



# A Polymorphism rs6726395 in Nrf2 Contributes to the Development of Emphysema-Associated Age in Smokers Without COPD

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## Abstract

**Introduction** Several studies have reported that single nucleotide polymorphisms (SNPs) in the gene encoding NF-E2-related factor 2 (Nrf2) contribute to airflow limitations in smokers without COPD. Although small airway lesions and emphysema contribute cooperatively to airflow limitation, the relationship between Nrf2 SNPs and the development of emphysema in smokers without COPD is not well understood.

**Methods** Healthy subjects who underwent an annual health checkup with computed tomography (CT) of the chest at Osaka City University Hospital were prospectively recruited. The percentage of low-attenuation area (%LAA) on chest CT was quantified, and correlations between %LAA, Nrf2 SNP [rs6726395 (G/A)] genotypes, and clinical characteristics were examined.

**Results** A total of 245 subjects without COPD [non-/light-smoker: 153 (62.4%) and smoker: 92 (37.6%)] were enrolled. The %LAA in the upper lung field was higher than that in the lower lung field ( $p < 0.001$ ). The %LAA in smokers was significantly higher than that in non-/light-smokers ( $p = 0.021$ ). The %LAA showed significant but weak correlation with age in all subjects ( $r = 0.141$ ,  $p = 0.028$ ). Divided by genotype, the %LAA of the upper lung field was significantly correlated with age in smokers with genotype GG (wild type) ( $r = 0.333$ ,  $p = 0.022$ ), but was not significantly correlated with age in smokers with genotype AG/AA. These correlations were not observed in non-/light smokers.

**Conclusion** A polymorphism rs6726395 in Nrf2 can contribute to the development of emphysema-associated aging in smokers. The Nrf2 SNP may be a predictive factor for smoking-induced emphysema, and genotyping of Nrf2 SNP may serve as biomarker for emphysema prevention.

**Keywords** Emphysema · COPD · Single nucleotide polymorphism · NF-E2-related factor2 · Percentage of low-attenuation area

## Introduction

Chronic obstructive pulmonary disease (COPD) is a high ranking cause of death, and COPD risk assessment is an important part of primary prevention. Small airway lesions and emphysema contribute cooperatively to airflow limitations in COPD. Although emphysema can occur without the airflow limitation that defines COPD [1], pathological emphysema has been identified both in patients with COPD

and in healthy adults with normal lung function. It has been demonstrated that emphysema is correlated with the development of airflow limitations in healthy adults, including those who have never smoked [2]. The most important environmental factor in development of emphysema is cigarettes smoke, which contain high concentrations of oxidants [3]. Oxidative stress promotes inflammatory gene expression through NF- $\kappa$ B signaling, which induces apoptosis of airway epithelial and vascular endothelial cells and leads to emphysema [4, 5].

NF-E2-related factor 2 (Nrf2) is a transcription factor and a master regulator of antioxidant responses to oxidative damage resulting from injury and inflammation. It has been reported that Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema [6]. Moreover, we have recently reported that expression of Nrf2 in bronchial

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and alveolar epithelial cells was significantly decreased in patients with COPD [7]. These studies indicate that there is a correlation between the Nrf2-regulated antioxidant defense system and oxidant-induced inflammation in the lungs.

Some studies have reported that single nucleotide polymorphisms (SNPs) in Nrf2 contribute to annual decline in forced expiratory volume in one second (FEV1) in healthy smokers [8, 9]. Because COPD in the Japanese population is predominantly emphysematous, and little is known about the role of Nrf2 SNPs on the pathology of emphysema, studying Nrf2 SNPs has the potential to provide insights into the nature of emphysema in Japanese patients and reveal new targets for therapies. Here, we focused on one Nrf2 SNP (rs6726395) shown to be related to a decline in lung function and conducted a study in the general Japanese population to evaluate correlations between this SNP and the extent of emphysema.

## Methods

### Study Subjects

A total of 273 healthy subjects who underwent an annual health checkup at the MedCity21 clinic of Osaka City University Hospital between May 2017 and December 2017 were prospectively recruited into this study. All subjects completed medical interviews, including medical history and cigarette smoking history (Pack year), physical examinations, routine blood studies, pulmonary function test (PFT), and high-resolution computed tomography (CT) of the chest. Based on data from PFT, 28 subjects were excluded from the study because they had already been diagnosed with COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [10]. A total of 245 subjects were enrolled for analysis. In this study, ‘non-/light-smoker’ was defined as a participant who smoked less than 10 Pack year, and ‘smoker’ was defined as a participant who smoked more than or equal to 10 Pack year. This study was approved by the ethics committee of Osaka City University Hospital (approval 3758), and all patients provided informed consent for their participation. All procedures were performed according to the guidelines of the Declaration of Helsinki.

### The Percentage of Low-Attenuation Areas (%LAA)

The chest CT was performed using a Whole Body X-ray CT System [Supria (16ch), Hitachi, Ltd., Tokyo, Japan]. The scan time was 0.75 s, and the image matrix was 512 × 512 pixels. We reconstructed 1-mm-thick thin-slice CT images for all lung fields, and all images were analyzed using Airway Inspector software (Surgical Planning Laboratory at

Brigham and Women’s Hospital, Boston, MA, USA). Areas with attenuation less than –950 Hounsfield units were defined as low-attenuation area (LAA), suggesting emphysema, and the ratio of LAA to the total area was defined as %LAA. We quantified %LAA for the upper-, middle-, lower-, and total lung areas, as previously described [11].

### Single-Nucleotide Polymorphism (SNP) Selection and Genotyping

Genomic DNA was extracted from blood samples obtained from each participant using the Gentra Puregene Blood Kit Plus (Qiagen NV, Venlo, the Netherlands). SNP (rs6726395) genotyping in the Nrf2 gene was carried out for each participant with 50 ng genomic DNA, and the predesigned TaqMan allele-specific polymerase chain reaction (PCR) assays using a GeneAmp PCR System (Applied Biosystems, Foster City, CA). All genotypes (GG (major allele homozygotes), AG, and AA) of all subjects were detected.

### Statistics

Correlations between %LAA, clinical characteristics, and rs6726395 (G/A) genotypes in Nrf2 gene were examined. Data are presented as mean ± standard deviation (SD), unless otherwise stated. Statistical analyses were performed using JMP version 10.0.0 software for Windows (SAS Institute, Cary, NC, USA). In all statistical analyses, a  $p < 0.05$  was considered significant. We performed univariate analysis to evaluate the correlations of clinical characteristics with %LAA or Nrf2 gene SNP (rs6726395). Fisher’s exact test was used for comparisons of categorical variables such as gender, smoking status, and genotype. For continuous variables, such as age, body mass index (BMI), and PFT measurements, the correlation with %LAA was assessed by Pearson’s correlation coefficient analysis. For comparisons of categorical variables and continuous variables, parametrically unpaired Student’s *t*-tests were used for comparison of two categorical group means. The correlations of rs6726395 genotypes with %LAA were analyzed by multivariate linear regressions adjusted for potential confounding factors, such as age and BMI. We did not include other variables (gender and PFT measurements) as confounding factors in a consideration of multicollinearity.

## Results

The characteristics of the 245 subjects without COPD [non-/light-smoker: 153 (62.4%) and smoker: 92 (37.6%)] in this study are summarized in Table 1. The comparison of %LAA in clinical variables and each lung area is shown in Table 2. The mean value of %LAA in the total lung field of males

**Table 1** Characteristics of the study subjects

Characteristics	Subjects ( <i>n</i> = 245)
Age (years)	53.6 ± 11.7
Gender— <i>n</i> (%)	
Male	150 (61.2)
Female	95 (38.8)
BMI (kg/m <sup>2</sup> )	22.9 ± 2.7
FVC (L)	3.70 ± 0.85
FVC % predicted (%)	102.9 ± 12.7
FEV <sub>1</sub> (L)	2.93 ± 0.67
FEV <sub>1</sub> /FVC (%)	79.6 ± 5.11
FEV <sub>1</sub> % predicted (%)	97.8 ± 12.4
Pack year— <i>n</i> (%)	
< 10	153 (62.4)
10 ≤	92 (37.6)

Data are presented as mean ± SD or *n* (%)

**Table 2** Comparison of %LAAs in clinical variables and areas in each lung

Variables	% LAA* (mean ± SD)	<i>p</i> Value
Gender		< 0.001
Male	21.2 ± 5.3	
Female	16.6 ± 4.5	
Pack year		0.021
< 10	18.8 ± 5.4	
10 ≤	20.5 ± 5.4	
Lung area		
Upper	20.6 ± 5.2**	
Middle	19.5 ± 5.5	
Lower	18.2 ± 6.2**	
Total	19.4 ± 5.5	

\*%LAA the ratio of low-attenuation areas to all lung areas

\*\**p* < 0.001 (upper vs lower)

was significantly higher than in females (*p* < 0.001). As expected, the mean value of %LAA in the total lung field of smokers was significantly higher than in non-/light-smokers (*p* = 0.021). The mean %LAA in the upper lung field was significantly higher than in the lower lung field (*p* < 0.001). Dividing the subjects into smoker and non-/light-smoker groups produced similar results in both groups (*p* < 0.001; data not shown).

We performed univariate analyses to assess correlations between %LAA and clinical variables in Table 3. The %LAA showed significant but weak correlation with age in all subjects, including smokers and non-/light-smokers (*r* = 0.141, *p* = 0.028). There was no significant correlation between %LAA and BMI. Regarding pulmonary function, %LAA was significantly correlated with FVC (*r* = 0.427, *p* < 0.001),

**Table 3** Correlation between %LAA and clinical variables

Variables	Correlation coefficient	<i>p</i> Value
Age	0.141	0.028
BMI (kg/m <sup>2</sup> )	−0.004	n.s
FVC (L)	0.427	< 0.001
FVC % predicted (%)	0.301	< 0.001
FEV <sub>1</sub> (L)	0.345	< 0.001
FEV <sub>1</sub> % predicted (%)	0.089	< 0.001
FEV <sub>1</sub> /FVC (%)	−0.286	n.s

**Table 4** Classification of clinical variables with respect to rs6726395 genotypes

Variables	Nrf2 gene SNP (rs6726395)		<i>p</i> Value
	GG	AA/AG	
Subjects	116 (47.3)	129 (52.7)	
Age	53.5 ± 11.7	53.7 ± 11.8	n.s
Gender— <i>n</i> (%)			n.s
Male	73 (48.7)	77 (51.3)	
Female	43 (45.3)	52 (54.7)	
BMI (kg/m <sup>2</sup> )	23.1 ± 2.7	22.7 ± 2.7	n.s
Pack year— <i>n</i> (%)			n.s
< 10	69 (45.1)	84 (54.9)	
10 ≤	47 (51.1)	45 (48.9)	
FVC (L)	3.72 ± 0.85	3.67 ± 0.84	n.s
FVC % predicted (%)	102.4 ± 12.8	103.4 ± 12.7	n.s
FEV <sub>1</sub> (L)	2.96 ± 0.68	2.92 ± 0.65	n.s
FEV <sub>1</sub> /FVC (%)	79.5 ± 5.2	79.7 ± 5.0	n.s
FEV <sub>1</sub> % predicted (%)	97.0 ± 12.0	98.4 ± 12.7	n.s
%LAA (upper)	20.1 ± 5.3	20.9 ± 5.1	n.s
%LAA (middle)	19.0 ± 5.7	20.0 ± 5.3	n.s
%LAA (lower)	17.6 ± 6.5	18.7 ± 6.0	n.s
%LAA (total)	18.9 ± 5.6	19.9 ± 5.3	n.s

Data was expressed mean ± SD otherwise indicated

%FVC (*r* = 0.301, *p* < 0.001), FEV<sub>1</sub> (*r* = 0.345, *p* < 0.001), and %FEV<sub>1</sub> (*r* = 0.089, *p* < 0.001). However, there was no significant correlation between %LAA and FEV<sub>1</sub>/FVC.

Genotype at SNP (rs6726395) in the Nrf2 gene was successfully detected for all subjects. The numbers of subjects with each genotype, GG, AG, and AA, were 116, 109, and 20, respectively. The group of genotype AA was very small, and was combined with group AG and designated group AG/AA. Relationships between the genotypes and clinical variables are shown in Table 4. There were no significant differences between any of the measured variables (age, gender, BMI, smoking status, the parameters of PFT, and %LAA) and genotypes (GG and AG/AA). Although the difference did not reach the statistical significance, the %LAA of genotype AG/AA was higher than that of genotype GG.

As emphysema in the upper lung field was prominent, the %LAA of the upper lung field in smokers was analyzed. Multivariate linear regressions of correlations between Nrf2 gene SNP genotypes (rs6726395) and %LAA of the upper lung field in smokers are shown in Table 5. In univariate analysis, the %LAA in the upper lung field showed a significant correlation with age ( $r=0.333$ ,  $p=0.022$ ) and BMI

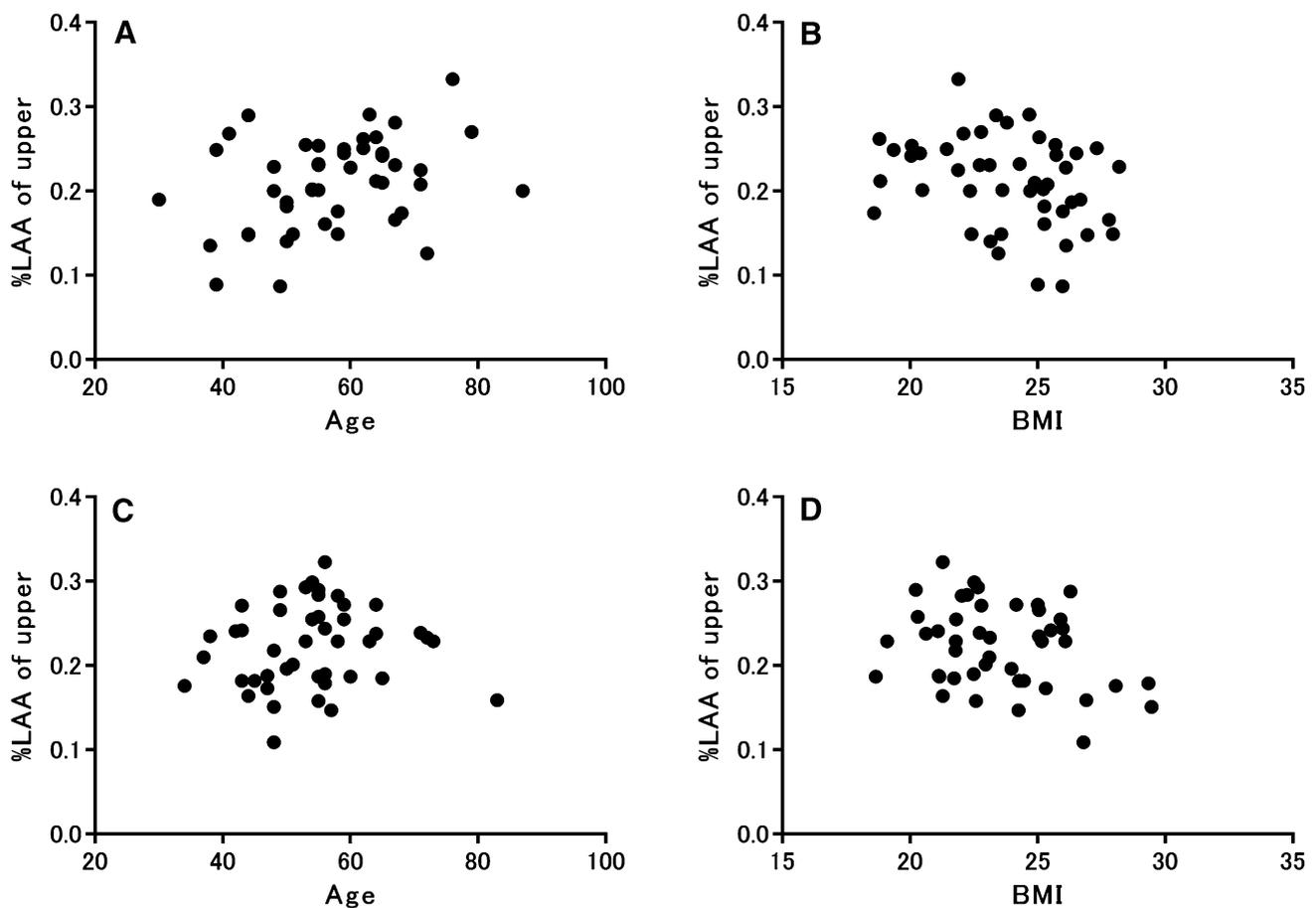
**Table 5** Multivariate linear regressions for correlation between ages or BMIs in each genotype at rs6726395 and %LAA of upper lung area in smokers

Genotype	Variable	Correlation coefficient	<i>p</i> Value	Corrected <i>p</i> value
GG	Age	0.333	0.022	<0.05 (0.0497)
	BMI	-0.298	0.042	n.s
AA/AG	Age	0.099	n.s	n.s
	BMI	-0.293	n.s	n.s

( $r=-0.298$ ,  $p=0.042$ ) in smokers without COPD with genotype GG (Fig. 1a, b), while the %LAA in the upper lung field showed no correlation with age and BMI in smokers without COPD with genotype AG/AA (Fig. 1c, d). Furthermore, adjusted for potential confounding factors as BMI, %LAA in the upper lung field was significantly correlated with age in smokers with genotype GG ( $p=0.0497$ ), while the %LAA was not significantly correlated with age in smokers with genotype AG/AA ( $p=0.582$ ). These correlations were not observed in non-/light-smokers with either genotype.

## Discussion

In the present study, we determined that a polymorphism of Nrf2 gene accelerated progress of upper lung emphysema-associated aging in smokers without COPD. We showed a



**Fig. 1** Correlation between %LAA in upper lung field and age or BMI in smokers without COPD with each genotype (GG and AG/AA). **a** %LAA in upper lung field showed a significant correlation with age ( $r=0.333$ ,  $p=0.022$ ) in smokers without COPD with genotype GG. **b** %LAA in upper lung field showed a significant correlation with BMI ( $r=-0.298$ ,  $p=0.042$ ) in smokers without COPD

with genotype GG. **c** %LAA in upper lung field showed no correlation with age ( $r=0.099$ ,  $p=0.519$ ) in smokers without COPD with genotype AG/AA. **d** %LAA in upper lung field showed no correlation with BMI ( $r=-0.293$ ,  $p=0.051$ ) in smokers without COPD with genotype AG/AA

correlation between a SNP of the Nrf2 gene and the extent of emphysema-associated aging in the general Japanese population, and our results indicate the presence of a gene–environment interaction in %LAA of the upper lung field. Several studies have implicated Nrf2 SNPs in some pulmonary diseases, such as lung adenocarcinoma, acute lung injury, and bronchial asthma [12–14]. Moreover, in some studies of healthy individuals from the general population, Nrf2 SNPs were shown to be correlated with FEV1 [8, 9]. We therefore hypothesized that Nrf2 SNPs could play some role in the development of emphysema-associated aging due to cigarette smoking. To investigate this, we evaluated correlations between a Nrf2 SNP and the extent of emphysema-associated aging in the general Japanese population.

Cigarette smoke contains various toxic substances, including high concentrations of oxidants [3]. Oxidants induce local inflammation, which develops apoptosis in airway epithelial and vascular endothelial cells and leads to emphysema [4, 5]. For this reason, the lungs of smokers easily become emphysematous. In the present study, the mean %LAA in smokers was significantly higher than that in non-/light-smokers. Emphysema is generally found in the upper lung field in COPD patients [11, 15], and it has been suggested that the cause of this distributional heterogeneity is the ventilation–perfusion mismatch between the upper and lower lobes [16]. In the current study, the mean %LAA in upper lung field was higher than that in lower lung field, even in smokers without COPD.

The initiation and development of emphysema can be impacted aging of the lung [17]. It has also been reported that cellular senescence-associated aging promotes abnormal cell turnover and development of emphysema [18]. We have also previously reported that levels of soluble  $\alpha$ -klotho, identified as a putative aging-suppressor gene, were significantly lower in smokers with COPD [19]. Therefore, aging is also a risk factor for emphysema, and the extent of emphysema was found to be weakly correlated with age in the current study.

Although recent large-scale genome-wide association studies have identified several loci related to measures of pulmonary function [20, 21], SNPs related to Nrf2 gene were not listed as whole subjects in these reports. Based on a report about the correlation between Nrf2 SNPs and FEV1 which focused on Japanese people with COPD, we have selected SNP (rs6726395) with relatively high minor allele frequency as the target SNP in this study [8]. The frequency of this allele differs by race, and the frequency of allele A is low in Asians [22]. The rs6726395 SNP is located in the first intron of the Nrf2 gene, and the specific functions of this SNP are unclear. In our study, the %LAA of the upper lung field was significantly correlated with age in smokers with genotype GG but not in smokers with genotype AG/AA. In major allele homozygotes (GG), our results reflect the general concept that cigarette smoke accelerates development

of upper lung emphysema-associated aging. By contrast, in genotype AG/AA with minor allele (A), this correlation was not observed. The rs6726395 SNP with allele A have been reported to have lower expression of Nrf2 compared with the allele G [23, 24], and genotype AG/AA might susceptible to oxidant-induced lung damage, i.e. emphysema. Interestingly, %LAA in genotype AG/AA was higher than in genotype GG with marginal significance. This tendency supports the reports of the lower expression of Nrf2 in allele A. In general, about 20% of smokers develop clinical COPD [25], but it is unclear what fraction of smokers develops smoking-induced emphysema-associated aging. In the current study, genotyping showed that 52.7% (AA/AG) of the study population was sensitive to smoking-induced emphysema. COPD is thought to have multiple genetic and environmental factors related to pathogenesis, which might explain this discrepancy.

PFT is recommended for COPD diagnosis [10] and early screening for unattributed or potential COPD subjects identified in health check-ups. PFT for COPD screening is comparatively easy and less invasive than other types of testing because it does not require irradiation. Recently low-dose chest CT has proved to be an efficient method for lung cancer screening [26]. Although the settings of chest CT and reconstruction algorithms need to be adjusted, chest CT screening at health check-ups can be conducted for both lung cancer and emphysema screening. Genotyping of the Nrf2 gene is less invasive than PFT or chest CT, because only sampling of genomic DNA is necessary for such screening. Moreover, genotyping can assess future risk of emphysema/COPD, while PFT and chest CT can only assess the lung at the present time.

Prevention of diseases can be achieved primarily through education and secondarily through early detection. Recently, earlier prevention has been advocated because environmental adjustment is important for public health. Incidence of inflammatory lung diseases like COPD is increased by noxious gases, mainly cigarette smoking. In addition to cigarette smoking, the issues of environmental pollution from second-hand smoke or air pollution must be considered as contributors to inflammatory lung diseases. Since genotyping can be used to determine susceptibility, it may be a tool that can be used in early prevention efforts. Results from the current study can contribute to development of this prevention concept.

Our study population had a limited sample size, and all subjects were enrolled from single center. As a result of this sample size limitation, we identified only a small number of smokers with genotype AA, and so this group was combined with the genotype AG group. For the same reason, we have not assessed other SNPs in the Nrf2 gene. Also, the functional effect of the Nrf2 SNP (rs6726395) in this study is still unknown. However, in the present study, we showed

correlations between a SNP (rs6726395) and the extent of upper lung field emphysema-associated aging in Japanese smokers. This might contribute to personalized medicine or prevention from COPD development, and Nrf2 could be an intervention target for COPD prevention and treatment. Further studies using larger populations will be required to confirm our findings.

## Conclusion

A polymorphism rs6726395 in Nrf2 can contribute to the development of emphysema-associated aging in smokers. The Nrf2 SNP may be a predictive factor for smoking-induced emphysema, and genotyping of Nrf2 SNP may serve as biomarker for emphysema prevention.

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## Compliance with Ethical Standards

**Conflict of interest** The authors have stated explicitly that there is no conflict of interest in connection with this article.

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