

Original article

Distinct association of genetic variations of vascular endothelial growth factor, transforming growth factor- β , and fibroblast growth factor receptors with atopy and airway hyperresponsiveness

Background: Recent studies showed that high levels of transforming growth factor (TGF)- β 1 in the airways reduced airway responsiveness, which was reversed in conditions of basic fibroblast growth factor (FGF2) deficiency, whereas high levels of vascular endothelial growth factor (VEGF) enhanced airway sensitization to allergens and airway hyperresponsiveness (AHR).

Objective: We investigated the effect of single-nucleotide polymorphisms (SNPs) in the VEGF, TGF- β 1, and FGF2 receptors on the expression of atopy and AHR in the general population.

Methods: Atopy and AHR were evaluated in a cohort of 2055 children and adolescents. Direct sequencing was used to identify informative SNPs (minor allele frequency > 5%) in the receptors of candidate genes. Tagging SNPs were scored using the high-throughput single-base pair extension method, and the statistical significance of these scores was assessed via haplotype analysis.

Results: Informative SNPs were identified for VEGF receptors 1 (*Flt-1*); TGF- β receptor 3 (*TGFBR3*); and FGR receptors 1, 2, and 4 (*FGFR1*, *FGFR2*, and *FGFR4*), and 13 tagging SNPs were scored in the cohort. Atopy was significantly associated with haplotypes of *TGFBR3*, *FGFR1*, and *FGFR2*. Meanwhile, AHR was significantly associated with haplotypes of *Flt-1*, *FGFR1*, and *FGFR4*. However, atopy was not associated with genetic variations of *Flt-1* and *FGFR4*, whereas AHR not associated with *TGFBR3* and *FGFR2*.

Conclusion: The expression of atopy and AHR is distinctly associated with genetic variations in VEGF, TGF- β 1, and FGFR in the Korean population.

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Key words: airway hyperresponsiveness; atopy; fibroblast growth factor; transforming growth factor- β ; vascular endothelial growth factor.

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Accepted for publication 14 October 2007

Asthma is a chronic inflammatory disorder of the airways that is associated with airway hyperresponsiveness (AHR) to nonspecific stimuli and phenotypically a complex genetic disorder (1). The development of asthma is significantly associated with intermediate phenotypes, such as atopy and AHR that have provided useful objective alternatives in genetic and epidemiologic studies (2). Atopy is defined as a genetic predisposition to an enhanced IgE responses to common environmental allergens, and is considered to be the factor that most strongly predisposes an individual to asthma development (3). On the other hand, AHR is believed to be an essential component of asthma development, and can be used to differentiate asthma from other airway diseases (4).

Interleukin-13 (IL-13) is a pleiotropic cytokine that is produced by activated T helper 2 lymphocytes in large quantities; it is also a central mediator in the development

of allergic asthma (5). Lung-specific transgenic animal experiments indicate that high levels of IL-13 in the airways induce structural and functional alterations, such as sub-epithelial myofibroblast hyperplasia, fibrosis, and AHR, all of which are found in asthma patients (6, 7). Vascular endothelial growth factor (VEGF) and transforming growth factor- β 1 (TGF- β 1) are key downstream molecules in the development of IL-13-mediated asthma phenotypes (8, 9). Our previous lung-specific transgenic experiments have shown that high levels of VEGF in the airways induce asthma-like phenotypes, which include lung inflammation, vascular remodeling and edema, sub-epithelial myofibroblast hyperplasia, and AHR (9). Moreover, our previous data indicate that VEGF enhances allergen sensitization (9), and that genetic variation of VEGFR2 (KDR) is associated with the prevalence of atopy in the Korean population (10).

Transforming growth factor- β 1 is a key factor in wound healing that promotes the transformation of fibroblasts to myofibroblasts in lung fibrosis (11, 12). This is consistent with previous results showing that high levels of IL-13 in the airways induce sub-epithelial and lung parenchymal fibrosis, mainly via TGF- β 1-dependent pathways; in addition, lung-specific overexpression of TGF- β 1 in transgenic mice resulted in marked fibrosis with sub-epithelial myofibroblast hyperplasia (8). Interestingly, our recent study indicated that airway remodeling induced by TGF- β 1 was associated with decreased airway responsiveness to methacholine in lung-specific TGF- β 1-overexpressing transgenic mice (13). We recently demonstrated that basic fibroblast growth factor (FGF2) is overexpressed in the TGF- β 1-overexpressing transgenic mice (13). Fibroblast growth factor 2 plays a role in the proliferation of vascular endothelial and smooth muscle cells during angiogenesis, the migration and proliferation of fibroblasts, the reversal of myofibroblast phenotypes, and the migration and proliferation of airway epithelial cells during wound healing (14). The expression of FGF2 messenger RNA is very low in the fetal lung but increases markedly after birth. In neonates, FGF2 expression is the highest in epithelial cells of large airways that also express FGF receptor (FGFR) 1, 2, and 4 and in alveolar cells that express FGFR2, 3, and 4 (15). We recently demonstrated that FGF2 is critical for the maintenance of TGF- β 1-suppressed airway responsiveness and that exogenous administration of FGF2 inhibits AHR in an asthma mouse model (13).

We hypothesized that genetic variation in the VEGF, TGF- β 1, and FGF2 signaling pathways is related with the expression of atopy and AHR, which are key intermediate phenotypes of asthma. We examined genetic variation in VEGFRs, TGF- β 1 receptors (TGFRs), and FGFRs and determined whether it is associated with atopy and AHR in the general population.

Methods

Subjects and clinical phenotyping

A total of 2108 children and adolescents living in a rural area (Jeju Island, Korea) were enrolled, as previously described (10), and phenotyping and genotyping were performed on 2055 of them. All subjects responded to a questionnaire concerning respiratory symptoms and risk factors, as previously described (10). To evaluate atopy, skin prick testing to 11 common aeroallergens (Allergopharma, Reinbek, Germany) was performed as previously described (10). To evaluate AHR, subjects with spirometric lung function of < 70% of the predictive value and those with a history of use of β 2 agonists or upper respiratory tract infection during the previous 2 weeks were excluded from the methacholine bronchial challenge. The methacholine challenge was performed as previously described, and the PC₂₀ was measured for each subject (16). An AHR-positive response was defined as a PC₂₀ value of < 16 mg/ml. All parents provided written informed consent. The study protocol was approved by the Ethics Committee of Seoul National University Hospital.

Informative SNP identification and scoring of candidate genes

To identify unknown single-nucleotide polymorphisms (SNPs) for our candidate genes in the Korean population, we used direct polymerase chain reaction sequencing to analyze exons, nearby exon-intron junctions, and the putative promoter area of each candidate gene in 24 healthy subjects. The eight genes analyzed were *VEGFR1* (fms-like tyrosine kinase, or *Flt-1*); *TGFR1*, 2, and 3; and *FGFR1*, 2, 3, and 4. After identifying informative SNPs (minor allele frequency > 5%), tagging SNPs were scored using the high-throughput single base pair extension method (SNP-IT™ assay) on a SNPstream25K system (Orchid Biosciences, Princeton, NJ, USA), which had been customized, as previously described, to perform fully-automated SNP genotyping of DNA samples in 384-well plates and provide a colorimetric readout (9, 17).

Statistical analysis

For this population-based case-control study, haplotypes were examined as a three-level factored variable (i.e. AA, AB, and BB, where A is the allele of the major frequency haplotype and B is the allele of the minor frequency haplotype). To determine whether one of the homozygous haplotypes had an effect that differed from the common effect of the heterozygous or other homozygous haplotypes [AA vs AB + BB (dominant model), or BB vs AB + AA (recessive model)], chi-squared tests were performed using a 2 × 2 contingency table. Then *P*-values for atopy and AHR were obtained by logistic analysis after controlling for age, sex, family history of allergic disease, and passive smoking history, and after controlling for age, sex, atopy, rhinitis, family history of allergic disease, and passive smoking history, respectively. Compliance with the Hardy-Weinberg equilibrium was tested using chi-squared tests. Haplotypes and their frequencies were estimated using an expectation-maximization algorithm. All statistical analyses were performed using SAS software (version 8.1; SAS Institute, Cary, NC, USA). A *P*-value < 0.05 was regarded as statistically significant.

Results

Genotyping

The DNA sequencing analysis identified a total of 46 informative SNPs. Ten were in *Flt-1*: -2534C/T, -2021G/A, -59C/T(5'UTR), IVS2 + 65A/C, IVS12 - 84C/T, IVS14 + 67T/G, IVS23 + 76C/G, P1068P, IVS26 + 37A/G, and 5533T/G(3'flanking). Eighteen were in *TGFR3*: -38G/A, S15P, A72A, IVS4 - 40C/T, IVS5 + 103G/A, IVS5 - 118G/A, IVS5 - 81T/C, IVS5 - 77T/C, S173S, IVS6 - 71T/C, P401P, T748T, G764R, 2572G/A(3'UTR), 2710G/A(3'UTR), 2753G/A(3'UTR), 3010A/G(3'UTR), and 3836A/G(3'UTR). Five were in *FGFR1*: -994A/G, -636C/T(5'UTR), IVS2 + 27319G/A, IVS3 + 389G/C, and IVS7 + 369C/T. Ten were in *FGFR2*: -236A/C(5'UTR), -157(5'UTR), IVS1 + 62G/C(5'UTR), IVS4 + 54T/C, M186T, IVS13 - 125del[T], IVS17 + 15C/T, IVS17 + 1829G/A, IVS17 + 1839G/A, and 2725T/C(3'UTR). Three were in *FGFR4*: V10I, T179A, and G388R. No informative SNP was identified in *TGFR1*, *TGFR2*, or *FGFR3*. Of the 46 informative

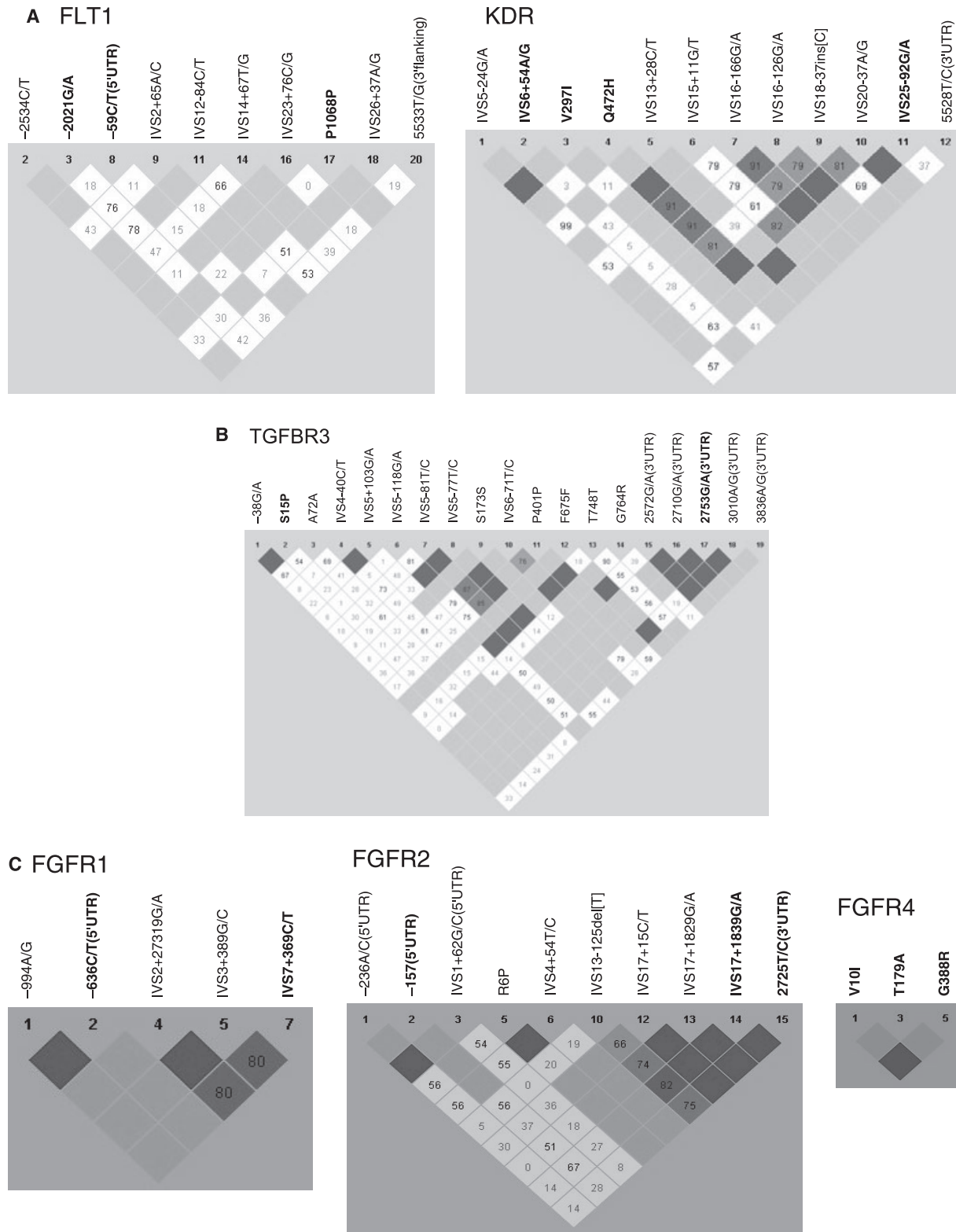


Figure 1. Linkage disequilibrium of informative SNPs in candidate genes. (A) *Flt-1*. (B) *TGFR2*. (C) *FGFR1* (right panel), *FGFR2* (middle panel), and *FGFR4* (right panel). Tagging SNPs selected for scoring are shown in bold.

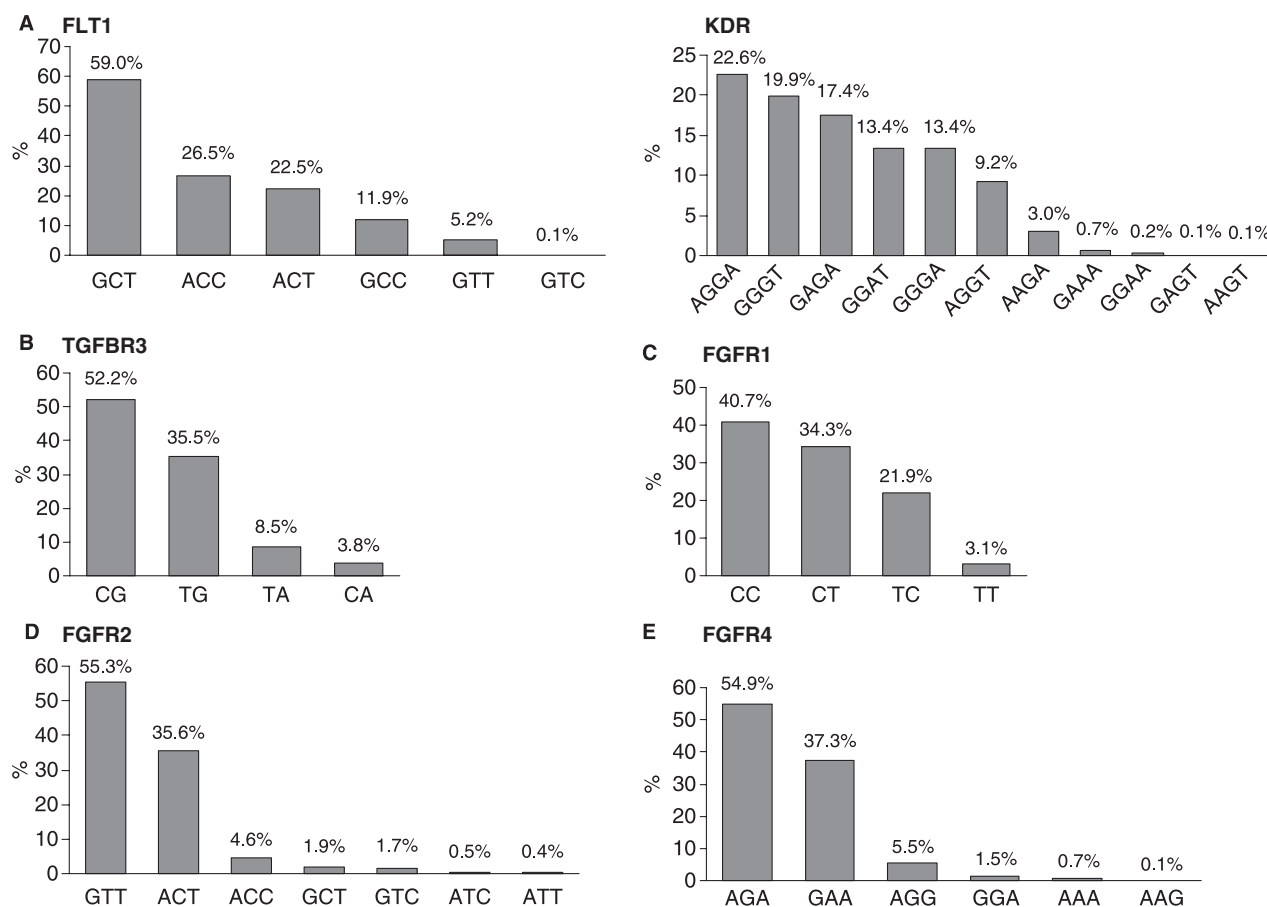


Figure 2. Haplotype frequencies of genes scored in the cohort. (A) *Flt-1*. (B) *TGFB3*. (C) *FGFR1*. (D) *FGFR2*. (E) *FGFR4*.

SNPs, 13 tagging SNPs in *Flt-1*, *TGFB3*, *FGFR1*, *FGFR2*, and *FGFR4* were scored to identify the haplotypes present in the cohort (Fig. 1). The haplotype frequency of each candidate gene is shown in Fig. 2. The 13 tagging SNPs examined in this study followed the Hardy–Weinberg equilibrium.

Association between genetic variation in the candidate genes and atopy in the general population

Each subject underwent skin prick test to common aeroallergens, and 767 (37.3%) of the subjects were positive, as previously described (10). We found significant positive associations between atopy and the TGF- β 1 receptors *TGFB3* and FGF2 receptors *FGFR1* and *FGFR2* [*P*-value, odds ratio (OR), and 95% confidence interval (CI) are shown] (Tables 1 and 2). The *TGFB3* + 44C_2753A haplotype was significantly associated with atopy [*P* = 0.04 (dominant model), 1.44 (1.01–2.06)]. For *FGFR1*, the –636C_IVS7 + 369C haplotype was significantly associated with atopy [*P* = 0.003 (dominant model), 0.74 (0.60–0.90)], as was the *FGFR2* IVS17 + 1839G_2725C_ + 557T [*P* = 0.01 (dominant model), 1.43 (1.11–1.83)]. However,

no significant association was found between atopy and genetic variations of *Flt-1* and *FGFR4*.

Association between genetic variation in VEGF and TGF- β 1 receptors and AHR in the general population

Each subject underwent the methacholine bronchial-provocation test, and 525 (25.6%) of the subjects were positive for AHR. We found significant positive associations of AHR with the VEGF receptors *Flt-1*, but not with *TGFB3* (Table 3). The *Flt-1* –2021G_– 59C_ + 3204T haplotype was significantly associated with AHR [*P* = 0.04 (recessive model), OR (95% CI) = 1.26 (1.01–1.57)], as was the *Flt-1* –2021A_–59C_ + 3204T haplotype [*P* = 0.04 (dominant model), OR (95% CI) = 0.80 (0.64–0.99)].

Association between genetic variations in FGF2 receptors and AHR in the general population

Airway hyperresponsiveness was significantly associated with two haplotypes of *FGFR1*, –636C_IVS7 + 369C [*P* = 0.02 (dominant model), OR (95% CI) = 0.77 (0.61–0.96)], as was the –636T_IVS7 + 369T [*P* = 0.01

Table 1. Association between haplotypes of VEGF and TGF- β 1 receptors genes and atopy in the general population

Phenotypes	Atopy		<i>P</i> -value*	OR (95% CI)
	+	–		
Haplotypes	(<i>n</i> = 525)	(<i>n</i> = 1530)		
FLT1 [–2021G>A_–59 C>T_P1068P (+3204T>C)]				
GCT	899 (37.1%)	1524 (62.9%)	0.65	1.03 (0.90–1.19)
ACT	336(36.2%)	592 (63.8%)	0.62	0.96 (0.82–1.13)
GCC	177(36.6%)	307 (63.4%)	0.82	0.98 (0.79–1.21)
TGFBFR3 [S15P (+44C>T)_2753G>A]				
CG	799 (37.2%)	1346 (62.8%)	0.06	1.27 (0.99–1.62)
TG	529 (43.4%)	937 (56.6%)	0.37	0.95 (0.82–1.09)
TA	127 (36.1%)	223 (63.9%)	0.75	0.96 (0.75–1.23)
CA	69 (36.3%)	90 (63.7%)	0.04**	1.44 (1.01–2.06)

Atopy was defined as positive skin test responses to one or more common allergens.

**P*-values using logistic analysis after adjusting confounding factors (history of allergic disease, and passive smoking history).

***P*-value is from the dominant model.

Table 2. Association between haplotypes of FGF2 receptors genes and atopy in the general population

Phenotypes	Atopy		<i>P</i> -value*	OR (95% CI)
	+	–		
Haplotypes	(<i>n</i> = 525)	(<i>n</i> = 1530)		
FGFR1 (–636C>T_IVS7 + 369C>T)				
CC	581 (34.5%)	1105 (65.5%)	0.003**	0.74 (0.60–0.90)
CT	536 (38.6%)	851 (61.4%)	0.10	1.23 (1.05–1.50)
TC	339 (38.3%)	546 (61.7%)	0.25	1.10 (0.94–1.30)
TT	52 (43.3%)	68 (56.7%)	0.49	1.15 (0.77–1.70)
FGFR2 [IVS17 + 1839G>A_2725C>T_T186M (+557T>C)]				
GCT	886 (38.4%)	1424 (61.6%)	0.01**	1.43 (1.11–1.83)
ATT	498 (35.0%)	925 (65.0%)	0.27	0.92 (0.80–1.06)
FGFR4 [I10V (+28A>G)_R388G (+1162G>A)_T179A (+529A>G)]				
AGA	848 (37.3%)	1428 (62.7%)	0.94	1.00 (0.87–1.14)
GAA	552 (36.7%)	954 (63.3%)	0.69	1.03 (0.90–1.18)
AGG	84 (37.0%)	143 (63.0%)	0.76	1.05 (0.78–1.40)

Atopy was defined as positive skin test responses to one or more common allergens.

**P*-values using logistic analysis after adjusting confounding factors (history of allergic disease, and passive smoking history).

***P*-value is from the dominant model.

(dominant model), OR (95% CI) = 1.73 (1.12–2.65)]. Airway hyperresponsiveness was also found to be significantly associated with two haplotypes of *FGFR4*, +28A_+529G_+1162A [*P* = 0.004 (dominant model), OR (95% CI) = 0.71 (0.59–0.90)] and +28A_+529G_+1162G [*P* = 0.02 (recessive model), OR (95% CI) = 1.37 (1.04–1.80)]. However, AHR was not significantly associated with the *FGFR2* haplotype (Table 4).

Discussion

Understanding the genetic origin of AHR is key to understanding the pathogenesis of asthma (1). Airway

Table 3. Association between haplotypes of VEGF and TGF- β 1 receptors genes and airway hyperresponsiveness in the general population

Phenotype	AHR		<i>P</i> -value*	OR (95% CI)
	+	–		
Haplotype	(<i>n</i> = 525)	(<i>n</i> = 1530)		
No. (%)				
<i>Flt-1</i> –2021G>A_–59 C>T_P1068P (+3204T>C)				
GCT	634 (26.5)	1760 (73.5)	0.04***	1.26 (1.01–1.57)
ACT	218 (23.8)	698 (76.2)	0.04**	0.80 (0.64–0.99)
GCC	127 (26.3)	355 (73.7)	0.50	1.09 (0.86–1.37)
<i>TGFBFR3</i> S15P (+44C>T)_2753G>A				
CG	532 (25.0)	1599 (75.0)	0.41	0.94 (0.82–1.09)
TG	368 (25.4)	1080 (74.6)	0.92	0.99 (0.86–1.15)
TA	93 (27.1)	250 (72.9)	0.47	1.10 (0.85–1.41)
CA	47 (30.1)	109 (69.9)	0.40	0.94 (0.82–1.09)

AHR+, PC₂₀ methacholine level of <16 mg/ml; AHR–, a level $\geq$ 16 mg/ml.

**P*-values using logistic analysis after adjusting confounding factors (atopy, history of allergic disease, and passive smoking history).

***P*-value is from the dominant model.

****P*-value is from the recessive model.

Table 4. Association between haplotypes of FGF2 receptors genes and airway hyperresponsiveness in the general population

Phenotype	AHR		<i>P</i> -value*	OR (95% CI)
	+	–		
Haplotype	(<i>n</i> = 525)	(<i>n</i> = 1530)		
No. (%)				
<i>FGFR1</i> –636C>T_IVS7 + 369C>T				
CC	404 (24.1)	1271 (75.9)	0.02**	0.77 (0.61–0.96)
CT	358 (26.0)	1018 (74.0)	0.49	1.02 (0.87–1.19)
TC	222 (25.6)	664 (74.4)	0.52	1.03 (0.85–1.24)
TT	42 (35.9)	75 (64.1)	0.01**	1.73 (1.12–2.65)
<i>FGFR2</i> IVS17 + 1839G>A_2725C>T_T186M (+557T>C)				
GCT	582 (25.5)	1702 (74.5)	0.97	1.00 (0.86–1.15)
ATT	362 (25.7)	1047 (74.3)	0.84	1.02 (0.88–1.18)
<i>FGFR4</i> I10V (+28A>G)_R388G (+1162G>A)_T179A (+529A>G)				
AGA	545 (24.2)	1703 (75.8)	0.004**	0.71 (0.59–0.90)
GAA	406 (27.1)	1089 (72.9)	0.77	1.05 (0.77–1.42)
AGG	58 (26.4)	162 (73.6)	0.02***	1.37 (1.04–1.80)

AHR+, PC₂₀ methacholine level of <16 mg/ml; AHR–, a level $\geq$ 16 mg/ml.

**P*-values using logistic analysis after adjusting confounding factors.

***P*-value is from the dominant model.

****P*-value is from the recessive model.

hyperresponsiveness is a complex genetic phenotype and requires genetic variation in several candidate genes to create a predisposition (18). We investigated the relationship between various haplotypes of the genes of VEGF, TGF- β 1, and FGF2 receptors and AHR in the general population. This is the first report demonstrating that genetic variation in the VEGF and FGF2 signaling pathways is significantly associated with AHR in the general population.

Recent evidences suggest that VEGF, TGF- β 1, and FGF2 play a critical role in the expression of AHR

initiated by IL-13-mediated airway inflammation (9, 13). The role of IL-13 in the development of AHR is partly mediated by the production of VEGF in airway epithelial cells and inflammatory cells (9). Vascular endothelial growth factor is a potent mediator of chronic inflammation and angiogenesis in asthma, and a recent study showed that VEGF and Flt-1 expression correlate with airway caliber and AHR (19). Previously, we observed that AHR expression was markedly enhanced in mice positive for lung-specific VEGF overexpression (9). Our data indicate that the prevalence of AHR is associated with the genetic variation of *Flt-1*, but not with *KDR* in the Korean population (10). These findings suggest that the VEGFR1 signaling pathway may partly determine genetic predisposition to AHR.

Our previous study indicated that AHR is significantly reduced by high concentrations of TGF- β 1 in the airways (13). In addition, decreased responsiveness to methacholine as a result of high TGF- β 1 concentrations in the airways is dependent on the presence of FGF2, suggesting that FGF2 is necessary to mediate or modulate the AHR-suppressive effects of TGF- β 1 (13). FGF2 binds to and activates the FGF receptors 1, 2, 3, and 4 (20). Until now, the genetic effects of TGF- β 1 and FGF2 receptors on the predisposition to AHR are unclear. In terms of the genetic effects of individual TGF- β 1 and FGFR receptors, we showed that AHR is significantly associated with genetic variation in FGF2 receptors, but not with TGF- β 1 receptors. These findings suggest that genetic variations of FGF2 receptor signaling rather than TGF- β 1 receptors signaling might be important roles in the expression of AHR.

Atopy is considered to be the factor that most strongly predisposes an individual to asthma development (3) and known to be complex phenotypes (21). Genetic variations of many genes have been reported to be genetically associated with atopy (22–24). Our

previous data indicate that VEGF enhances airway sensitization to allergens (9) and that genetic variations of *KDR* were significantly associated with atopy prevalence (10). However, the present study showed that genetic variations of *Flt-1* were not significantly associated with atopy. In terms of genetic effects of VEGFR signaling, our present data suggest that genetic predisposition to atopy and AHR was distinctly determined.

Transforming growth factor- β 1 is well known to induce immune tolerance to allergens, although it is key downstream molecule of IL-13 (25). Indeed, our present data showed that genetic variations of TGF- β 1 receptors were significantly associated with atopy. These findings suggest that genetic variations of TGF- β 1 signaling are important in the predisposition to atopy. Meanwhile, the genetic effects of FGF2 signaling on the expression of atopy are wholly unknown. Interestingly, our present data showed that genetic variations of FGF2 receptors were significantly associated with atopy. These findings suggest that FGF2 receptor signaling is also involved in the development of atopy, although further studies will be needed.

In summary, the expression of atopy is significantly associated with genetic variations in the TGF- β 1 (*TGFBR3*) and FGF2 (*FGFR1* and *FGFR2*) receptors, whereas AHR with VEGF (*Flt-1*) and FGF2 (*FGFR1* and *FGFR4*) receptors, which suggests that signaling by each, and the interaction of such signaling, may influence genetic susceptibility to atopy or AHR.

Acknowledgments

We thank Ji-In Lim and Jung-Mi Paek for secretarial assistance. This study was supported in part by grants from the Korean Ministry of Health & Welfare (03-PJ10-PG13-GD01-0002 & A050288) and by the Pohang University of Science and Technology (POSTECH) research fund (1RC0660301).

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