Genome-wide assessment of gene-by-smoking interactions in COPD

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Cigarette smoke exposure is a major risk factor in chronic obstructive pulmonary disease (COPD) and its interactions with genetic variants could affect lung function. However, few gene-smoking interactions have been reported. In this report, we evaluated the effects of gene-smoking interactions on lung function using Korea Associated Resource (KARE) data with the spirometric variables—forced expiratory volume in 1 s (FEV1). We found that variations in FEV1 were different among smoking status. Thus, we considered a linear mixed model for association analysis under heteroscedasticity according to smoking status. We found a previously identified locus near SOX9 on chromosome 17 to be the most significant based on a joint test of the main and interaction effects of smoking. Smoking interactions were replicated with Gene-Environment of Interaction and phenotype (GENIE), Multi-Ethnic Study of Atherosclerosis-Lung (MESA-Lung), and COPDGene studies. We found that individuals with minor alleles, rs17765644, rs17178251, rs11870732, and rs4793541, tended to have lower FEV1 values, and lung function decreased much faster with age for smokers. There have been very few reports to replicate a common variant gene-smoking interaction, and our results revealed that statistical models for gene-smoking interaction analyses should be carefully selected.

The spirometric measurement forced expiratory volume in the first second (FEV1) reflects the physiological and functional state of the lungs; this measure has been used as the gold standard for diagnosing a lung disease, classifying its severity, assessing its progression over time, and monitoring the treatment response1. Furthermore, this parameter is a predictor of other morbidities and mortality in the general population, even independent of smoking history2,3. Reduced FEV1 is a characteristic of chronic obstructive pulmonary disease (COPD), a leading cause of mortality and morbidity worldwide4,5. The prevalence and burden of COPD are expected to increase in the coming decades owing to continued exposure to COPD risk factors and the aging population6.

Multiple risk factors for COPD have been identified, and smoking has been recognized as the major risk factor for a rapid decline in lung function and consequent development of COPD. However, only a minority of smokers develop COPD7, and there are substantial differences in the sensitivity to smoking among individuals. These differences are partly attributable to genes and/or their interactions with smoking. Heritability estimates of lung function range from 39% to 66%7,8. Moreover, a hereditary severe deficiency in alpha-1 antitrypsin, encoded by SERPINA1 on chromosome 149, is the best known genetic risk factor for the development of COPD. However, severe alpha-1 antitrypsin deficiency accounts for only about 1% of the patients with COPD10. Thus, improving our understanding about disease pathogenesis and progression would require studies on genetic susceptibility loci and their interactions with smoking.
Recently, many genome-wide association studies (GWASs) have been conducted to identify the genetic loci associated with lung function levels, and many genome-wide significant loci have been identified. The necessity of replication across populations with diverse ethnic or environmental characteristics has been reported. However, loci identified from GWASs have often failed to be replicated in different populations. There are many reasons for this inconsistency, and several studies have shown a partial relation with gene-environment interactions. Smoking has a strong effect on lung function, and the effects of gene-smoking interactions on lung function have been repeatedly highlighted. However, the effects of gene-smoking interactions on variability in lung function in different ethnicities are not clear. Furthermore, because COPD is expected to be the fifth most common disease worldwide, with the third highest mortality rate in 2020, and because its burden, including financial cost, is predicted to increase, early prediction of lung function may be important for developing individualized therapeutic strategies, and further studies are required to identify genetic factors that predict the risk for a subsequent rapid decline in lung function across ethnicities.

In this study, we aimed to identify the genetic variants interacting with smoking on FEV$_1$, using genome-wide interaction studies (GWISs). We considered various models in terms of smoking-related variables and variance-covariance structure, and the best model was chosen by Akaike information criterion (AIC) for each dataset. GWISs were conducted using the Korea Associated Resource (KARE) data. We detected that SNPs with the smallest P-values are located near SOX9. The SOX9 has been reported to be involved in lung branching morphogenesis and recovery of lung function after lung injury. It should be noted that genetic association of SOX9 was firstly detected by Hankcock et al., but their interaction P-values were larger than 0.05. We replicated interactions in the Gene-Environment of Interaction and phenotype (GENIE), Multi-Ethnic Study of Atherosclerosis-Lung (MESA-Lung), and COPDGene studies. Our findings provide important insights into our understanding about lung disease prevention and control.

Methods

Data description. Our analyses consisted of two phases—discovery and replication. For the discovery phase, we conducted GWISs on FEV$_1$ using KARE data. For the replication phase, we considered GENIE, MESA-Lung, and COPDGene data, and replicated the significant results identified from the filtering step. Detailed procedure for genotyping, quality controls (QC), and imputations for each data are described in the Supplementary Text 1.

KARE. Data collected by the KARE project were used for GWASs. Participants were recruited from the rural Ansan and urban Ansan cohorts. Initiated in 2001 as part of the Korean Genome Epidemiology Study (KoGES), the initial samples included 5,018 and 5,020 participants aged 40–69 years from Ansan and Ansan areas, respectively. After QC of genotypes, there were 8,534 participants between the age of 40 and 69 years with at least one spirometry test and genotype data (see Supplementary Text 1 for detailed procedures about QC). Among these participants, 4,001 were men and 4,533 were women. The values of FEV$_1$ were observed up to three times every two years, and 19,557 measurements were used for the analyses. Smoking history was obtained through a questionnaire, and smoking status and pack years were used for association analyses as covariates. Smoking status was categorized as never smokers, former smokers, and current smokers. Never smokers were defined as individuals who had never smoked, and former smokers who had smoked previously, but stopped smoking prior to the survey. Current smokers were individuals who stated that they currently smoked during the investigation, or who had a record of smoking and did not belong to the other two categories. According to our categorization, there were 4,926 never smokers, 1,742 former smokers, and 1,866 current smokers in our cohort.

GENIE. GENIE data were used to replicate SNPs identified from GWISs using KARE data. The GENIE cohort consisted of 7,999 participants, who had visited Seoul National University Gangnam Center during 2014–2016. They agreed to participate in genetic studies and donated blood samples, and after QC, there were 5,971 participants (3,404 men and 2,567 women) with spirometry and smoking-related variables (see Supplementary Text 1 for detailed procedures about QC). Spirometry and smoking-related variables were repeatedly measured up to 11 times. Smoking-related variables were obtained by questionnaire. Based on their responses, smoking status was categorized into three groups, similar to the smoking status categories used in the KARE data. The numbers of never smokers, former smokers, and current smokers were 3,396, 1,804, and 771, respectively.

MESA-Lung non-Hispanic whites. MESA-Lung data were used to replicate SNPs identified from GWISs using KARE data. MESA was a prospective cohort initiated to investigate cardiovascular diseases. Participants consisted of 6,814 men and women aged 45–84 years. As a subgroup of the MESA cohort, the MESA-Lung study enrolled 3,965 participants who were sampled from the MESA cohort, and agreed to participate in the genetic analysis and to measure their lung functions. The MESA-Lung study was composed of four populations—non-Hispanic whites (NHWs; 35%), African-Americans (AAs; 26%), Hispanics (23%), and Chinese-Americans (16%)—and we considered only NHWs. After QC of genotypes, 1,033 participants had both spirometry and smoking-related variables with genotype data, including 459 never smokers, 468 former smokers, and 106 current smokers (see Supplementary Text 1 for detailed procedures about QC).

COPDGene. COPDGene was a multi-center study on smokers with and without COPD, and included AAs and NHWs. All participants had at least 10 pack years of smoking, and their ages were between 45 and 80 years. Pre- and post-bronchodilator spirometric data were obtained for all participants with standardized spirometry (EasyOne Spirometer; Zurich, Switzerland); to be consistent with other studies, we focused on pre-bronchodilator spirometry. After QC of genotypes, 7,760 NHWs and 3,300 AAs were enrolled for replication studies (see Supplementary Text 1 for detailed procedures about QC). AAs consisted of 2,643 current smokers and 657 former smokers, and NHWs consisted of 2,616 current smokers and 4,054 former smokers.
**Statistical analysis.** Smoking has a significant effect on respiratory function, and it was found that variances of FEV₁ can differ by smoking status. Linear mixed models which allow heteroscedasticity according to smoking status were computationally very intensive and we consider a two step approach; (i) filtering step and (ii) testing step. In the filtering step, participants were stratified according to the smoking status and a likelihood ratio test with 3 degrees of freedom which allow heteroscedasticity according to smoking status were applied to select the most significantly associated SNPs. Filtering step was considered only for discovery data, and the likelihood ratio test will be called 3 DF test in the remainder of this article. For testing step, we consider several variance-covariance structures for the linear mixed models, and the best model was chosen with AIC. Then former, current, and never smokers were pooled, and linear mixed models with the smallest AIC were applied to the SNPs selected from the filtering step.

**Filtering step: genome-wide interaction studies (GWISs) with KARE data.** We considered FEV₁ (mL) as spirometric measures, which were used to identify the genetic variants interacting with smoking. GWISs were conducted using KARE data. We found that there were no substantial differences in spirometric measures between current smokers and former smokers for KARE data with AIC, and both groups were combined into a single group; ever smokers. To handle heteroscedasticity, we conducted stratified analyses which applied linear mixed models to never smokers and ever smokers separately for GWISs. For both groups, sex, age at baseline measurement, height, body mass index (BMI), elapsed time from the baseline measurement, and interaction of age and sex were included as covariates. It was reported that lung function decline accelerates after he or she becomes 35 years old[^23], and all participants in KARE data were around 40–69 years old at the baseline. Thus, effect of ages on the baseline pulmonary function measurement was that of elapsed time on their decrement were expected to be substantially different, and it is a main reason why the baseline age and elapsed time from the baseline measurement were considered as different covariates. To adjust for population substructure strictly, principal component (PC) analyses were applied to the genetic relationship matrix, and the first 10 PC scores were included as covariates[^23]. FEV₁ of each participant was measured up to 3 times, and FEV₁ at each time point was included as response variables. The similarities among repeated spirometric measures for each participant were handled with a random intercept. We let \( y_{ij} \) be FEV₁ values for participant \( i \) at time point \( j \), and they were assumed to follow multivariate normal (MVN) distribution. We denote elapsed time from baseline measurement and PC scores for participant \( i \) and component \( k \) by \( time_{ij} \) and \( pc_{ik} \). Then the linear mixed model for ever smokers becomes

\[
y_{ij} = \beta_0 + \beta_1 age_i + \beta_2 sex_i + \beta_3 BMI_i + \beta_4 height_i + \beta_5 time_{ij} + \beta_6 pack\, year_i + \beta_7 sex_i \cdot age_i + \beta_8 SNP_i + \beta_9 SNP \cdot pack\, year_i + \sum_{k=1}^{10} \tau_k pc_{ik}^k + b_i + \epsilon_{ij}, \quad (\epsilon_{ij1}, ..., \epsilon_{ijn})^T \sim MVN (0, \Sigma), \quad b_i \sim iid \, MVN (0, \sigma^2)
\]  

(1)

For never smokers, we consider

\[
y_{ij} = \beta_0' + \beta_1' age_i + \beta_2' sex_i + \beta_3' BMI_i + \beta_4' height_i + \beta_5' time_{ij} + \beta_6' sex_i \cdot age_i + \beta_7' SNP_i + \sum_{k=1}^{10} \tau_k pc_{ik}^k + \beta_i' + \epsilon'_{ij}, \quad (\epsilon'_{ij1}, ..., \epsilon'_{ijn})^T \sim MVN (0, \Sigma'), \quad \beta_i' \sim iid \, MVN (0, \sigma_i'^2)
\]  

(2)

It should be noted that pack years are 0 for never smokers and were included as covariates, respectively. We compared several structures for \( \Sigma \) and \( \Sigma' \), and selected an unstructured covariance structure. The proposed models were applied to detect gene-smoking interaction of FEV₁ average levels. To identify SNPs interacting with smoking on spirometric measures, we considered \( H_0: \beta_7 = \beta_8 = \beta_9 = 0 \). This could be tested by summing a likelihood ratio test with 2 degrees of freedom (DF) for ever smokers and a likelihood ratio test with 1 DF for never smokers. The summed likelihood ratio test followed the chi-square test with 3 DF under the null hypothesis, and this statistic would be denoted as the 3 DF test. The most significant SNPs were selected for further analyses of gene-smoking interaction effects.

We compare the results from 3 DF test with the homoscedasticity model. For homoscedasticity model, ever and never smokers were pooled and linear mixed model was fitted. We assume the same variances between never smokers and ever smokers, and the same coefficients of covariates as follows:

\[
y_{ij} = \beta_0 + \beta_1 age_i + \beta_2 sex_i + \beta_3 BMI_i + \beta_4 height_i + \beta_5 time_{ij} + \beta_6 pack\, year_i + \beta_7 sex_i \cdot age_i + \beta_8 smoking\, status_i + \beta_9 sex_i \cdot smoking\, status_i + \beta_{10} SNP_i + \beta_{11} SNP \cdot smoking\, status_i + \beta_{12} SNP \cdot pack\, years_i + \sum_{k=1}^{10} \tau_k pc_{ik}^k + \gamma_i pc_{1i} + \gamma_i pc_{1i}^1 \cdot smoking\, status_i + b_i + \epsilon_{ij}, \quad (\epsilon_{ij1}, ..., \epsilon_{ijn})^T \sim MVN (0, \Sigma), \quad b_i \sim iid \, MVN (0, \sigma^2)
\]  

(3)

Then P-values for homoscedasticity model were obtained by likelihood-ratio tests with 3 degrees of freedom for \( H_0: \beta_7 = \beta_8 = \beta_9 = 0 \). This will be called homoscedasticity test in the remainder of this article.
Testing step: estimating the effects of SNPs and SNP-smoking interaction effects with KARE data. The 3 DF test provides P-values for overall effects about the main and interaction effects. However, it could not identify interaction effects of SNPs with smoking on lung function because never and ever smokers were separately analyzed. Furthermore, stratified analyses are usually less powerful compared to analyses using pooled data. Thus, former, current, and never smokers in KARE data were pooled and analyzed by a linear mixed model.

While building the linear mixed model, we first assessed variance-covariance structures by smoking status. If heteroscedasticity is not correctly taken into account, the false positive rates cannot be controlled with P-values [25]. Furthermore, modeling the relationship between smoking-related variables and FEV1 is not clear. If lung function of the participants worsens, they tend to quit smoking, and such indication biases make the relationships complicated. Thus, the appropriate choice of smoking-related variables may depend on sampling strategies. We considered various models with the choices of smoking-related variables and variance-covariance structure by smoking status for each dataset, and the best model was selected with the AICs. Notably, SNPs were not included for the model selection, and this step did not violate any statistical inference.

Supplementary Table S1 presents AICs for various models fitted to FEV1 for KARE data. Supplementary Table S1 shows that KARE data has the smallest AIC when smoking status had two levels (never versus ever smokers) instead of three levels, and different variances between never smokers and ever smokers were assumed. All covariates used for GWISs of KARE data were also included as covariates in our linear mixed model. We found that coefficients of some covariates differed according to smoking status, and interactions of some covariates and smoking-related variables were considered. For example, interactions between smoking status and time effects were significant at the 0.05 level and included as covariate. Furthermore, to control the effects of confounders on interaction effects, the interaction between confounders and environmental factors of interest should be considered for gene-environmental analyses [26]. Therefore, we considered interactions between PC scores for adjusting the population substructures and smoking status regardless of significance at 0.05 level. We considered g = 1 and 2 indicate ever smokers and never smokers, respectively, and nj is the number of repeatedly observed measurements for participant j. The best model selected with AIC was as follows:

\[
y_{ij} = \beta_0 + \beta_{age} x_{ij} + \beta_{sex} x_{ij} + \beta_{BMI_{ij}} + \beta_{height_{ij}} + \beta_{time_{ij}} + \beta_{pack\ years_{ij}}
+ \beta_{age} \cdot smoking\ status_{ij} + \beta_{sex} \cdot smoking\ status_{ij} + \beta_{BMI_{ij}} \cdot smoking\ status_{ij} + \beta_{height_{ij}} \cdot smoking\ status_{ij} + \beta_{time_{ij}} \cdot smoking\ status_{ij}
+ \sum_{k=1}^{10} \tau_k p_{ij}^k + \tau_1 p_{ij}^1 \cdot smoking\ status_{ij} + b_{ij} + e_{ij}, \quad \mathcal{E} \sim MVN(0, \Sigma_1), \quad b_{ij} \sim iid MVN(0, \sigma_y^2)
\]

This model includes two smoking-related variables—smoking status, and pack years. Therefore, both were used to define the interactions between SNP and smoking. To test gene and environment interactions, we considered

\[
y_{ij} = \beta_0 + \beta_{age} x_{ij} + \beta_{sex} x_{ij} + \beta_{BMI_{ij}} + \beta_{height_{ij}} + \beta_{time_{ij}} + \beta_{pack\ years_{ij}}
+ \beta_{age} \cdot smoking\ status_{ij} + \beta_{sex} \cdot smoking\ status_{ij} + \beta_{BMI_{ij}} \cdot smoking\ status_{ij} + \beta_{height_{ij}} \cdot smoking\ status_{ij} + \beta_{time_{ij}} \cdot smoking\ status_{ij} + \beta_{SNP} \cdot pack\ years_{ij} \cdot smoking\ status_{ij}
+ \sum_{k=1}^{10} \tau_k p_{ij}^k + \tau_1 p_{ij}^1 \cdot smoking\ status_{ij} + b_{ij} + e_{ij}, \quad \mathcal{E} \sim MVN(0, \Sigma_2), \quad b_{ij} \sim iid MVN(0, \sigma_y^2)
\]

Replication studies. SNPs selected from GWISs using KARE data were replicated with GENIE, MESA-Lung, and COPDGene data. For each dataset, we considered various variance-covariance structures, and the best model was selected with AICs. Supplementary Tables S2–S4 show AICs for GENIE, MESA-Lung, and COPDGene data, respectively. We considered various models with the choices of smoking-related variables and variance-covariance structure by smoking status for each dataset, and the best model was selected with the AICs. Notably, SNPs were not included for the model selection, and this step did not violate any statistical inference.

Data availability. All data analyzed in this article were utilized in previously published articles (KARE: Cho, Go et al. 27; GENIE: Choe, Lee et al. 26; MESA-Lung: Hankinson, Kawt et al. 21; COPDGene: Castaldi, Cho et al. 22).

Results Descriptive statistics. Table 1 shows baseline characteristics of participants in the KARE, GENIE, MESA-Lung, and COPDGene data. KARE and GENIE data were from the Korean population and included both baseline and longitudinal data. MESA-Lung and COPDGene were cross-sectional data, and participants in MESA-Lung were NHWs, whereas COPDGene data consisted of AAs and NHWs. In the KARE data, there were 8,534 participants, of which 47% were men. In the KARE data, participants were 40–69 years old, and the percentage of never smokers, former smokers, and current smokers were 58%, 20%, and 22%, respectively. The GENIE cohort...
composed of participants who were regularly screened for health, and their average lung function values were expected to be better than those of the general population. There were 5,971 participants, of which 57% were men. Participants were repeatedly measured an average of 3.13 times. In the GENIE data, participants were 30–84 years old, which explained the largest range of FEV1. In the GENIE data, the percentages of never smokers, former smokers, and current smokers were 56.9%, 30.2%, and 12.9%, respectively. MESA-Lung data consisted of 1,033 participants, of which 50.5% were men. These participants were 45–84 years old, and 44.4%, 45.3%, and 10.3% were never, former, and current smokers, respectively. Lastly, COPDGene data consisted of AAs and NHWs. The number of AAs was 3,300, of which 56% were men; these participants were 45–80 years old, and the percentages of former and current smokers were 20% and 80%, respectively. The number of NHWs was 6,670, of which 52% were men. Participants were 45–80 years old, and 61% and 39% were former and current smokers, respectively. Unlike KARE, GENIE, and MESA-Lung data, there were no never smokers in the COPDGene data, and participants in the COPDGene data had the lowest mean FEV1 and the highest pack years.

### Heterogeneity of FEV1 decline along ages. Figure 1 shows the estimated FEV1 according to age and their 95% confidence intervals. This generalized additive models were applied for MESA-Lung and COPDGene data. KARE and GENIE data have the repeated measures of FEV1 levels, and the generalized additive mixed models were used. According to Figure 1, there were substantial differences among FEV1 according to the smoking status for each dataset. Current smokers in KARE and MESA-Lung data tended to have the lowest FEV1 values, followed by former smokers. For GENIE data, there were no differences in FEV1 values among never, former, and current smokers. Interestingly, for NHWs and AAs in COPDGene data, average FEV1 values of former smokers were smaller than those of current smokers, even though the differences were quite small for NHWs. This difference likely relates to selection bias, because participants with the worse lung function tended to quit smoking[8,29], and only heavy smokers were considered for COPDGene. These results suggest that the same model could not be applied to different data to identify SNPs interacting with smoking, and the best model for the choice of smoking-related covariates and variance-covariance structures were selected with AICs.

### GWISs of FEV1 with KARE data. In the discovery phase, associations of 310,515 SNPs were tested by applying the 3 DF test to KARE data. For the GWISs of FEV1, we included ten PC scores to adjust population substructure, and they explain 0.41% of genetic variances. Scatter plot for the first two PC scores and scree plot are provided in Supplementary Figure S1. Figure 2A presents the QQ-plot for 3 DF tests, and it shows that the proposed 3 DF tests generally preserve nominal significance levels. Variance-inflation factors (VIF) were estimated test was also applied[20] and Figure 2B shows its QQ plot. Figure 2B reveals evidence of some inflation, and VIF of homoscedasticity model, 1.22, is substantially larger than 1. Supplementary Figure S2 is a Manhattan plot of results from the 3 DF test. We further checked the effects of heteroscedasticity according to smoking status on FEV1/FVC by applying the same methods, and there were no significant results (Supplementary Table S5). Supplementary Figure S3A,B are based on their results from 3 DF test and homoscedasticity test, respectively. VIFs for both models were 1.01 and 1.12. Therefore, we can conclude that heteroscedasticity according to smoking status should be carefully addressed for identifying the interaction effects between SNPs and smoking on lung function.

Table 2 shows the most significant SNPs from results of 3 DF tests for FEV1. These selected SNPs were located in the upstream region of SOX9 on chromosome 17 and had similar minor allele frequencies (MAFs). Furthermore the linkage distribution plot in Figure 3A reveals that these factors were highly correlated, which indicates that four significant SNPs actually indicate same association signal. Figure 3B shows the regional plot for this region. Those 4 SNPs are located within topologically associated domains (TAD) of SOX9 region (68.67–70.45 Mb of chromosome 17 in hg19)[31–33]. DNA sequences within a TAD physically interact with each other more frequently than with sequences outside the TAD, and thus our most significant SNPs may affect the expression of SOX9. Therefore, our results indicate that SOX9 may be functionally related with FEV1.

### Table 1. Descriptive statistics Means of variables and their 95% confidence intervals are calculated.
Effects of SNPs and SNP-smoking interactions on spirometric measures in KARE data. 3 DF tests cannot separately estimate the main effects of SNPs and their interaction effects with smoking. In addition, ever and never smokers were separately analyzed, and such stratified analyses were less powerful than analyses with pooled data. Thus, all participants were pooled and analyzed with linear mixed models. Supplementary Table S1 shows AICs for the various models, and the selected model assumed different variances between never and ever smokers, corresponding to the linear mixed model eq. (5). This method was computationally very intensive and was applied to the most significant SNPs from GWISs. The selected models are summarized in Supplementary...
Table S6, and the Wald test of selected models for rs17765644, which has the smallest P-value in filtering step, are provided in Table 3. These results were obtained with PROC MIXED in SAS (version 9.4). The results for the other three SNPs are provided in Supplementary Tables 7–9. The results also showed that P-value for testing $H_0: \beta_{SNP} = 0$ was close to the Bonferroni-adjusted 0.05 significance level ($1.61 \times 10^{-7}$), and their SNP-smoking group interactions were significant at the 0.05 significance level, even though there were no significant interactions between SNPs and pack years. Coefficients for both the main association of rs17765644 and its interaction with smoking status were $-0.025$ and $-0.029$, respectively. This implies that the FEV$_1$ of never smokers tends to be lower around (number of minor alleles) $\times 0.025$ and ever-smokers are further lower around (number of minor alleles) $\times 0.029$. Figure 4 shows the interacting effect of SNP with smoking status.

Replication studies with GENIE, MESA-Lung, and COPDGene data. To validate the potential association of lung function with smoking, the four SNPs selected with KARE data were replicated in GENIE, MESA-Lung, and COPDGene data. According to the results from KARE data, the main effects of those four SNPs were negative, and their effects were more profound for ever smokers. Based on these factors, we conducted one-tailed tests for the main and interaction effects at the 0.05 significance level for replication studies.

Table 3 shows the results of replication studies for rs17765644. The best model for GENIE data was selected with AICs, and the selected model is shown in the eq. (1) of Supplementary Text 2 and Supplementary Table S6. According to Table 3, the main effect of rs17765644 and its interaction with smoking status were $-0.025$ and $-0.029$, respectively. This implies that the FEV$_1$ of never smokers tends to be lower around (number of minor alleles) $\times 0.025$ and ever-smokers are further lower around (number of minor alleles) $\times 0.029$. Figure 4 shows the interacting effect of SNP with smoking status.

| SNP        | Chromosome | Associated gene | Minor/Major alleles | MAFs | P-values for HWE test | P-values for 3 DF tests |
|------------|------------|----------------|---------------------|------|-----------------------|-------------------------|
| rs17765644 | 17         | SOX9           | C/T                 | 0.384| 0.604                 | $4.45 \times 10^{-7}$   |
| rs17178251 | 17         | SOX9           | G/C                 | 0.383| 0.572                 | $5.21 \times 10^{-7}$   |
| rs11870732 | 17         | SOX9           | G/A                 | 0.384| 0.636                 | $6.15 \times 10^{-7}$   |
| rs1703541  | 17         | SOX9           | C/T                 | 0.391| 0.324                 | $7.63 \times 10^{-7}$   |

Table 2. Results from GWISs with KARE data. 3 DF tests are conducted and the most significant SNPs were summarized. - Definition of Abbreviations: SNP means single-nucleotide polymorphism, MAF means minor allele frequency, and HWE means Hardy-Weinberg equilibrium.

Figure 3. Linkage disequilibrium among the four most significant SNPs and regional plots. Figure 3A shows the LD plot generated with Haploview software and D’ values were used. Figure 3B is a regional plot r$^2$ around rs17765644 SNPs created with LocusZoom. SOX9 TAD is located between 68.67 to 70.45 Mb.
was proportional. Results for the other SNPs had a pattern similar to that of rs17765644 because they were highly correlated (Figure 3). These results are shown in Supplementary Tables 7–9.

Lastly, AAs and NHWs in COPDGene data for rs17765644 were utilized to replicate the main effect and its interaction with smoking. For COPDGene data, there were no never smokers, and smoking status had two levels, former and current smokers. We found that former and current smokers had different FEV1 values, and the best model was selected with AIC. The selected models for AAs and NHWs are shown in the eqs (3) and (4) of Supplementary Text 2, respectively, and summarized in Supplementary Table S6. Former smokers were used as the reference level for smoking status. However, because there were no never smokers, and the directions of regression coefficients from the KARE data could not be considered, we used two-tailed tests. For AAs, the interaction effects of rs17765644 were significant at the 0.1 significance level (P-value = 0.082). Interestingly, rs11870732 (Supplementary Table S8) had significant interactions with smoking status (P-value = 0.037). For NHWs, the main effect of rs17765644 was significant at the 0.05 significance level, and the regression coefficient was −0.066, which was consistent with the data from other studies. However, its interaction effect with smoking status had the opposite direction (P-value = 0.054). This result may be attributable to the selection bias, and former smokers may have smoked more than the current smokers. The results for the other SNPs showed patterns similar to those of rs17765644.

### Table 3. Results for rs17765644

| Data          | Minor/Major alleles | MAF | HWE | β_{SNP} (P-value) | β_{SNP-SM1} (P-value) | β_{SNP-SM2} (P-value) | β_{SNP-SM3} (P-value) | β_{SNP-PY} (P-value) | Overall effects |
|---------------|---------------------|-----|-----|-------------------|-----------------------|-----------------------|-----------------------|-------------------|-----------------|
| Discovery KARE (Koreans) | C/T | 0.384 | 0.604 | −0.025 (2 × 10^{−4}) | −0.029 (0.043) | | | 0.0004 (0.185) | 2.70 × 10^{−7} |
| Replication GENIE (Koreans) | C/T | 0.380 | 0.164 | −0.004 (0.336) | −0.018 (0.052) | −0.024 (0.049) | | | |
| MESA-Lung (NHWs) | C/T | 0.438 | 0.521 | −0.064 (0.008*) | 0.078 (0.941) | | | −0.0021 (0.014*) | 0.0037 |
| COPDGene (AAs) | C/T | 0.177 | 0.377 | 0.042 (0.499) | | | | 0.0005 (0.555) | 0.2205 |
| COPDGene (NHWs) | C/T | 0.459 | 0.433 | −0.066 (0.020) | | | | 0.0006 (0.200) | 0.0746 |

β_{SNP} indicates the coefficient of the main effect of SNP. The smoking status was coded as dummy variables and never smokers were used as reference level. If three levels were defined, then two dummy variables are used. SM1 indicates the dummy variable which is coded as 1 for former smokers, and otherwise 0. SM2 indicates the dummy variable which is coded as 1 for current smoker and otherwise 0. SM3 is utilized only for COPDGene because there are no never smokers. 1 and 0 are for current and former smokers respectively. β_{SNP-SM1}, β_{SNP-SM2} and β_{SNP-SM3} are the coefficients for the interaction between SNP and the corresponding dummy variables respectively. Since KARE and MESA-Lung data chose the smoking status with two levels (never vs ever smokers), β_{SNP-SM1} and β_{SNP-SM2} are shown. β_{SNP-PY} indicates the coefficient for the interaction between SNP and pack years. For GENIE, MESA-Lung data, we conducted one-tailed P-value based on the coefficients from KARE data and *indicates the results of one-tailed P-value. Overall effects indicate P-values for testing the null hypotheses β_{SNP} = β_{SNP-smoking} = β_{SNP-PY} = 0 by F test.

### Figure 4. SNP-smoking interaction

Figure 4 visualize the effects of rs17765644 by smoking status. X axis presents the number of minor alleles, and left and right of Y axis represent mean FEV1 values of never smokers, and ever smoker, respectively.
Discussion

We conducted GWISs of lung function (FEV₁) to identify genetic variants interacting with cigarette smoking. We identified such an interaction using a joint test near the previously described SOX9 locus on chromosome 17 in KARE. These findings were further explored in GENIE, MESA-Lung, and COPDGene. In this report, firstly, we replicated the main effects of SOX9 on FEV₁ values in Koreans, as shown in NHWs. Secondly, we found some evidence for SOX9 gene-smoking interaction effects on FEV₁, and former/current smokers with minor alleles of the selected SNPs near SOX9 tended to have lower FEV₁ values, even though the interaction effects were not strong. According to our results, the coefficient of gene-smoking interaction showed the same direction as the main SNP effect and its amount was almost the same. There have been very few studies showing a significant gene-smoking interaction effect, which could be replicated in other populations, though there were inconsistencies in the interaction effects for different ethnicities. Finally, we highlighted that the statistical model for the analysis of gene-smoking interactions should be carefully selected. The effects of smoking on FEV₁ were very strong, and the means and variances could differ according to smoking status. Notably, when the mis-specified variance-covariance matrices were taken into account, QQ plots were inflated, and type-1 errors were not controlled.

SOX9 has been extensively studied and shown to have pivotal roles in the lung epithelium during branching morphogenesis. The epithelial-mesenchymal transition (EMT) is the process through which epithelial cells become mesenchymal-like, playing crucial roles in tissue repair and several pathalogical processes, including tissue fibrosis, tumor invasiveness, and metastasis. EMT is involved in specific steps in embryogenesis and organ development; however, this concept has been challenged by recent evidence showing that terminally differentiated epithelium can be changed to mesenchymal cells, even in adulthood. This process can be activated by tissue injury or pathological stresses, and inappropriate control of processes may induce tissue fibrosis and cancer. SOX9 induces migratory fibroblasts responsible for extracellular matrix (ECM) deposition and tissue destruction by EMT. SOX9 induces lung fibrosis mediated by transforming growth factor (TGF)→1 repair signaling, characterized by inappropriate ECM deposition; this can result in the destruction of tissue architecture and function. Recent studies have suggested that SOX9 activation is essential for the recovery of lung function after acute lung injury, and SOX9 inhibition induces impaired recovery. Smoking, which includes exposure to several oxidants and free radicals, causes numerous pulmonary diseases through inflammatory processes, leading to cell recruitment to the lung, activation of signaling pathways, and upregulation of proteins, consequently contributing to disruption of the lung ECM. This process varies from person to person; therefore, genetic susceptibility and gene-smoking interactions have been suggested to contribute to disease progression. Previous genome-wide joint meta-analyses of SNPs by smoking interactions on FEV₁ and FEV₁/FVC across 19 studies (total N = 50,047) demonstrated that SOX9 was associated with FEV₁ and was expressed at higher levels in the airway epithelium in smokers than in non-smoking adults of the European ancestry. Our findings were consistent with the results of a previous study, and the SOX9 gene-smoking interaction effects were verified in several ethnicities (Korean, NHWs, and AAs).

However, despite our interesting findings, some inconsistencies were observed in our replication results. For example, an interaction between smoking status and SNPs was found for KARE and GENIE data; however, for MESA-Lung data, the interaction between pack years and SNPs was significant. For COPDGene data, there were no never smokers, and former/current smokers were compared. Coefficients of smoking status and SNP interactions were significant at the 0.1 significance level, but had different directions for NHWs and AAs. There were multiple explanations for these phenomena, including ethnic differences. Genetic ancestry itself is not assumed to be a cause for this difference, but could account for differences in lung function and susceptibility to smoking. For example, the structures of smoking experience vary by population implying complicated relationships between genetics, ethnicity, smoking, and lung function. Therefore, the effects of gene-smoking interactions can be heterogeneous among different populations. In our replication study, we considered diverse ethnicities. According to our results, the replication with GENIE data was quite consistent with that of KARE data, and both were based on the Korean population. However, MESA-Lung and COPDGene data consisted of NHWs and AAs. Small differences between GENIE and KARE data could be explained by the characteristics of the participants. For example, KARE data were based on rural and urban community populations, and GENIE data were composed of participants who underwent regular health screening and received routine medical care. Medical care and routine health check-ups are often positively related to socioeconomic status.

Furthermore, the effects of smoking on FEV₁ are substantial; however, modeling its relationship with FEV₁ is not clear. For example, we found that the effects of gene-smoking status were significant in the KARE and GENIE data, and in the MESA-Lung data, the effects of gene-pack years were significant. Smoking does not have a linear relationship with FEV₁, and non-linear relationships have been reported frequently. The importance of various factors, such as smoking cessation time, smoking behavior, duration, and total dose, has been shown to explain the effects of smoking. In this report, we utilized the smoking status and pack years based on a self-reported smoking history. The prevalence of smoking from self-reporting surveys is usually underestimated, and the degree of underestimation varies among different countries. To minimize this heterogeneity among different studies, we selected the best model with AIC. However, the appropriate definition for smoking is still unclear, and further studies are still necessary.

COPD is expected to be the third leading cause of mortality worldwide within a few years, and identifying genetic variants interacting with smoking would be beneficial in terms of social burden, aging, and the growing importance of personalized medicine. However, statistical models that correctly model the effects of gene-smoking interactions based on lung function are complicated, and successful gene-smoking interaction analyses have been very limited. The proposed method illustrates the complexity of gene-smoking interaction analyses, and to identify consistent gene-smoking interactions, a statistical model should be developed that considers the non-linear relationships between smoking history and lung function.
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Boram Park, So-My Koo, Hyeon-Jong Yang and Sungho Won wrote the main manuscript text, and Boram Park, Jaehoon An and MoonGye Lee analyzed and interpreted the results. Hae Yeon Kang, Dandi Qiao, Michael H. Cho, Joohon Sung and Edwin K. Silverman provided the data. Michael H. Cho, Joohon Sung, Edwin K. Silverman, Hyeon-Jong Yang and Sungho Won revised this paper critically for important intellectual content. All authors reviewed the manuscript.

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