

Associations between *TMEM196* polymorphisms and NSAID-exacerbated respiratory disease in asthma

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Background We previously found differences in the minor allele frequency (MAF) of single-nucleotide polymorphisms (SNPs) in transmembrane protein 196 (*TMEM196*) between 995 patients with aspirin-tolerant asthma (ATA) and 141 asthmatic patients with NSAID-exacerbated respiratory disease (NERD). In this study, we statistically analyzed the distributions of the genotypes and haplotypes of these SNPs to determine the exact association between *TMEM196* genetic variants and the risk for NERD.

Materials and methods Lewontin's D' and r^2 values were used to measure linkage disequilibrium between the biallelic loci having MAFs more than 0.05, and haplotypes were inferred using the PHASE algorithm (version 2.0). The genotype distribution was analyzed by logistic regression models using age of onset, smoking status (nonsmoker = 0, ex-smoker = 1, smoker = 2), and BMI as covariates. Regression analysis of the association between SNPs and the risk of NERD was analyzed using SPSS version 12.0 and PLINK version 1.9.

Results The MAF of *rs9886152* C > T was significantly lower in NERD than in ATA [24.8 vs. 34.0%, odds ratio = 0.64 (0.48–0.85), $P = 2.07 \times 10^{-3}$, $P_{\text{corr}} = 0.048$]. The rate of the *rs9886152* C > T minor allele was significantly lower in NERD than in ATA [44.0 vs. 56.4% in the codominant model, $P = 0.002$, $P_{\text{corr}} = 0.049$, odds ratio = 0.64 (0.48–0.85)]. An additional three SNPs (*rs9639334* A > G, *rs9638765* A > G, and *rs2097811* G > A) showed similar associations with the risk of NERD. NERD patients had lower frequencies of the *rs9639334* A > G minor allele (51.1 vs. 64.4%, $P = 0.002$,

$P_{\text{corr}} = 0.043$), *rs9638765* A > G (49.7 vs. 64.2%, $P = 0.001$, $P_{\text{corr}} = 0.017$), and *rs2097811* G > A (51.1 vs. 64.5%, $P = 0.002$, $P_{\text{corr}} = 0.04$) compared with ATA patients. Patients homozygous for the minor alleles of the four SNPs showed significantly less of an aspirin-induced decrease in forced expiratory volume in one second compared with those homozygous for the common alleles ($P = 0.003$ – 0.012).

Conclusion The minor alleles of the four SNPs in *TMEM196* may exert a protective effect against the development of NERD and may be useful genetic markers to predict the risk of NERD. *Pharmacogenetics and Genomics* 00:000–000 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Aspirin [acetylsalicylic acid (ASA)] hypersensitivity includes ASA-induced or other NSAID-induced respiratory diseases (NERDs), such as bronchoconstriction and nasal symptoms and skin manifestations [1]. The airway of patients with NERD is characterized by inflammatory cell infiltration and epithelial cell proliferation and disruption [2,3]. Altered production of arachidonate metabolites by these cells accounts for the development of NERD [4]. However, NERD is underdiagnosed because of inadequate diagnostic tools and insufficient awareness of the relationship between

ingestion of aspirin and development of asthma [4–7]. Several genes involved in arachidonate generation, immune response, and inflammatory reactions have been identified as genetic risk factors for NERD; however, the genetic effects of these variants do not fully explain the differences in NERD manifestations among individuals [8]. To identify additional genes involved in the pathophysiology of NERD, we performed three genome-wide association studies (GWASs) [9–11]. The GWASs revealed that the risk of NERD was strongly associated with single-nucleotide polymorphisms (SNPs) in several genes including *HLA-DPB1*, *MEIS1*, *NAB1*, *NRP2*, *CLDN1*, *DCDC2*, transmembrane protein 196 (*TMEM196*), *AK8* [C9orf98], *TSC1*, *ZFYVE1-RBM25*, *PCSK6*, *ILVBL*, *GP6*, and *RDH13*, with P value more than 0.05 [12]. Among

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these SNPs, *rs9886152* C>T in *TMEM196* showed significant associations with the risk of NERD and with the decrease in forced expiratory volume in one second (FEV₁) after oral aspirin challenge [odds ratio (OR)=0.54 (0.39–0.76), $P=3.09 \times 10^{-4}$] [12]. Recently, *TMEM196* was shown to regulate autophagy and apoptosis of cancer and inflammatory cells [13]. Furthermore, disruption of the balance between autophagy and apoptosis induces hyperplasia of the airways, a typical feature of nasal polyps and airway eosinophilia in NERD [14–16]. This prompted us to perform additional analyses of the previously genotyped SNPs in *TMEM196* [12] to investigate the association between the SNPs and haplotypes of the *TMEM196* gene and the risk for NERD.

Materials and methods

Genotypes of the single-nucleotide polymorphisms in transmembrane protein 196 in the study participants

The clinical and demographic characteristics of 141 NERD and 995 aspirin-tolerant asthma (ATA) patients were described in our previous publication [12], with the exception of two patients with failed *TMEM196* locus genotyping. Written informed consent was provided by all patients, and the protocol was approved by the Ethics Committee of Soonchunhyang University Hospital (approval no. SCHBC-IRB-2010-005). Genotyping was performed using purified DNA from the patients, and SNPs were selected, as described in [12]. SNPs located in the region from 2 kb upstream to 0.5 kb downstream of *TMEM196* were selected using Asian population data from the International HapMap Project database (<http://hapmap.ncbi.nlm.nih.gov/>) and the NCBI database (<http://www.ncbi.nlm.nih.gov>). Linkages between SNPs with more than 5% minor allele frequencies (MAFs) were analyzed, and one representative SNP was selected from cases of almost absolute linkage disequilibrium (LD) [Lewontin's D' ($|D'|$) = 1 and $r^2 > 0.95$]. Finally, a total of 51 SNPs were genotyped by the GoldenGate assay using VeraCode microbeads (Illumina Inc., San Diego, California, USA) [17].

Statistics

We used $|D'|$ and r^2 to measure LD between the biallelic loci [18]. Haplotypes of each individual were inferred using the PHASE algorithm (version 2.0). The genotype distribution was analyzed by logistic regression models using age of onset (continuous variable), smoking status (non-smoker=0, ex-smoker=1, smoker=2), and BMI (continuous variable) as covariates. Haplotype associations were estimated using HaploScore (<http://www.biostat.wustl.edu/genetics/geneticssoft>). Differences in the rate of FEV₁ decrease following ASA challenge among the genotypes, and haplotypes were examined using a type III generalized linear model. The normality of the distributions of the variables was evaluated using the Kolmogorov–Smirnov test and Shapiro–Wilk test. Before the statistical analyses, the percentage of blood eosinophils, PC20 methacholine concentration, and total IgE level were log transformed

because of their skewed distributions. The data were analyzed using SAS version 9.1 (SAS Inc., Cary, North Carolina, USA) and SPSS version 12.0 (SPSS Inc., Chicago, Illinois, USA). The SNPspD software (<http://genepi.qimr.edu.au/general/daleN/SNPspD>) was used to calculate the corrected P values for multiple testing. The data are expressed as the mean $\pm$ SEM. P values less than 0.05 were deemed statistically significant.

Results

The frequency, heterozygosity, and Hardy–Weinberg equilibrium values of single-nucleotide polymorphisms in transmembrane protein 196

A total of 1136 patients were recruited, and their clinical characteristics are summarized in Table 1. There were significant differences in age, smoking status, BMI, and rates of nasal polyposis and cloudy appearance in the sinuses between the NERD and ATA groups ($P < 0.05$; Table 1). Thus, these parameters were used as covariates in the analyses of genetic associations. Among the 51 SNPs, 22 are located upstream of the gene, 27 in the introns, one in the 3'-untranslated region (UTR), and one downstream of the gene (Fig. 1a). MAFs and Hardy–Weinberg equilibrium values of the 51 SNPs are summarized in Supplemental Table 1, Supplemental digital content 1, <http://links.lww.com/FPC/B341>. The LD parameters $|D'|$ and r^2 of the SNPs revealed that *TMEM196* is parsed into six LD blocks (BLs) (Fig. 2b and c). We excluded haplotypes with frequencies less than 0.05 (haplotypes 5 and 6 on BL1, 5 on BL2, 5–7 on BL3, 5–9 on BL4, 5 on BL5, and 4 on BL6) and those having equivalent SNPs (BL1 haplotype 2 equivalent to rs1024029 and rs6951739, BL1 haplotype 3 equivalent to rs6946520, BL2 haplotype 1 equivalent to rs4721822, BL2 haplotype 2 equivalent to rs9886152, BL2

Table 1 Clinical characteristics of the study participants

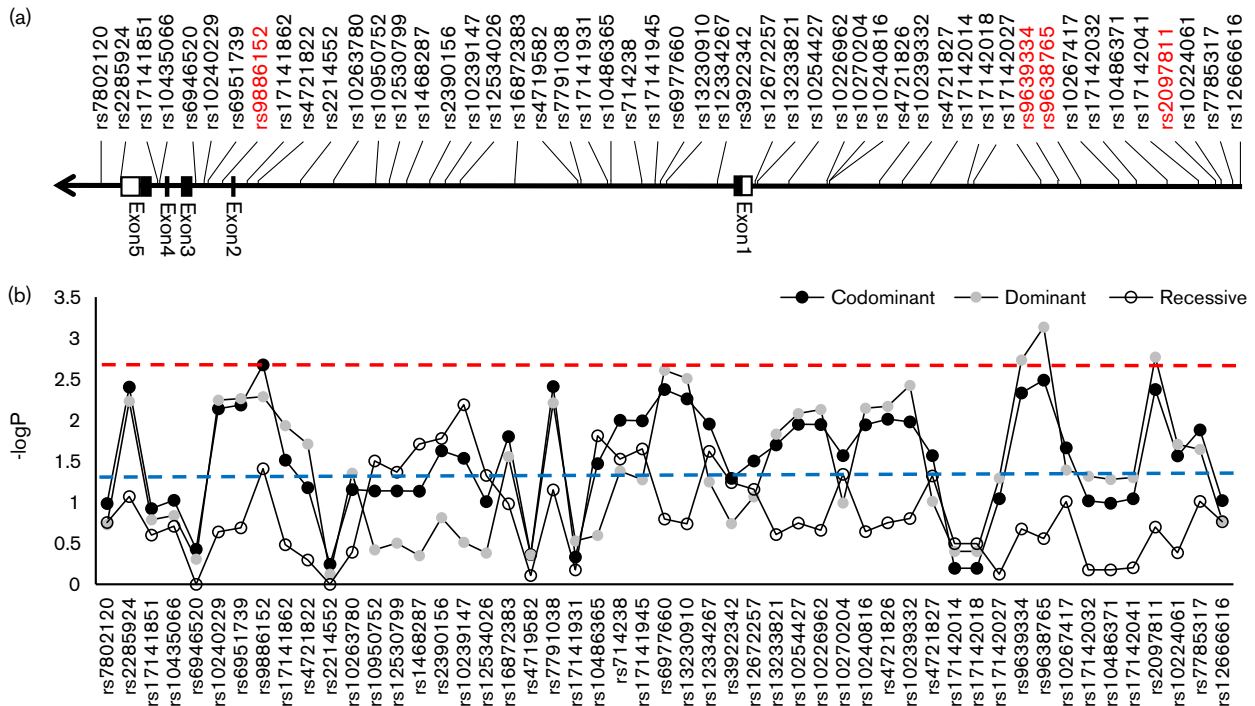
Variables	ATA	NERD	P
Number	995	141	–
Sex (male) (%)	38.5	38.3	0.965
Age (years)	44.8 $\pm$ 0.49	42.39 $\pm$ 1.26	0.082
Age of onset (years)	38.82 $\pm$ 0.53	34.5 $\pm$ 1.53	0.007
Smoking status (NS/ES/SM) (%)	69.6/16.6/13.7	79.4/5.0/15.6	0.002
BMI (kg/m ²)	24.38 $\pm$ 0.11	23.65 $\pm$ 0.3	0.023
FEV ₁ before ASA challenge (% predicted)	83.42 $\pm$ 0.63	80.52 $\pm$ 1.76	0.124
Decline of FEV ₁ after ASA challenge (%)	3.83 $\pm$ 0.16	32.42 $\pm$ 1.08	1.14 $\times 10^{-53}$
Log (PC20 methacholine) (mg/ml)	0.36 $\pm$ 0.02	–0.02 $\pm$ 0.07	1.13 $\times 10^{-7}$
Atopy (yes) (%)	51.9	48.2	0.419
Serum total IgE (kU/l)	393.02 $\pm$ 20.22	411.2 $\pm$ 60.71	0.768
Nasal polyp (yes) (%)	20.5	30.7	4.61 $\times 10^{-17}$
Cloudy Water's view (yes) (%)	34.7	59.6	1.14 $\times 10^{-8}$

Numeric data are presented as mean $\pm$ SE.

ASA, acetylsalicylic acid; ATA, aspirin-tolerant asthmatic patients; ES, ex-smokers; NERD, NSAIDs-exacerbated respiratory disease; NS, never smokers; SM, current smokers.

P values were obtained using independent t -test or χ^2 -test and considered significant when less than 0.05.

Fig. 1



Associations between the minor allele frequencies of transmembrane protein 196 (*TMEM196*) single-nucleotide polymorphisms (SNPs) and the risk of NSAID-exacerbated respiratory disease. (a) A schematic figure of the *TMEM196* gene map (chromosome 7p21) and SNPs. Black blocks represent the coding exons, and white blocks represent the 5' and 3'-untranslated regions. (b) $\text{Log}_{10} P$ values for the association between *TMEM196* SNP genotypes and the risk of aspirin intolerance. A horizontal linear line indicates a P -value of 0.05, and a horizontal dotted line indicates a corrected P -value of 0.05 for multiple testing using the SNPSpD software (<http://genepi.qimr.edu.au/general/daleN/SNPSpD>).

haplotype 3 equivalent to rs2214552, BL3 haplotype 1 equivalent to rs10950752, rs12530799, rs1468287, rs2390156, and rs12534026, BL5 haplotype 2 equivalent to rs10224061, and BL5 haplotype 4 equivalent to rs17142041).

Associations between single-nucleotide polymorphisms in transmembrane protein 196 and the risk of NSAID-induced respiratory disease in asthmatic patients

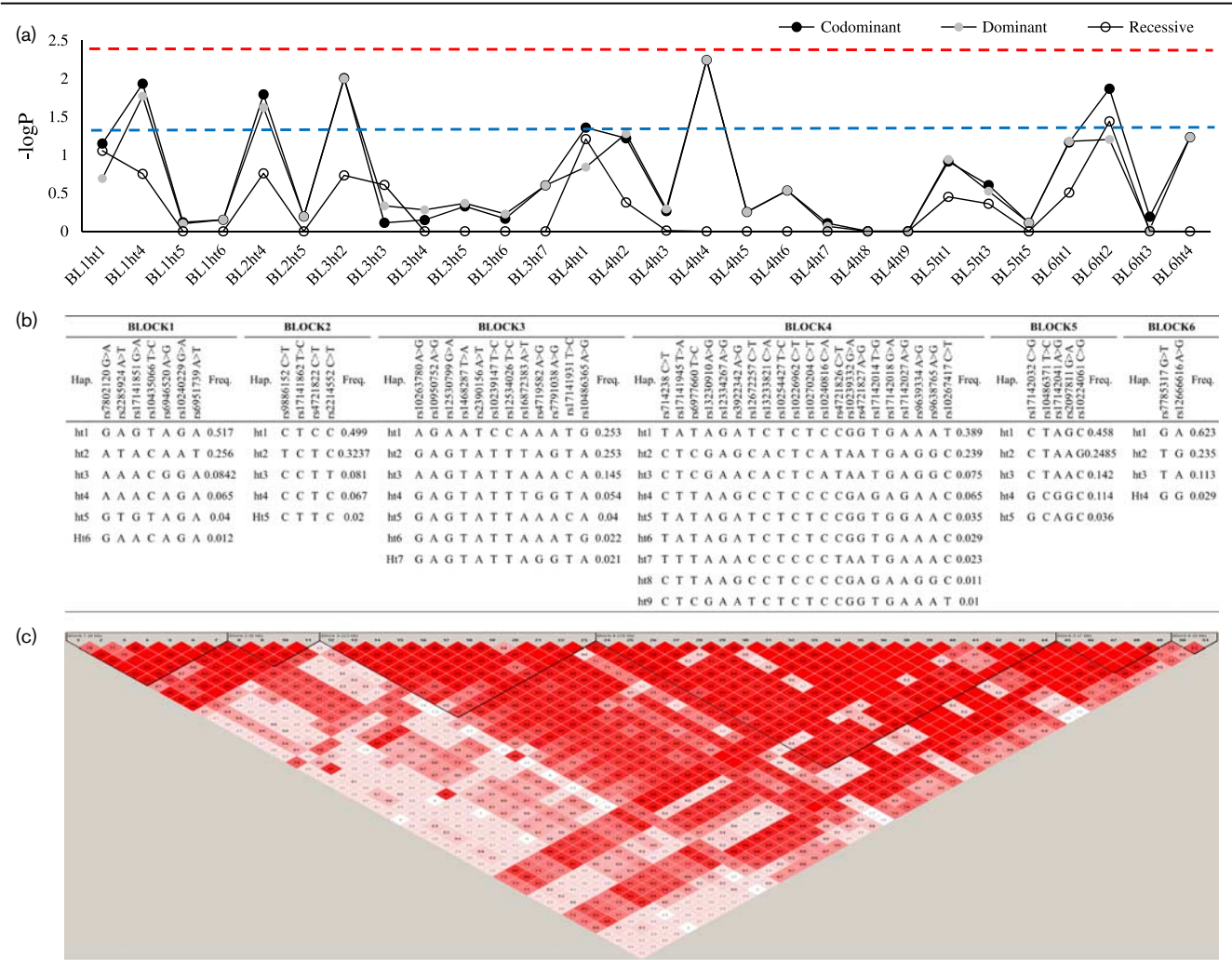
The *TMEM196* SNPs and haplotypes were analyzed for their associations with the risk of NERD using multiple logistic regression models. Comparison analysis showed that the MAF of rs9886152 C > T was significantly lower in patients with NERD than in those with ATA [24.8 vs. 34.0%, OR = 0.64 (0.48–0.85), $P = 2.07 \times 10^{-3}$, $P_{\text{corr}} = 0.048$; Supplemental Table 2, Supplemental digital content 1, <http://links.lww.com/FPC/B341>]. The distributions of the genotypes were compared between the NERD and ATA patients (Supplemental Table 3, Supplemental digital content 1, <http://links.lww.com/FPC/B341>), which revealed that the MAF of rs9886152 C > T located in the intron was significantly lower in NERD than in ATA patients [44.0 vs. 56.4% in the codominant model, $P = 0.002$, $P_{\text{corr}} = 0.049$, OR = 0.64 (0.48–0.85); Table 2 and Fig. 1b]. An additional three SNPs (rs9639334 A > G, rs9638765 A > G, and rs2097811 G > A in the 5'UTR) also showed similar associations with the risk of

NERD (Fig. 1b and Table 2). In the dominant model, NERD patients, compared with ATA patients, had lower MAFs of rs9639334 A > G (51.1 vs. 64.4%, $P = 0.002$, $P_{\text{corr}} = 0.043$), rs9638765 A > G (49.7 vs. 64.2%, $P = 0.001$, $P_{\text{corr}} = 0.017$), and rs2097811 G > A (51.1 vs. 64.5%, $P = 0.002$, $P_{\text{corr}} = 0.04$). No haplotype showed a significant difference between the NERD and ATA patients (Supplemental Table 3 and 4, Supplemental digital content 1, <http://links.lww.com/FPC/B341> and <http://links.lww.com/FPC/B342> and Fig. 3).

Comparison of the acetylsalicylic acid challenge-induced decrease in the FEV₁ among the rs9886152 C > T, rs9639334 A > G, rs9638765 A > G, and rs2097811 G > A genotypes

Because the ASA-induced decrease of FEV₁ is the most important parameter for NERD in asthmatic patients, we compared the declines of FEV₁ following ASA challenge among the genotypes of the four SNPs (Supplemental Table 5, Supplemental digital content 1, <http://links.lww.com/FPC/B341>). Minor allele homozygotes of rs9886152 C > T, rs9639334 A > G, rs9638765 A > G, and rs2097811 G > A showed significantly lower values of ASA-induced decrease of FEV₁ com-

Fig. 2



Associations between haplotype frequencies in the transmembrane protein 196 (*TMEM196*) gene and the risk of NSAID-exacerbated respiratory disease. (a) $\log_{10} P$ values for the association between *TMEM196* single-nucleotide polymorphism (SNP) haplotypes and the risk of aspirin intolerance. A horizontal linear line indicates a P -value of 0.05, and a horizontal dotted line indicates a corrected P -value of 0.05 for multiple testing using the SNPSpD software (<http://genepi.qimr.edu.au/general/daled/SNPSpD>). (b) Haplotype of *TMEM196* SNPs and (c) linkage disequilibrium blocks.

pared with common allele homozygotes ($P=0.003\text{--}0.012$) (Table 3).

Discussion

In the present study, we found that the minor alleles *rs9886152* C > T, *rs9639334* A > G, *rs9638765* A > G, and *rs2097811* G > A in *TMEM196* were significantly lower in NERD than in ATA patients, and that the patients homozygous for the minor alleles of these SNPs showed significantly less airway obstruction after aspirin challenge compared with those homozygous for the common alleles. These data suggest that these SNPs may be protective against NERD.

Because the *rs9886152* C > T SNP is located in an intron, we performed in-silico analysis of this SNP using Human

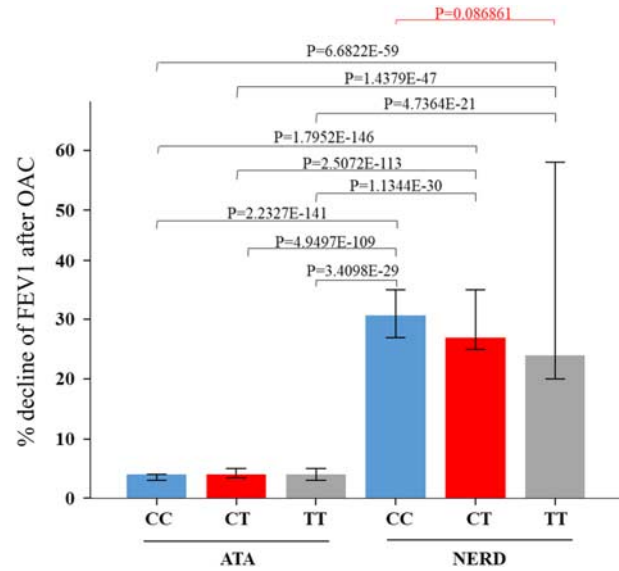
Splicing Finder [19] and MicroRNA.org (<http://www.microrna.org>). This locus is 265 bp from a splicing donor site and does not encode any microRNAs. Additional analysis of the RNA two-dimensional structure using RNAsnp (<https://rth.dk/resources/rnasnp/>) showed no difference in the minimum free energy between the global wild-type and mutant-type sequences (-95.70 and -93.40 kcal/mol).

The other three SNPs in the 5' upstream region, *rs9639334* A > G, *rs9638765* A > G, and *rs2097811* G > A, were excluded from the transcription factor binding analysis (PROMO; <http://algen.lsi.upc.edu/>), because they are located 25589, 27395, and 38818 bp from the transcription start site of *TMEM196*, respectively (Supplemental Fig. 1, Supplemental digital content 2, <http://links.lww.com/FPC/B342>). DNase I

Table 2 Comparison of genotypes' frequency of four single-nucleotide polymorphisms on the *TMEM196* gene between the patients with NSAIDs-exacerbated respiratory disease and those with aspirin-tolerant asthma

Locus	Diagnosis	Genotype			Codominant			Dominant			Recessive		
		RR	CR	CC	Total	<i>P</i> *	<i>P</i> _{corr} **	OR (95% CI)	<i>P</i> *	<i>P</i> _{corr} **	OR (95% CI)	<i>P</i> *	<i>P</i> _{corr} **
rs9886152	NERD	8 (5.7)	54 (38.3)	79 (56.0)	141 (100)	0.002	0.049	0.64 (0.48–0.85)	0.005	0.121	0.6 (0.42–0.86)	0.039	0.905
	ATA	115 (11.6)	446 (44.8)	434 (43.6)	995 (100)								
rs9639334	NERD	17 (12.1)	55 (39.0)	69 (48.9)	141 (100)	0.005	0.108	0.68 (0.52–0.89)	0.002	0.043	0.57 (0.4–0.81)	0.211	1
	ATA	155 (15.6)	484 (48.8)	353 (35.6)	992 (100)								
rs9638765	NERD	17 (12.1)	53 (37.6)	71 (50.4)	141 (100)	0.003	0.076	0.67 (0.51–0.87)	0.001	0.017	0.54 (0.38–0.77)	0.277	1
	ATA	150 (15.1)	487 (49.1)	355 (35.8)	992 (100)								
rs2097811	NERD	17 (12.1)	55 (39.0)	69 (48.9)	141 (100)	0.004	0.098	0.68 (0.52–0.88)	0.002	0.040	0.56 (0.39–0.81)	0.201	1
	ATA	157 (15.8)	485 (48.7)	353 (35.5)	995 (100)								

ATA, aspirin-tolerant asthmatic patients; C, common allele; CI, confidence interval; NERD, NSAID-exacerbated respiratory disease; OR, odds ratio; R, rare allele; SNP, single-nucleotide polymorphism.

P* values were obtained using logistic regression analysis controlling for age of onset, smoking status, and BMI as covariates.Corrected *P* values for multiple comparison using SNPSpD.**Fig. 3**

Percentage decrease in FEV₁ after oral aspirin challenge (OAC) in the *rs9886152* genotypes of transmembrane protein 196 (*TMEM196*) in patients with NSAID-exacerbated respiratory disease (NERD) and in those with aspirin-tolerant asthma (ATA). *P* values were obtained using the Mann-Whitney *U*-test.

hypersensitive sites (DHSs) represent diverse classes of *cis*-regulatory regions, such as enhancers. However, these three intergenic SNPs were not associated with DHSs according to DHSpred (<http://www.thekeelab.org/DHSpred.html>).

Although the four SNPs were not associated with genetic functions predicted using *in silico* analysis, rs9639334 and rs9638765, which showed significant associations with the risk of NERD in the dominant model, are in LD ($r^2 \geq 0.8$ in CHB and JPT population) with rs10254427, rs12672257, and rs13233821. These three SNPs were predicted as potential binding sites for transcription factors such as autoimmune regulator 1, breast cancer 1, activating transcription factor, and peroxisome proliferator activated receptors. rs10254427 is located 2.19 kb upstream from the transcription start site of *TMEM196*, and rs12672257 and rs13233821 are located +588 and +417 bp downstream from the transcription start site, respectively. These findings suggest that the observed associations of rs9639334 and rs9638765 may be a consequence of LD with functional SNPs involved in transcriptional (rs10254427) or translational (rs12672257 and rs13233821) regulation. *rs9639334* A>G and *rs9638765* A>G also showed associations with mRNA expression of TWIST Neighbor (TWISTNB; $P = 3.0 \times 10^{-8}$ and $P = 7.7 \times 10^{-8}$, respectively) in the tibial artery in an expression quantitative trait loci database using GTEx (<http://www.gtexportal.org/>). TWISTNB is a DNA-dependent RNA polymerase catalyzing the transcription of DNA into RNA using the four ribonucleoside

Table 3 Comparison of decline of FEV₁ after oral acetylsalicylic acid challenge test among the four genotypes in all study participants

Locus	Genotypes			Codominant		Dominant		Recessive	
	RR	CR	CC	P*	P _{corr} **	P*	P _{corr} **	P*	P _{corr} **
rs9886152	5.23±7.93 (123)	6.48±10.07 (502)	8.05±12.17 (513)	0.003	0.077	0.007	0.156	0.053	1
rs6977660	5.28±8.33 (163)	6.85±10.21 (527)	7.92±12.37 (448)	0.009	0.217	0.033	0.769	0.033	0.769
rs9639334	5.48±8.72 (172)	6.87±10.29 (544)	7.92±12.34 (422)	0.012	0.290	0.040	0.940	0.042	0.983
rs9638765	5.53±8.82 (167)	6.71±9.96 (545)	8.08±12.61 (426)	0.007	0.163	0.016	0.373	0.055	1

C, common allele; R, rare allele.

*P values were obtained using linear regression analysis controlling for age of onset, smoking status, and BMI as covariates.

**Corrected P values for multiple comparison using SNPSpD.

triphosphates as substrates and is a component of RNA polymerase I, which synthesizes ribosomal RNA precursors (UniProtKB/Swiss-Prot: Q3B726). In addition, RNA polymerase I is involved in autophagy [20]. Thus, the SNPs in the present study may be related to the dysregulated autophagy observed in NERD.

TMEM196 belongs to the transmembrane protein family, which plays important roles in cellular processes including cell migration, differentiation, aggregation, adhesion, dissolving, and signal transduction regulation [21–24]. A GWAS reported associations between *TMEM196* SNPs (*rs17142039* and *rs10486376*) and various phenotypes including longitudinal lung functions [25] and longevity and certain age-related phenotypes [26]. In another study, *rs10230207* was associated with ischemic stroke and a subtype of large vessel occlusive ischemia [27]. However, these SNPs were not associated with the risk of NERD in the present study.

Recently, several other transmembrane proteins have been implicated in tumorigenesis [28–31].

TMEM16A inhibits cell growth both *in vitro* and *in vivo* by the ERK/MAPK pathway [28] and also inhibits autophagy and apoptosis of cancer and inflammatory cells [13,32]. NERD is characterized by inflammation involving infiltration of immune and inflammatory cells in nasal polyps [2,33]. As autophagy is decreased in nasal polyps [34], we evaluated the association between *TMEM196* polymorphisms and the presence of nasal polyps but found no associations (data not shown).

The present study had several limitations. First, when we designed this study, there was no available NGS data for eastern Asians (CHB and JPT) in the 1000 Genome Project. Thus, the SNPs in this study were selected from among the polymorphisms in the dbSNP and HapMap project databases. There are 222 SNPs in the region 40 kb upstream and 2 kb downstream of the *TMEM196* gene in the database. Of these, 94 were located upstream of the gene, 116 in introns, 10 in the 3'-UTR, and two downstream of the gene. There are no SNPs in the coding region. Among the 222 SNPs, 141 showed a MAF more than 0.05 (71 located upstream, 62 in introns, five in the 3'-UTR, and two downstream). In the recent 1000 genome database (build GRCh38.p12), no exonic

polymorphisms have an MAF more than 0.05; the SNP with the highest MAF (*rs61738917*) among the coding SNPs was only 0.004. Second, we cannot exclude the possibility of population stratification bias. However, because the Korean population is characterized by a relatively high degree of genetic homogeneity [35], we consider population stratification bias to be unlikely in our sample.

Conclusion

We found associations of *rs9886152* C>T, *rs9639334* A>G, *rs9638765* A>G, and *rs2097811* G>A in *TMEM196* with the risk of NERD. Although further investigation of the biological roles of these SNPs in the molecular mechanism of NERD is required, these SNPs are promising genetic markers of NERD.

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Conflicts of interest

There are no conflicts of interest.

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