with the development of acute lung injury [16]. Recently, one SNP (rs2364723) in the first intron of *Nrf2* has been shown to be related to a lower FEV_1 [17]. All of these findings indicate that an Nrf2-dependent adaptive response is important in inhibiting the oxidant-induced lung inflammation that results in a rapid decline in lung function.

Therefore, we conducted a longitudinal retrospective cohort study of a general Japanese population in order to analyze associations between *Nrf2* polymorphisms and annual decline in FEV_1. We also assessed whether an interaction between the *Nrf2* polymorphisms and smoking status affects FEV_1 decline.

Methods

Subjects

A retrospective cohort study was conducted. We recruited 1,507 full-blooded Japanese subjects from a general population who visited the Tsukuba Medical Center for annual health checkup from June 2008 to May 2009 (Figure 1). Detailed information on the cohort is available in a previous report [18]. The individuals completed questionnaires concerning respiratory health, medical history, lifestyle, and exposure to environmental irritants (e.g. cigarette smoke, allergens, and air pollution). All 1,507 subjects participated in a medical interview, a physical examination, routine blood studies, a chest roentgenogram, and PFTs. Based on detailed data from these analyses, we excluded 36 subjects from the study because of a preexisting lung ailment; 12 were diagnosed as having preexisting tuberculosis, 3 also had a diagnosis of asthma, and 24 individuals with asthma or COPD who had been treated by inhaled corticosteroids, leukotriene receptor antagonists, and/or bronchodilators such as β-adrenoceptor stimulants and anticholinergic agents. From among the remaining 1,471 subjects, 915 participants who took at least 4 valid PFT measurements over a period of at least 4 years were selected for this study in order to ensure a reliable estimate of longitudinal decline in FEV_1. The final study population of 915 participants included 47 subjects with asthmatic history who had not taken asthma medication during the retrospective study period.

The Institutional Review Boards of the University of Tsukuba (IRB No. 136) and the Tsukuba Medical Center (IRB No. 2008-01-31) approved the study, and each subject provided written informed consent.

Pulmonary Function Test (PFT)

Spirometry was performed with an electronic spirometer (Autospiro SYSTEM7; Minato Medical Science Co., Ltd., Osaka, Japan) according to the standards recommended by the Japanese Respiratory Society (JRS) [19]. The patients performed the maneuvers without any bronchodilators. The highest value for the sum of FVC and FEV_1 was selected as the measurement for each PFT. FVC and FEV_1 were expressed as a percentage of predicted values approved by the JRS [19]. All available longitudinal data for each participant were collected retrospectively to estimate the annual decline in FEV_1.

Single-Nucleotide Polymorphism (SNP) Selection and Genotyping

Using JPT (Japanese in Tokyo, Japan) genotype data (PhaseIII/Rel#2, Feb09, on NCBI B36 assembly, dbSNP b126) from the International HapMap project http://hapmap.org/, four tag SNPs (rs2001350, rs6726395, rs1962142, rs2364722) were identified in the 34.38 kb *Nrf2* gene region (chromosome 2, position 177,803,285-177,837,663). We used the multi-marker predictor method implemented in the Tagger program [20]. Tag set was generated using a threshold r^2 of 0.8 and a minor allele frequency of > 0.1. Genomic DNA was extracted from samples of whole blood from each of the 915 participants by an automated DNA extraction system (QuickGene-610L, FUJIFILM, Tokyo, Japan). Genotyping of these 4 bi-allelic SNPs were attempted for each participant by the pre-designed TaqMan allele-specific polymerase chain reaction (PCR) assays according to the manufacturer's instruction (Applied Biosystems, Foster City, CA).

It has been reported that 3 SNPs (rs6721961, rs6706649, and rs35652124) located in the promoter region of the *Nrf2* gene affect the transcriptional activity

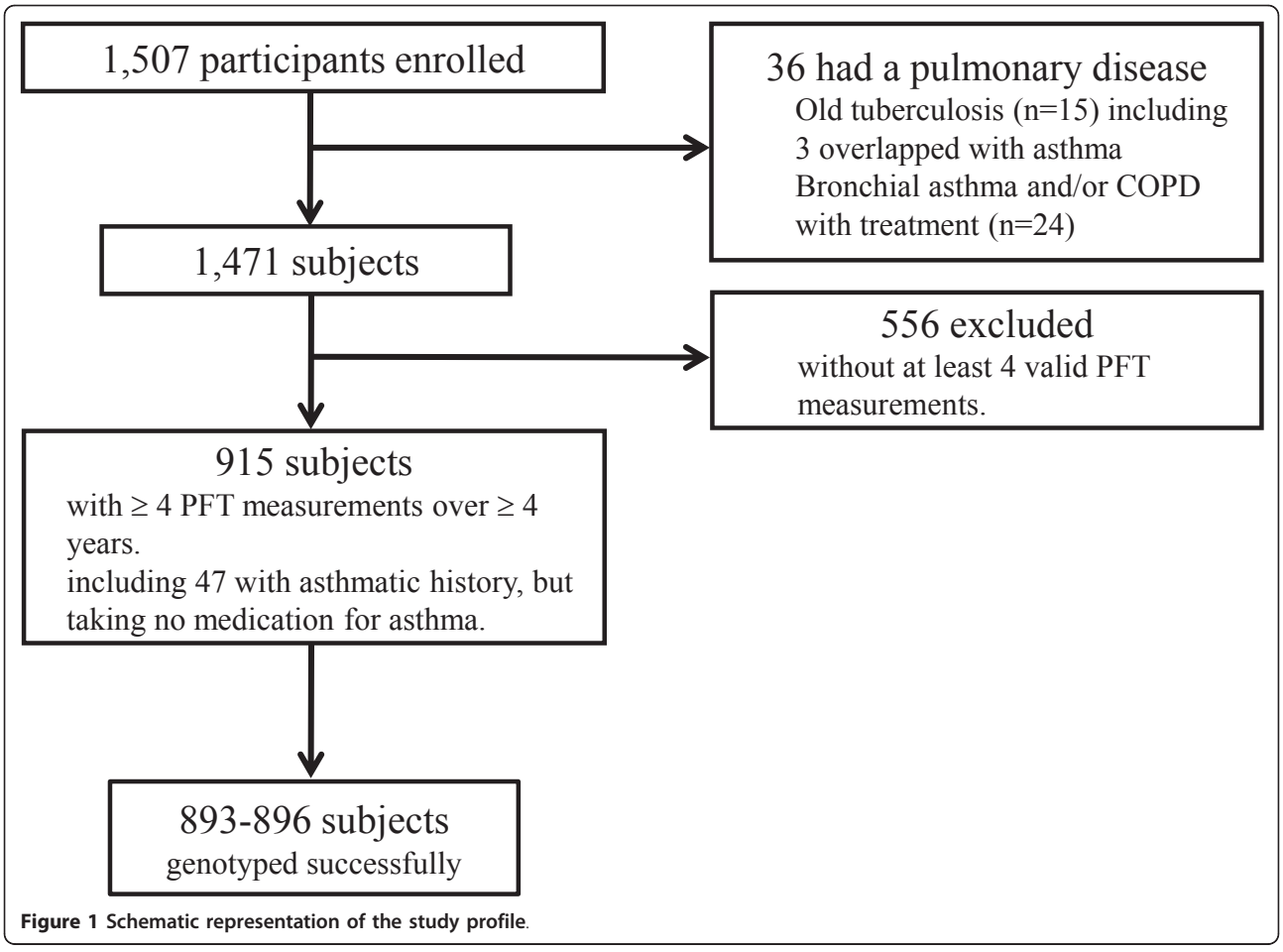

Figure 1 Schematic representation of the study profile.

of *Nrf2* [16]. Genotyping for rs6721961 was carried out for each participant by the TaqMan technique using a pair of primers and a pair of oligonucleotide probes designed and synthesized by Applied Biosystems. The sequences of the primers were as follows: forward, 5'-CAGTGGGCCCTGCCTAG-3'; reverse, 5'-TCAGGGTGACTGCGAACAC-3'. The TaqMan fluorescence-labeled oligonucleotide probes were 5'-[VIC]-TGGACAGCGCCGGCAG-3' and 5'-[FAM]-TGTGGACAG CTCCGGCAG-3'. Because rs6706649 and rs35652124 are only 2 base pairs apart, the allele-specific probe technique was not appropriate for genotyping. Instead, for these 2 SNPs, direct DNA sequencing analysis was performed for 50 subjects (25 major allele homozygotes and 25 minor allele homozygotes for rs6726395 SNP). PCR amplification was carried out with 50 ng genomic DNA and a pair of primers flanking the 2 SNPs by a GeneAmp PCR System (Applied Biosystems). The primer sequences were as follows: forward, 5'-AGAGGTTC TCTTGGGGTTCC-3'; reverse, 5'-AGAACCTTGCCCT GCTTTTA-3'. The amplified 343-bp PCR DNA products were sequenced using the same primers and the dideoxynucleotide chain termination method available as a fluorescent sequencing kit (DNA Sequencing Kit; Applied Biosystems) and an automated sequencer (ABI PRISM 3130; Applied Biosystems) according to the manufacturers' instruction.

Statistics

Data are expressed as mean ± SD, unless otherwise stated. Statistical analysis was performed using SYSTAT software, version 13 (Systat Software, Inc., Chicago, IL). Statistical tests with a p value < 0.05 were considered significant.

Values of annual FEV_1 decline were computed for each individual across the repeated measurements using a linear mixed-effect model. We used a random intercept to take into account the heterogeneity across subjects and the correlation induced by having repeated observations on the same subjects.

We performed univariate analysis to evaluate association of annual decline in FEV_1 with clinical characteristics. For categorical variables such as gender and smoking status, Student's *t* tests and one-way analyses of

variance with Bonferroni *post hoc* correction were used for comparisons of 2 and 3 group means, respectively. For continuous variables such as age, body mass index (BMI), PFT measurements, and total serum IgE levels, the correlation with annual decline in FEV_1 was assessed by Pearson correlation coefficient analysis.

All polymorphisms were tested for Hardy-Weinberg equilibrium using Haploview 4.2 software http://www.broadinstitute.org/haploview[21]. Estimates of pairwise linkage disequilibrium (LD) between the loci were calculated using r^2 [22]. The associations of genotypes with annual decline in FEV_1 were analyzed by multivariate linear regressions adjusted for potential confounding factors such as sex, age, BMI, FEV_1/FVC ratio, total serum IgE levels, smoking status (never, ex, or current), smoking index (0, 0-200 or > 200), and affection of bronchial asthma. Correction for multiple comparisons was done by the Bonferroni's method. The interaction effect of genotypes and smoking status on the annual decline in FEV_1 was analyzed using general linear models adjusted for the same confounding factors except for smoking behavior.

Association of the rs6726395 genotypes with the mRNA expression levels of *Nrf2* was analyzed using GENEVAR database http://www.sanger.ac.uk/humgen/genevar/[23], which shows mRNA expression profiles of 3 cell types (fibroblast, lymphoblastoid cell line and T-cell) derived from umbilical cords of 75 Geneva GenCord individuals [24].

For analyses of association between haplotypes and annual FEV_1 decline, we used the Haplo. score program http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm, which adjusts for the same covariates and calculates simulation p values for each haplotype [25].

Results

Characteristics of the study cohort, 915 Japanese individuals from a general population, are provided in Table 1. The average number of visits for routine health checkups over the study period per participant was 9.1 ± 3.7 times during 11.1 ± 4.6 years. The mean age at the recruitment was 52.1 years (31-78 years). All the participants were over 25 years of age at the first visit. Of the participants, 63% were never-smokers, 23% were ex-smokers, and 14% were current-smokers. The average of serum IgE levels was 1.78 (log IU/mL) (normal, < 2.23). The mean annual decline in FEV_1 was 23.8 mL/year. The final study population included 47 subjects with a history of asthma, but each of these subjects had not been treated with asthma medications.

Univariate analysis was performed to evaluate relationships between annual decline in FEV_1 and clinical variables (Table 2). There was no significant difference in FEV_1 decline between men and women. As expected,

Table 1 Characteristics of the participants[a]

Characteristic	Participants (N = 915)
Age (years)	52.1 ± 8.3
Male sex - N (%)	418 (45.7)
Total duration of follow-up (years)	11.1 ± 4.6
Number of visits	9.1 ± 3.7
BMI	23.1 ± 3.0
FVC (L)	3.12 ± 0.78
FVC % predicted (%)	100.5 ± 13.6
FEV_1 (L)	2.56 ± 0.64
FEV_1/FVC (%)	82.2 ± 5.6
FEV_1 decline (mL/year)	23.8 ± 26.3
Serum IgE (Log IU/mL)	1.78 ± 0.59
Smoking habits - N (%)	
Never-smokers	576 (63.0)
Ex-smokers	210 (23.0)
Current-smokers	129 (14.1)
Smoking index[b] - N (%)	
0	576 (63.0)
0-200	114 (12.5)
> 200	225 (24.6)
Asthma - N (%)	47 (5.1)

[a] Data are presented as mean ± SD or n (%).

[b] Smoking index was calculated for current and past smokers by multiplying smoking dose (cigarettes per day) and duration (years smoked).

the association between annual FEV_1 decline and smoking behavior was statistically significant. The mean value of annual FEV_1 declines in current-smokers was significantly larger than that in never-smokers. We calculated smoking index (cigarettes/day × years smoked) for all the participants and divided the study population into 3 categories; 0, 0-200, and > 200. Mean FEV_1 declines in the 0-200 and > 200 groups were significantly larger than that in the 0 group. The value of FEV_1 decline showed a weak inverse correlation with FEV_1/FVC ($r = -0.278$, $p < 0.001$) and FEV_1 % predicted ($r = -0.263$, $p < 0.001$). As for the pairwise correlation between annual FEV_1 decline and the other pulmonary function measurements, the correlation coefficients were -0.146 for FVC % predicted ($p < 0.001$), -0.144 for FEV_1 ($p < 0.001$), and -0.069 for FVC ($p = 0.037$). The correlation coefficients for annual FEV_1 decline and age was 0.105 ($p = 0.001$) and that for annual FEV_1 decline and total IgE levels was 0.097 ($p = 0.003$).

Genotype analysis at 4 tag SNPs (rs2001350, rs6726395, rs1962142, rs2364722) and one previously-reported functional SNP (rs6721961) [16] in the *Nrf2* promoter region was attempted for the each participant. Genotyping of rs2001350 and rs6726395 was successful for 895 subjects; similarly, rs1962142 and rs2364722 genotyping was successful for 896 subjects, and rs6721961 was for 893 subjects. The overall success rate was 97.6-97.9%. All the analyzed SNPs were

Table 2 Univariate analysis comparing annual decline in FEV_1 and clinical variables

Variables	Correlation coefficient[a]	Annual decline in FEV_1 Mean ± SD (mL/yr)	P value
Categorical variables			
Gender			0.205
Male		25.0 ± 28.2	
Female		22.8 ± 24.5	
Smoking habits			0.005
Never-smokers		21.7 ± 24.5	
Ex-smokers		26.7 ± 27.6	
Current-smokers		28.5 ± .30.5*	
Smoking index[b]			0.006
0		21.7 ± 24.5	
0-200		28.2 ± 29.6	
> 200		27.0 ± 28.3*	
Continuous variables			
Age	0.105		0.001
BMI	0.038		0.245
FVC (L)	-0.069		0.037
FVC % predicted (%)	-0.146		< 0.001
FEV_1 (L)	-0.144		< 0.001
FEV_1 % predicted (%)	-0.263		< 0.001
FEV_1/FVC (%)	-0.278		< 0.001
Serum IgE (Log IU/mL)	0.097		0.003

[a] Pearson coefficient.

[b] Smoking index was calculated for current and past smokers by multiplying smoking dose (cigarettes per day) and duration (years smoked).

* $p < 0.05$ compared with never-smokes (smoking index 0) after Bonferroni *post hoc* correction.

in Hardy-Weinberg equilibrium. Relationships between the genotypes and the annual FEV_1 decline are shown in Table 3. Annual FEV_1 declines adjusted for potential confounding factors were significantly different among rs6726395 genotypes ($p_{corr} = 0.016$). The mean annual FEV_1 declines in major allele homozygotes (G/G), heterozygotes (G/A), and minor allele homozygotes (A/A) for the rs6726395 SNP were 26.2, 22.3, and 20.8 mL/year, respectively. In contrast, the GENEVAR database did not show a significant difference in *Nrf2* mRNA expression levels based on rs6726395 genotypes (data not shown).

Table 3 Multivariate linear regressions[a] for association between genotypes at the *Nrf2* SNPs and decline in FEV_1

SNP	Genotype	N	Annual decline in FEV_1 Mean ± SD (mL/year)	P value (corrected p value)
rs2001350	CC	43	24.7 ± 7.5	0.101
	CT	296	24.2 ± 7.4	
	TT	551	23.8 ± 7.9	
rs6726395	GG	431	26.2 ± 8.3	0.0031 (0.016)
	GA	378	22.3 ± 7.4	
	AA	86	20.8 ± 8.8	
rs1962142	AA	23	28.1 ± 10.4	0.194
	AG	277	23.6 ± 6.8	
	GG	591	24.0 ± 8.0	
rs2364722	AA	157	24.9 ± 7.9	0.277
	AG	421	24.0 ± 7.4	
	GG	313	23.4 ± 8.1	
rs6721961	GG	496	23.6 ± 7.8	0.900
	GT	344	24.5 ± 7.7	
	TT	53	24.4 ± 7.3	

[a] Adjusted for sex, age, BMI, FEV_1/FVC ratio, serum IgE levels, smoking status, smoking index, and asthma diagnosis.

Pairwise LD (r^2) values among the 5 SNPs are shown in Figure 2. In the present study, the observed LD values among the 4 tag SNPs detected in the HapMap project corresponded well with the LD structure that is expected based on the patterns in the JPT (Japanese) population of the HapMap group. All the r^2 values among the 5 SNPs studied did not exceed 0.61, indicating that these 5 SNPs were not in tight LD with each other.

Previous results indicate that 3 SNPs (rs6721961, rs6706649 and rs35652124) in the promoter region of *Nrf2* are functionally relevant [16]. Although it has been reported that the minor allele of rs6721961 SNP diminishes promoter activity of the *Nrf2* gene, there was no significant association between this SNP and annual FEV_1 decline in the present study (Table 3). We estimated the extent of linkage disequilibrium between rs6726395 and the other 2 functional SNPs (rs6706649 and rs35652124) by sequencing 50 subjects. The SNP rs6726395 was not in tight LD with rs6706649 (r^2 = 0.02) or with rs35652124 (r^2 = 0.67), suggesting that genetic effects of these 2 functional SNPs do not underlie the association of rs6726395 with FEV_1 decline. The SNP rs35652124 was in tight LD (r^2 = 0.92) with rs2364722, which did not have a significant association with annual decline in FEV_1 in the present study (Table 3).

Next, we analyzed effect of interaction between the bi-allelic SNP rs6726395 and cigarette smoking status on annual decline in FEV_1 (Figure 3). In ex- and current-smokers, annual FEV_1 decline was significantly different between individuals with and without the rs6726395 G allele for FEV_1 decline. The mean decline values were 27.8 mL/year and 12.5 mL/year in individuals with the G/G + G/A genotype and those with the A/A genotype, respectively (p = 0.010). In contrast, the annual FEV_1 decline was not significantly different between individuals carrying the G/G + G/A and the A/A genotypes (22.7 and 22.8 mL/year, respectively) for individuals who were never-smokers. The p value for interaction between rs6726395 and smoking status on the annual decline in FEV_1 was 0.011. These results indicated that the genetic influence of *Nrf2* was modified by smoking status, suggesting the presence of a gene-environment interaction in annual decline in FEV_1.

We constructed haplotypes composed of the 4 tag SNPs and rs6721961, and analyzed association of the haplotypes with annual FEV_1 decline (Table 4). The distribution of the haplotypes was significantly related to annual FEV_1 decline with the global simulation p value of 0.004. We identified 4 common haplotypes covering 86.8% of the whole genotyped population. The haplotype most strongly associated with annual FEV_1 decline was rs2001350T/rs6726395A/rs1962142A/rs2364722A/rs6721961T with a haplotype score of -2.988 and a

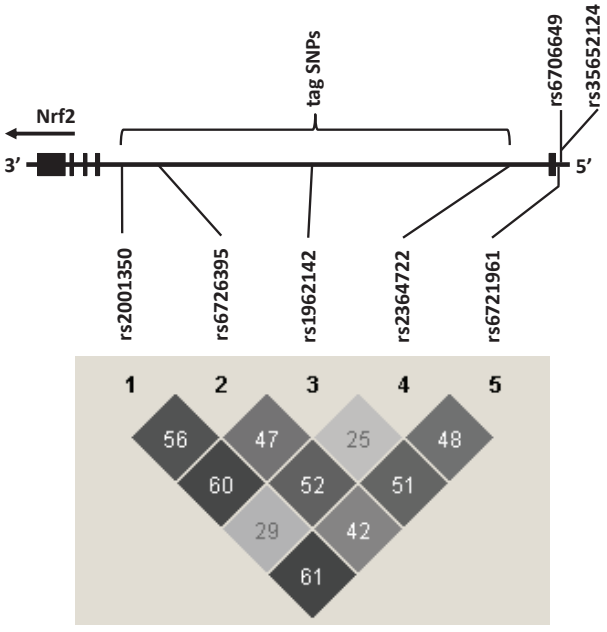

Figure 2 Genomic organization of the *Nrf2* gene and linkage disequilibrium (LD) map for single-nucleotide polymorphisms (SNPs). Exons are shown as black boxes. Note that the reference sequence uses the complementary strand of DNA; thus, the 5′ region is on the right. The 4 tag SNPs are located in the first intron and the 3 functional SNPs are in the promoter region. Pairwise LD values ($r^2 \times 100$) for the 4 tag SNPs and rs6721961 were calculated based on genotypes of the whole study population. The intensity of the gray shading in squares of the LD map is proportional to r^2.

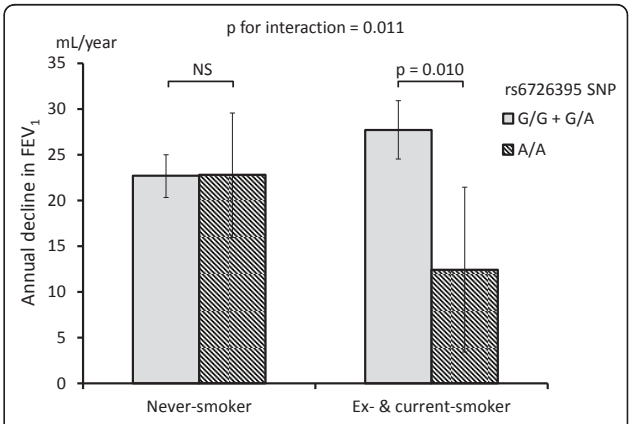

Figure 3 Effect of interaction between rs6726395 SNP and cigarette smoking status on annual decline in FEV_1. Data are presented as mean (95% CI). Light gray bar and striped bar represent rs6726395 G/G + G/A and A/A genotype groups, respectively. Only in ex- and current-smokers was the annual FEV_1 decline significantly different between the 2 groups. The interaction effect was analyzed by general linear models adjusted for sex, age, BMI, FEV_1/FVC ratio, serum IgE levels, and affection of bronchial asthma.

Table 4 Estimated haplotype frequencies and haplotype association with annual decline in FEV_1

Haplotype	rs2001350	rs6726395	rs1962142	rs2364722	rs6721961	Haplotype frequency	Haplotype-specific score	Simulation p value	Global simulation p value
1	T	A	A	A	T	0.082	-2.988	0.002	0.004
2	C	G	A	G	T	0.148	-1.319	0.182	
3	T	A	G	A	G	0.561	0.453	0.663	
4	T	A	A	A	G	0.077	1.184	0.235	

Haplotype frequencies were estimated using the Haplo. Stats program. Haplotype 1(rs2001350T/rs6726395A/rs1962142A/rs2364722A/rs6721961T) was associated with annual decline in FEV_1 with a haplotype score of -2.988 and a simulation p value of 0.002. Haplotye with frequencies less than 0.05 were excluded.

simulation p value of 0.002; this haplotype was associated with lower decline in FEV_1.

Discussion

Cigarette smoke (CS) contains a high concentration of oxidants and leads to oxidative stress [7]. In smokers, increased oxidative stress in the airways is a predominant cause of accelerated decline in lung function [26,27]. Nrf2 plays a central role in protecting the lung against CS-induced oxidative stress by up-regulating multiple genes encoding antioxidant and detoxification proteins, e.g., heme oxygenase-1, NADPH, and glutathione S-transferase [12,13]. CS-induced reactive oxygen species production via NADPH oxidase activation is involved in the positive regulation of the Nrf2/ARE pathway. NADPH oxidase, as a critical regulator of innate immunity, also limits lung inflammation by attenuating NF-κB and by activating Nrf2[28,29]. On the other hand, CS activates the NF-κB pathway, which participates in the negative regulation of Nrf2/ARE signaling[30,31]. In addition, protein carbonylation induced by CS is involved in the suppression of the Nrf2/ARE pathway[32]. Because several studies have demonstrated that an Nrf2-dependent adaptive response is important in preventing CS-induced lung inflammation and injury[15,33,34], we reasoned that *Nrf2* polymorphisms have a genetic impact on the CS-induced deterioration of lung function.

In the present study, we showed that a variant of the *Nrf2* gene was associated with accelerated decline in FEV_1 in a general population sample including non-smokers, moderate smokers, and heavy smokers. A stronger effect of the rs6726395 SNP on annual FEV_1 decline was observed in smokers than in never-smokers, indicating a gene-smoking interaction in FEV_1 decline. Such an interaction is reasonable because Nrf2 activation protects tissues against oxidative stress. The minor allele of rs6726395 in the homozygous state (A/A) was associated with a smaller FEV_1 decline; therefore, this allele was thought to be protective against FEV_1 decline.

The mechanisms mediating the relationship between rs6726395 and FEV_1 decline were not determined in this study. Although the rs6726395 SNP is located in the first intron of the *Nrf2* gene, rs6726395 variants did not correlate with different *Nrf2* mRNA levels according to the GENEVAR database. However, given that GENEVAR utilizes only three cell types (fibroblast, lymphoblastoid cell line and T-cell), the possibility remains that rs6726395 has some genetic influence on*Nrf2*transcriptional activity in alveolar macrophages because Nrf2 appears to exert its protective effects through the transcriptional activation of antiprotease and antioxidant genes in alveolar macrophages[14]and its mRNA expression is decreased in macrophages of COPD patients[15]. As the rs6726395 SNP was not in significant LD with any of the 3 functional SNPs known to reside in the promoter region, rs6726395 may be in LD with other causal SNPs in or nearby the *Nrf2* gene; the allele responsible for the protective effects observed in this study could be on the extended rs2001350T/rs6726395A/rs1962142A/rs2364722A/rs6721961T haplotype. We have not comprehensively assessed the genetic variation in *Nrf2*, and the functional impact of the *Nrf2* SNPs carried on different haplotypes is still unknown. The identification of functional variants in *Nrf2* loci will require fine mapping efforts using large populations. However, the lack of suitable Japanese cohorts with the measurements of annual decline in FEV_1 and genomic DNA samples available for genotyping prevented us from performing a replication of this study on the association of rs6726395 with FEV_1 decline.

In the present study, the annual declines in FEV_1 were estimated by longitudinal retrospective measurements. The natural course of FEV_1 over time is divided into three phases; a lung growth phase occurs during childhood and adolescence, this grow phase is followed by a plateau phase, and a decline phase begins at about 25 years of age [35]. The level of FEV_1 at a given time in adulthood is affected by any deterioration that occurred in any of these 3 phases. Because we planned to analyze the effects of oxidative stress caused by cigarette smoking on lung function, FEV_1 decline over time calculated in a longitudinal study was more valuable than absolute values of pulmonary function measurements in a cross-sectional study. As the ages of all the participants in this study were over 25 years at the first visit, all the subjects were thought to be in the decline phase of lung function.

Siedlinski et al. [17] have reported that the heterozygote genotype of rs2364723, which is in the first intron of *Nrf2*,

is associated with a lower level of FEV_1 in Caucasian smokers. However, they showed no relationship between rs2364723 and annual decline in FEV_1. Because rs2364723 is in complete LD with rs2364722 (r^2 = 1.00) in the JPT population of the HapMap group, the finding from the Siedlinski et al. investigation may be compatible with the result from the present study. Our results are also consistent with findings from a previous Japanese case-control association study that showed no relationship between the 3 previously identified functional SNPs in the promoter region (rs6721961, rs6706649, and rs35652124) and susceptibility to COPD [36]. Recently, two large-scale genome-wide association studies have identified several loci associated with FEV_1 and FEV_1/FVC ratio [10,11]. Reports from the studies included lists of the top 2,000 SNPs related to the pulmonary function measurements. SNPs in or nearby the *Nrf2* gene including the SNPs in the current study were not among these top 2,000 SNPs.

In order to ensure a reliable estimate of FEV_1 decline, we selected the subjects who provided at least 4 valid PFT measurements over a period of at least 4 years. Variation in the follow-up periods and the numbers of visits could contribute to bias in estimations of annual FEV_1 decline; therefore, we used a linear mixed-effects model to control for correlations among repeated measures from each subject. Moreover, inhaled corticosteroids, leukotriene receptor antagonists, and bronchodilators (e.g., β-adrenoceptor stimulants and anticholinergic agents) can improve FEV_1 measurements in asthmatic and/or COPD subjects; therefore, we excluded those subjects with a history of asthma and/or COPD treated with these medications during the retrospective study period. Nevertheless, because this study was retrospective, attrition could affect the estimation of FEV_1 decline. Subjects with accelerated decline in FEV_1 are more likely to drop out from the annual health checkup. However, as attrition is likely to result in an underestimate of annual FEV_1 decline, it would bias the study against finding an effect.

Conclusions

We demonstrated an association between a SNP (rs6726395) in the first intron of the *Nrf2* gene and annual decline in FEV_1. In smokers, individuals carrying the major allele of this SNP showed greater decline in FEV_1 than those homozygous for the minor allele; however, this effect was not seen in never-smokers. Although the direct functional effect of rs6726395 on the *Nrf2* gene is unknown, this risk allele may be useful as a clinical marker for identifying individuals particularly susceptible to loss of lung function due to cigarette smoking. Our study suggests that pharmacological activation of Nrf2 by chemopreventive and phytochemical agents may be the strategy capable of exerting protective effects against various stress conditions including increased annual decline in FEV_1 associated with CS.

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Authors' contributions

HM performed the subject recruitment, data collection, laboratory work, statistical analysis and manuscript writing. TN provided support in the development of the population. TS, HI and YK supervised clinical characterization and contributed to patient recruitment, the statistical analyses and the interpretation of clinical and genetic data. TH contributed to developing and performing the genotyping assays. MT contributed to overseeing the genotyping assays. EN contributed to overseeing the statistical methods and analysis of the genetic data. NH conceived the project design, supervised the study, discussed the results and finalized the manuscript. All authors contributed to and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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