EPIDEMIOLOGY

Multilocus analysis of atopy in Korean children using multifactor-dimensionality reduction

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Background: Atopy is considered to be a complex genetic trait and does not follow a simple mendelian pattern of inheritance. It is now well recognised that gene–gene interactions are important in complex genetic disease.
Aim: To analyse the influence of gene–gene interactions in the development of atopy.
Methods: A total of 2055 ethnically identical participants aged 10–18 years living in rural areas on Jeju Island, Korea, were randomly recruited. Atopy was defined as a positive skin prick test response to one or more common inhalant allergens. Gene–gene interactions among 12 polymorphic loci were analysed in the seven candidate genes of atopy using the multidimensionality-reduction method.
Results: A significant interaction was found between V297I in the gene coding vascular endothelial growth factor receptor 2 (*KDR*) and −308G→A in the gene coding tumour necrosis factor (*TNF*)α on the risk of atopy, with a cross-validation consistency of 10 out of 10 and a prediction error of 35.9% (p=0.001). Conventional logistic regression also revealed significant interactions between *KDR* and *TNF* for atopy. Individuals with the variant allele of −308G→A in *TNF* (GA or AA) and V297I in *KDR* (VI or II) had a significantly higher risk of atopy (OR 2.23; 95% CI 1.48 to 3.57).
Conclusion: *KDR* and *TNF* may synergistically influence the development of atopy through gene–gene interaction in Korean children and adolescents.

Atopy is defined as a genetic predisposition to induce enhanced IgE responses to common environmental allergens. It is considered to be a complex genetic trait and thus does not follow a simple mendelian pattern of inheritance. Instead, the genetic determination of atopy is probably due to several genes, each having a small, possibly synergistic, effect on the phenotype. Therefore, it is necessary to consider simultaneously the effect of several single-nucleotide polymorphism (SNP) genotypes at different loci. Such gene–gene interactions are traditionally evaluated using logistic regression. However, procedures for fitting logistic regression models are problematic, leading to an increase in type II errors and a decrease in power. In addition, sparseness of the data can be another problem in high dimensions.

To deal with these problems, multifactor-dimensionality reduction (MDR) has been developed.[1] MDR is a non-parametric and genetic model-free approach and is able to identify evidence for high-order gene–gene interactions in the absence of any statistically significant independent main effects in simulated data.[2 3] With MDR, gene–gene interactions have been revealed in complex genetic disorders such as hypertension,[4] type 2 diabetes mellitus,[5] atrial fibrillation,[6] myocardial infarction[7] and asthma.[8]

In this study, 12 loci in seven genes proved to be related with atopy or enhanced serum IgE were genotyped in Korean children and adolescents, and gene–gene interaction was examined with MDR.

METHODS

Study participants and atopy definition

All the participants enrolled in this study gave written informed consent, and the study protocol was approved by the institutional review board of Seoul National University Hospital, Seoul, Republic of Korea. A total of 2864 ethnically identical participants aged 10–18 years were randomly recruited through schools located on the southern part of Jeju Island in Korea, of whom 2055 (71.8%) were enrolled in this study. All of them resided in rural areas, and most of their parents lived by fishing or cultivating fruit trees. Skin prick testing with 11 common aeroallergens (*Dermatophagoides pteronyssinus*, *D farinae*, dog fur, cat fur, *Aspergillus*, *Alternaria*, tree pollen mixture, grass pollen mixture, mugwort, ragweed and cockroach; Allergopharma, Reinbeck, Germany) was performed as described previously.[9] Participants who had received oral antihistamines during the 5 days before the skin prick test or had dermographism were excluded. Atopy was defined as a positive skin prick test response (allergen/histamine ratio >1 and a mean weal size >4 mm) to one or more allergens.

Selection of genes and SNPs

On the assumption that cytokines play a crucial role in the widely used immunological model that explains the increasing prevalence of atopy by an altered balance between T helper (Th) 1 and Th 2 immune responses,[10] we selected six cytokine-related candidate genes using public databases—for example, PubMed and Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/Omim/). These have been characterised and potentially associated with atopy or enhanced serum IgE in an Asian population, and subsequent studies performed in a non-Asian population have witnessed this association.[11–25] The only exception was kinase insert domain-containing receptor (*KDR*) coding vascular endothelial growth factor (VEGF) receptor 2 which, in our previous study, was proved to be associated with an increased prevalence of atopy in a Korean population.[26] In addition to these cytokine-related genes, *MS4A2*

Abbreviations: KDR, kinase insert domain-containing receptor; MDR, multifactor-dimensionality reduction; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor

Table 1 Candidate genes and single-nucleotide polymorphism analysed in this study

Gene	SNP	rs Number	Association
IL4R	−3223T→C	rs2057768	Atopy,[11–13] IgE[14]
	Q576R	rs1801275	
	I75V	rs1805010	
IL13	−1510A→C	rs1881457	Atopy,[16 17] IgE[15 18]
	−1111C→T	rs1800925	
	R130Q	rs20541	
TNF	−308G→A	rs1800629	Atopy,[19 20] IgE[21]
IL12B	S226N	—	Atopy[22 23]
IL12RB1	M365T	rs375947	Atopy[24 25]
*KDR**	V297I	rs2305948	Atopy[26]
	H472Q	rs1870377	
MS4A2†	E237G	rs569108	Atopy,[27 28] IgE[27 28]

SNP, single-nucleotide polymorphism; rs, reference SNP.
**KDR*: gene coding vascular growth factor receptor 2.
†*MS4A2*: gene coding high-affinity IgE receptor β subunit.

coding high-affinity IgE receptor β subunit (FcεR1B) was included, which was also known to be distinctively associated with atopy.[27 28] Within these genes, 10 functional SNPs[11 12 14 17 18 29–31] were chosen for analysis. As for *KDR*, I297V and H472Q were selected because they were located in regions coding for the extracellular fourth and fifth immunoglobulin-like domains and thus possibly alter the signalling pathway as discussed in our previous study.[26] Table 1 shows the list of SNPs analysed in this study.

Genotyping

SNPs were scored using the high-throughput single base-pair extension method (SNP-IT assay) using an SNPstream25K system, which was customised to automatically genotype DNA samples in 384-well plates and provide a colorimetric readout (Orchid Biosciences, New Jersey, USA) as described previously.[26]

Statistical analysis

Allele frequencies were estimated by gene counting methods and the χ^2 test was used to examine the Hardy–Weinberg equilibrium. Association between atopy and each of the 12 loci was made with the Pearson χ^2 test using dominant, recessive and codominant genetic models. MDR (V.0.6.2; Computational Genetics Laboratory, Dartmouth Medical School, Hanover, New Hampshire, USA; http://www.epistasis.org) was performed as described previously.[1–3 5 8] The dataset is divided into 10 parts of equal size for 10-fold cross-validation (9/10 of the data for training and 1/10 of the data for testing). Next, a set of n SNP polymorphisms is selected that are represented in n-dimensional space. The ratio of cases to controls is then calculated for each combination, which is labelled "high risk" (>1) or "low risk" (<1). Consequently, n dimensional space was reduced to one dimension with two levels. Among all of the two-factor combinations, a single model that minimises classification error is chosen. To evaluate the predictive ability of the model, a prediction error is obtained through 10-fold cross-validation. In our study we set out to detect all two-locus interactions through five-locus interactions due to computation restrictions. From this set, the model with the combination of loci that maximises the cross-validation consistency and minimises the prediction error is selected. We determined the statistical significance of the final best model using 1000 permutation testings. The entire procedure is repeated for each, generating a distribution of predictive errors and cross-validation consistencies that could be expected by chance alone. The significance of the final model is determined by comparing the predictive error and cross-validation consistency of the final model to the distribution. A p value is extracted for the model by its theoretical location in the distribution. In addition, logistic regression analysis and χ^2 tests were performed to confirm the results from MDR analyses. A p value <0.05 was considered significant. The detection power of the sample in this study was 0.8 for atopy, if the relative risk for atopy in people carrying a putative risk allele is set to 2 compared with that in people without the allele. The exception was S226N in *IL12B* (detection power 0.6).

Table 2 Characteristics of study population

Characteristics	Number of positivity, n (%)
Mean (range) age (years)	14.6 (10–18)
Male	1004 (48.9)
Atopy*	767 (37.3)
History of passive smoking	1342 (65.3)
Family history of allergic disease	376 (18.3)
Vaccination history	1552 (75.5)

*Positive skin test responses to one or more common aeroallergens.

RESULTS

Study population

A total of 2055 children and adolescents were enrolled. The mean age was 14.6 (range 10–18) years and 48.9% were male. Table 2 shows the characteristics of the study population.

Association between atopy and individual SNPs

All 12 SNPs examined were in Hardy–Weinberg equilibrium. The minor allele frequencies of SNPs in this study in comparison with those previously reported are given in table S1 (available at http://thorax.bmj.com/supplemental). Among them, *MS4A2* E237G (p = 0.028 in a codominant model), *TNF* −308G→A (p = 0.031; odds ratio (OR) 1.31; 95% CI 1.02 to 1.69 in a dominant model) and *KDR* V297I (p = 0.048; OR 1.22; 95% CI 1 to 1.5 in a dominant model) showed significant associations with atopy (table 3).

MDR analysis

Table 4 summarises for each number of loci evaluated the average cross-validation consistency and average prediction error obtained from MDR analysis. A two-locus model had a minimum prediction error of 35.93% (p = 0.001) and a maximum cross-validation consistency of 10 out of 10. This two-locus model, which included *TNF* −308G→A and *KDR* V297I (fig 1), was regarded as the best model.

Logistic regression analysis

A significant interaction between *TNF* −308G→A and *KDR* V297I on the risk of atopy was also found by means of logistic regression analysis (p<0.001), adjusting for age, sex, passive smoking and family history of allergic disease as covariates. Individuals with the variant allele of *TNF* −308G→A (GA or AA) and *KDR* V297I (VI or II) had a significantly higher risk of atopy (OR 2.3; 95% CI 1.48 to 3.57). Table 5 shows the results.

DISCUSSION

Perhaps the toughest problem faced by the allergist is that of identifying genes carrying alleles affecting liability to asthma or atopy from the vast field of potential candidates. The problem becomes even harder if gene–gene interactions must be considered. In this study, a two-locus model involving SNPs

Table 3 Genetic effects of individual single-nucleotide polymorphisms on atopy

Genotype frequency				p Value*		
				Dominant†	Recessive†	Codominant†
IL4R						
−3223T→C						
	TT	TC	CC			
Atopy	278 (38%)	346 (47.3%)	108 (14.7%)	0.13	0.413	0.301
Control	515 (41.4%)	561 (45.1%)	167 (13.5%)			
Q576R						
	QQ	QR	RR			
Atopy	529 (69.2%)	214 (28%)	21 (2.8%)	0.831	0.426	0.728
Control	890 (69.1%)	357 (27.7%)	41 (3.2%)			
V75I						
	VV	VI	II			
Atopy	244 (31.9%)	378 (49.5%)	142 (18.6%)	0.789	0.283	0.214
Control	460 (35.7%)	615 (47.7%)	213 (16.6%)			
IL13						
−1510A→C						
	AA	AC	CC			
Atopy	381 (53.4%)	267 (37.5%)	65 (9.1%)	0.63	0.17	0.248
Control	646 (52.3%)	498 (40.3%)	91 (7.4%)			
−1111C→T						
	CC	CT	TT			
Atopy	478 (67.3%)	203 (28.6%)	29 (4.1%)	0.614	0.907	0.880
Control	805 (66.2%)	360 (29.6%)	51 (4.2%)			
R130Q						
	RR	RQ	QQ			
Atopy	364 (47.5%)	334 (43.5%)	69 (9%)	0.631	0.891	0.853
Control	628 (48.7%)	546 (42.4%)	114 (8.9%)			
TNF						
−308G→A						
	GG	GA	AA			
Atopy	606 (82.6%)	126 (17.2%)	2 (0.2%)	0.031	0.857	0.043
Control	1083 (86.2%)	170 (13.5%)	4 (0.3%)			
KDR						
V297I						
	VV	VI	II			
Atopy	543 (71.3%)	206 (27.1%)	12 (1.6%)	0.048	0.212	0.029
Control	972 (75.4%)	286 (22.2%)	30 (2.4%)			
H472Q						
	HH	HQ	QQ			
Atopy	256 (33.7%)	355 (46.8%)	148 (19.5%)	0.559	0.698	0.827
Control	451 (35.1%)	597 (46.4%)	240 (18.5%)			
IL12B						
S226N						
	SS	SN	NN			
Atopy	724 (94.4%)	43 (5.6%)	0 (0%)	0.744	–	0.748
Control	1230 (95.5%)	58 (4.5%)	0 (0%)			
IL12RB1						
M365T						
	MM	MT	TT			
Atopy	247 (32.8%)	351 (46.6%)	155 (20.6%)	0.845	0.555	0.767
Control	420 (32.4%)	624 (48.1%)	253 (19.5%)			
MS4A2						
E237G						
	EE	EG	GG			
Atopy	568 (80.8%)	120 (17.1%)	15 (2.1%)	0.076	0.129	0.028
Control	936 (77.4%)	259 (21.4%)	15 (1.2%)			

*p Values for logistic analyses controlling for age, sex, a family history of allergic diseases, passive smoking history and vaccination history.
†Dominant model (AA vs AB+BB), recessive model (AA+AB vs BB) and codominant model (AA vs AB vs BB), where A is the major frequency allele and B is the minor frequency allele.

in *TNF* and *KDR* was identified by MDR as being associated with atopy. Moreover, this was confirmed again by conventional logistic regression analysis.

To date, several investigators have witnessed gene–gene interactions in asthma or its related phenotypes using traditional procedures for fitting logistic regression models.[15 32]

Table 4 Summary of multiloci interaction for atopy by multifactor-dimensionality reduction analysis

Number of loci	Combination of SNPs	CV consistency	Prediction error (%)
2	−308G→A (*TNF*), V297I (*KDR*)	10	35.93
3	−308G→A (*TNF*), V297I (*KDR*), Q576R (*IL4R*)	3	35.44
4	E237G (*MS4A2*), V297I (*KDR*), I75V (*IL4R*), −1510A→C (*IL13*),	4	39.39
5	I75V (*IL4R*), R130Q (*IL13*), M365T (*IL12RB1*), H472Q (*KDR*), S226N (*IL12B*)	8	39.41

CV, cross-validation; SNP, single-nucleotide polymorphism.

However, logistic regression analysis can be problematic leading to an increase in type II errors and a decrease in power. For example, forward selection is limited because interactions are only tested for those variables that have a statistically significant independent main effect. Those SNPs that have an interaction effect but not a main effect will be missed. Similarly, only two SNPs with the strongest evidence for an association with asthma phenotypes were selected in previous studies. On the contrary, MDR is able to identify evidence for high order gene–gene interactions in the absence of statistically significant independent main effects in diseases.[2 3] No a priori assumption was made on whether there was an interaction between any specific combination of SNPs in this study. Along with this, MDR effectively detected two-locus interactions among 12 SNPs, which showed no significant association with atopy individually after correction for multiple testing in this study. We used 0.016 (0.05/3) as a Bonferroni-corrected p value because we analysed our findings at each locus under three models. The Bonferroni procedure is said to be conservative and, thus, will be unable to detect some of the actual differences. However, the MDR procedure in this study evidently finds genetic effects on atopy derived by gene–gene interactions, which is much stronger than those caused by individual SNPs.

The results of this study are of particular interest because previous studies have consistently reported that interactions between *IL4RA* and *IL13* markedly increased an individual's susceptibility to asthma.[8 15] Recently, Chan *et al*,[8] using MDR analysis, showed a significant interaction between I50V in the *IL4RA* gene and R130Q in the *IL13* gene for asthma that were also included for analysis in this study.[8] However, like us, they failed to demonstrate an interaction between *IL4RA* and *IL13* for total serum IgE concentration, another important intermediate phenotype of atopy. Taken together, these findings suggest that, although atopy is an important risk factor, additional or different genetic factors are needed for the development of asthma.

This study gives a new insight in the genetic basis for atopy—that is, a significant interaction between V297I in the *KDR* gene and −308G→A in the *TNF* gene. VEGF was originally described as a vascular permeability factor because of its ability to generate tissue oedema, and subsequently it was found to be a multifunctional angiogenic regulator that stimulates epithelial cell proliferation, blood vessel formation and endothelial cell survival.[33] VEGF receptor 2 is a major VEGF signalling receptor.[33]

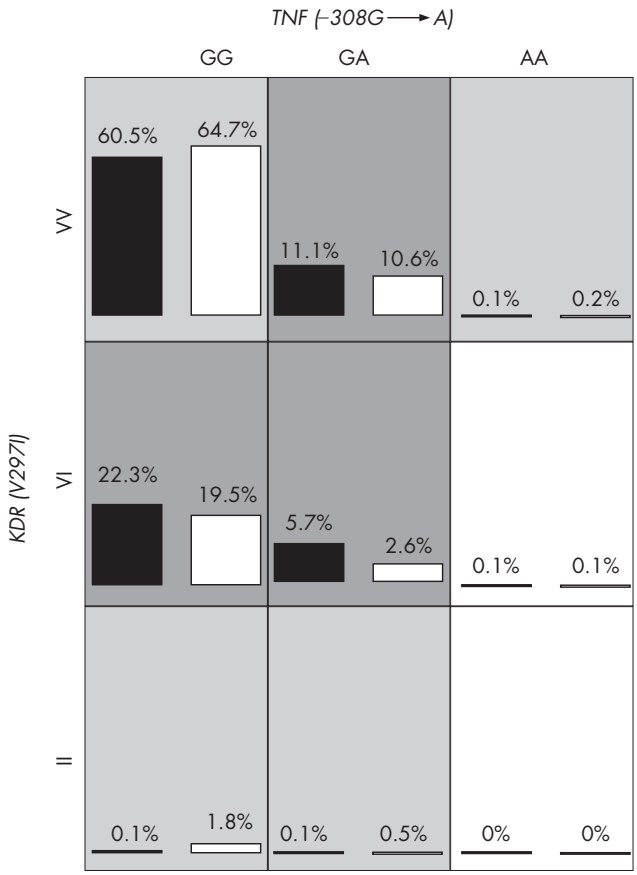

Figure 1 Distribution of high-risk and low-risk genotypes in the best two-locus model. This summary of the distribution shows high-risk (dark shading) and low-risk (light shading) genotypes associated with atopy in the two-locus interaction detected by multifactor dimensionality reduction analysis. The percentage of participants with atopy (left black bar in boxes) and control subjects (right hatched bar in boxes) is shown for each two-locus genotype combination. The white boxes are unclassified. *KDR*, kinase insert domain-containing receptor; *TNF*, tumour necrosis factor.

The ability of cockroach antigen to directly stimulate epithelial VEGF[34] may account for the impressive levels of sensitisation that are caused by even low-level exposure to this antigen.[35] Moreover, our previous study demonstrated that V297I causing amino acid change in regions in the *KDR* gene which is essential for maintaining the high association rate with VEGF and retention of the VEGF on the receptor, was significantly associated with the prevalence of atopy.[26] It is possible that VEGF contributes to the proclivity of individuals to become sensitised to respiratory antigens, and thus genetic variation in the *KDR* gene may have effects on the development of atopy. Tumour necrosis factor (TNF)α is a potent proinflammatory cytokine that is thought to be associated with a predisposition to atopy.[36] During early maturation of the infant's immune system, TNFα might be produced by antigen-presenting cells such as monocytes–macrophages, dendritic cells[37] and mast cells,[38] playing an important role in the interactions between innate and adaptive immunity. Innate immune cytokines, such as TNFα, are likely to be involved in priming the adaptive immune humoral responses. Notably, recent evidence has linked TNFα to the development of allergic rhinitis in mice.[39] Interestingly, it has been shown that TNFα and VEGF react with each other in an inflammatory site; TNFα induces VEGF[40] and vice versa.[41] Collectively, *TNF* and *KDR* may synergistically influence the development of atopy through gene–gene interaction.

Table 5 Interactions between *TNF* and *KDR* genotypes for atopy

Genotype		Phenotype			
TNF (−308G→A)	*KDR* (V297I)	Atopy, n (%)	Control, n (%)	p Value*	OR (95% CI)
GG	VV	434 (35)	806 (65)		1
GG	VI or II	81 (37.5)	135 (62.5)	0.53	1.11 (0.83 to 1.50)
GA or AA	VV	166 (38.5)	265 (61.5)	0.21	1.16 (0.93 to 1.46)
GA or AA	VI or II	47 (55.3)	38 (44.7)	<0.001	2.30 (1.48 to 3.57)

*p Values for logistic analyses controlling for age, sex, a family history of allergic diseases, passive smoking history and vaccination history.

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Supplementary table S1 is available at http://thorax.bmj.com/supplemental

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Multilocus analysis of atopy in Korean children using multifactor-dimensionality reduction

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Running title: Gene-gene interaction of atopy

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Abbreviations:

CV: Cross Validation

KDR: Kinase insert domain–containing receptor

MDR: Multifactor dimensionality reduction

VEGF~~R~~: Vascular endothelial growth factor

TNF: Tumor necrosis factor

SNP: Single nucleotide polymorphism

Summary

Background: Atopy is considered to be a complex genetic trait and do not follow a simple Mendelian pattern of heritance. It is now well recognized that gene-gene interactions are important in complex genetic disease.

Methods: A total of 2,055 ethnically identical subjects aged from 10 to 18 years living in rural areas on Jeju Island, Korea were randomly recruited. Atopy was defined as a positive skin prick test response to one or more common inhalant allergen. We analyzed gene to gene interactions among 12 polymorphic loci in the 7 candidate genes of atopy using multidimensionality reduction method.

Results: A significant interaction was found between V297I in the **gene coding** vascular endothelial growth factor receptor 2 (~~***VEGFR2***~~ ***KDR***) and -308G>A in the **gene coding** ~~***TNFα***~~ **tumor necrosis factor α (*TNF*)** on the risk of atopy, with a cross-validation consistency of 10 of 10 and a prediction error of 35.9% (P = **0**.001). Conventional logistic regression also revealed significant interactions between ~~***VEGFR2***~~ ***KDR*** and ~~***TNFα***~~ ***TNF*** for atopy. Individuals with the variant allele of -308G>A in ~~***TNFα***~~ ***TNF*** (GA or AA) and V297I in ~~***VEGFR2***~~ ***KDR*** (VI or II) had a significant higher risk of atopy [OR (95%CI) = 2.23~~97~~ (1.48~~79~~-3.57~~68~~)].

Conclusion: ~~***TNFα***~~ ***KDR*** and ~~***VEGFR2***~~ ***TNF*** may synergistically influence on the

development of atopy through gene-gene interaction in Korean children and adolescents.

Atopy is defined as a genetic predisposition to induce enhanced IgE responses to common environmental allergens. It is considered to be a complex genetic trait and thus do not follow a simple Mendelian pattern of heritance. Instead, the genetic determination of atopy is likely due to several genes, each having a small, possibly synergistic, effect on phenotype. Therefore it is necessary to consider simultaneously the effect of several single nucleotide polymorphism (SNP) genotypes at different loci. Such gene-gene interactions are traditionally evaluated using logistic regression. However procedures for fitting logistic regression model are problematic leading to an increase in type II errors and a decrease in power. In addition, sparseness of the data can be another problem in high dimensions.

To address these problems, multifactor dimensionality reduction or MDR has been developed[1]. MDR is a nonparametric and genetic model-free approach and is able to identify evidence for high-order gene–gene interactions in the absence of any statistically significant independent main effects in simulated data[2,3]. With MDR, gene-gene interactions has been revealed in complex genetic disorders, such as hypertension[4], type 2 diabetes mellitus[5], atrial fibrillation[6], myocardial infarction[7] and asthma[8].

In the present study, 12 loci in 7 genes proven to be related with atopy or enhanced serum IgE were genotyped in Korean children and adolescents and gene-gene

interaction was examined with MDR.

Methods

Study subjects and atopy definition

All the subjects enrolled in this study gave written informed consent, and the study protocol was approved by the Institutional review board of Seoul National University Hospital. ~~A total of 2,055 ethnically identical subjects aged from 10 to 18 years living in rural areas on Jeju Island, Korea were randomly recruited.~~ **A total of 2,864 ethnically identical subjects aged from 10 to 18 years were randomly recruited through school located on the southern part of Jeju Island in Korea, of whom 2,055 (71.8%) were enrolled in this study. All of them resided in rural area and most of their parents lived by fishing or cultivating fruit trees.** Skin prick test with 11 common aeroallergens (***Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog fur, cat fur, *Aspergillus*, *Alternaria*, tree pollen mixture, grass pollen mixture, mugwort, ragweed, and cockroach;** Allergopharma, Germany) was performed as previously described[9]. Subjects who had received oral antihistamines during the five days prior to skin prick test or had dermographism were excluded. Atopy was defined as a positive skin prick test response (allergen/histamine ratio > 1.0 plus a mean wheal size > 4 mm) to one or more allergens.

Selection of genes and SNPs

~~Using public databases, e.g. PubMed and Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/Omim/), we selected 7 candidate genes and 12 SNPs within these genes that have been characterized and potentially associated with atopy and enhanced serum IgE[10-24]. These are listed in Table 1.~~

On the assumption that cytokines play a crucial role in the widely used immunological model that explains the increasing prevalence of atopy by an altered balance between Th1 and Th2 immune responses[10], we selected 6 cytokine-related candidate genes using public databases, e.g. PubMed and Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/Omim/). All have been characterized and potentially associated with atopy or enhanced serum IgE in asian population and subsequent studies performed in non-asian population have witnessed this association[11-25]. The only exception was *KDR* (Kinase insert domain–containing receptor) coding vascular endothelial growth factor (VEGF) receptor 2 which, in our previous study, was proven to be associated with increased prevalence of atopy in Korean population[26]. In addition to these cytokine-related genes, *MS4A2* coding high affinity IgE receptor beta subunit (FcεR1B) was included which was also known to be significantly associated with atopy[27,28]. Within these genes, 10 functional SNPs[11,12,14,17,18,29-31] were chosen for analysis. As

for *KDR*, I297V and H472Q were selected because they were located in regions coding for the extracellular fourth and fifth immunoglobulin-like domains and thus possibly alter signaling pathway as discussed in our previous study[26].

Genotyping

SNPs were scored using the high throughput single base-pair extension method (SNP-IT™ assay) using an SNPstream25K system, which was customized to automatically genotype DNA samples in 384 well plates and provide a colorimetric readout (Orchid Biosciences, New Jersey, USA) as previously described~~[25]~~ [26].

Statistical analysis

Allele frequencies were estimated by gene counting methods and the $\chi 2$ test was used to examine the Hardy-Weinberg equilibrium. Association between atopy and each 12 loci was made with the Pearson $\chi 2$ test with use of dominant, recessive and co-dominant genetic models. MDR (version 0.6.2; Computational Genetics Laboratory, Dartmouth Medical School, Hanover, NH; http://www.epistasis.org) was done as described previously[1-3,5,8]. Briefly, the data set is divided into ten parts of equal size for 10-fold cross-validation (CV) **(9/10 of the data for training and 1/10 of the data for testing).** ~~**A training set composed of 9/10 of the data used to build the MDR model is selected. The remaining 1/10 of the data is used to compose the testing set.**~~ Next, a

set of *n* ~~genetic factors~~ **SNP polymorphisms** is selected which are represented in *n*-dimensional space. **Then the ratio of cases to controls is calculated for each combination, which is labeled 'high-risk' (> 1.0) or 'low-risk' (< 1.0).** ~~During the model selection process, each multifactor cell class in *n*-dimensional space is labeled as high-risk if the ratio of cases to controls meets or exceeds the threshold of 1.0 and as low-risk if the threshold is not exceeded. When the final best model is selected, a model for high-risk and low-risk genotype combinations is formed using an adjusted threshold that is equal to the ratio of cases and controls in the dataset.~~ **Consequently, *n* dimensional space was reduced to one dimension with two levels.** Among all of the two-factor combinations, a single model that ~~maximizes the cases to controls ratio is selected. This two-locus model has the minimum~~ **minimizes** classification error **is chosen.** ~~among the two-locus models.~~ To evaluate the predictive ability of the model, **a prediction error is obtained through 10-fold CV.** ~~the model is evaluated on the basis of the 1/10 of the data left out for testing. This procedure is done ten times, each time using a different 1/10 of the data for testing.~~ In our study we ~~we~~ set out to detect all two-locus interactions through five-locus interactions due to computation restrictions. ~~The result is a set of models, one for each model size considered.~~ From this set, the model with the combination of loci and/or discrete

environmental factors that maximizes the CV consistency and minimizes the prediction error is selected. ~~CV consistency is a measure of the number of times a particular set of loci and/or factors are identified in each possible 9/10 of the subjects. The proportion of subjects for whom an incorrect prediction was made is the prediction error. When CV consistency is maximal for one model and prediction error is minimal for another, statistical parsimony is used to choose the best model. Thus when the CV metric and the prediction error metric support different models, the model with the fewest loci/factors is selected. Hypothesis testing of this final best model can then be done by evaluating the magnitude of the prediction error.~~ We determined statistical significance **of the final best model** ~~by comparing the average prediction error from the observed data with the distribution of average prediction errors under the null hypothesis of no associations derived empirically from~~ **using** 1000 permutation~~s~~ **testing**. **The entire procedure is repeated for each, generating a distribution of predictive errors and CV consistencies that could be expected by chance alone. The significance of the final model is determined by comparing the predictive error and CV consistency of the final model to the distribution. A P value is extracted for the model by its theoretical location in the distribution.** ~~In this study, prediction error is evaluated for the best model identified by MDR in~~

~~each permuted dataset. The null hypothesis was rejected when the upper-tail Monte Carlo p-value derived from the permutation test was 0.05 or lower.~~ In addition, logistic regression analysis and $\chi 2$ tests were performed to confirm the results from MDR analyses. A P value of less than **0**.05 was considered statistically significant. **The detection power of the sample in the present study was 0.8 for atopy, if the relative risk for atopy in those persons carrying a putative risk allele is set to 2 compared with that in persons without the allele (The exception was S226N in *IL12B* whose detection power was 0.6).**

Results

Study population

A total of 2,055 children and adolescents were enrolled. The mean age was 14.6 (range; 10-18) and 48.9% of them were male. **The characteristics of study population are shown in Table 2.** ~~**The prevalence of atopy was 37.3% (767 subjects).**~~

Association between atopy and individual SNP

All 12 SNPs examined in the present study were in Hardy-Weinberg equilibrium. **The minor allele frequencies of SNPs in the present study with comparison to those previously reported are given in table S1 available online at the Thorax website http://www.thoraxjnl.com/supplemental.** Among them, ~~***FceR1B***~~ ***MS4A2*** E237G (*P* = **0**.028 in a co-dominant model), ~~***TNFa***~~ ***TNF*** -308G>A [*P* = **0**.031; odds ratio (95% CI) = 1.31~~5~~ (1.02~~4~~-1.6**9**~~86~~) in a dominant model] and ~~***VEGFR2***~~ ***KDR*** V297I [*P* =**0**.048; odds ratio (95% CI) = 1.22~~4~~ (1.00~~1~~-1.**50**~~498~~) in a dominant model] showed significant association with atopy (Table ~~2~~ **3**).

MDR analysis

Table ~~3~~ **4** summarizes, for each number of loci evaluated, the average CV consistency and average prediction error obtained from MDR analysis. One two-locus model had a minimum prediction error of 35.93% (*P* = **0**.001) and a maximum CV consistency of 10

out of 10. This two-locus model~~,~~ which included ~~the~~ ***TNF*** -308G>A ~~promoter SNP in the *TNFα* gene~~ and ~~the~~ ***KDR*** V297I ~~coding non-synonymous SNP in exon 7 of the *VEGFR2* gene~~ (~~Fig 1~~ **figure 1**) was regarded as the best model.

Logistic regression analysis

A significant interaction between ***TNF*** -308G>A ~~in *TNFα*~~ and ***KDR*** V297I ~~in *VEGFR2*~~ on the risk of atopy was also found by means of logistic regression analysis (P < **0**.001), adjusting for age, sex, passive smoking and family history of allergic disease as covariates. Individuals with the variant allele of ***TNF*** -308G>A ~~in *TNFα*~~ (GA or AA) and ***KDR*** V297I ~~in *VEGFR2*~~ (VI or II) had a significant higher risk of atopy [OR (96%CI) = 2.**30**~~297~~ (1.4**8**~~79~~-3.57~~68~~)]. ~~Figure 2~~ **Table 5** shows the results.

Discussion

Perhaps ~~a tough problem ever faced by allergist~~ **the toughest problem faced by the allergist** is that of identifying genes carrying alleles affecting liability to asthma or atopy from the vast field of potential candidates. The problem becomes even harder if gene-gene interaction must be considered. In the present study, a 2-locus model involving SNPs in ~~*TNFα*~~ **TNF** and ~~*VEGFR2*~~ **KDR** was identified by MDR as being associated with atopy. Moreover this was confirmed again by conventional logistic regression analysis.

So far, some investigators have witnessed gene-gene interactions in asthma or its related phenotypes using traditional procedures for fitting logistic regression models[~~16~~ 15,~~26 28~~ 32]. However, logistic regression analysis can be problematic leading to an increase in type II errors and a decrease in power. For example, forward selection is limited because interactions are only tested for those variables that have a statistically significant independent main effect. Those SNPs that have an interaction effect but not a main effect will be missed. Likewise only two SNPs with the strongest evidence for association with asthma phenotypes were selected in the previous studies. On the contrary, MDR is able to identify evidence for high order gene-gene interactions in the absence of the statistically significant independent main effects in diseases[2,3]. There was

no *a prior* assumption on whether there was interaction between any specific combination of SNPs in the present study. Along with this, MDR effectively detected 2-locus interaction among 12 SNPs which showed no significant association with atopy individually **after correction for multiple testing. We used 0.016 (0.05/3) as a Bonferroni corrected P value because we analyzed our findings at each locus under three models. The Bonferroni procedure is said to be conservative and thus will be unable to detect some of the actual differences. However, MDR procedure in the present study evidently finds genetic effects on atopy derived by gene-gene interactions which is much stronger than those caused by individual SNP.** ~~**(Association between atopy and individual SNP has become insignificant after multiple comparison).**~~

Results of the present study are of particular interesting in that previous studies consistently reported that interaction between IL4RA and IL13 markedly increased an individual's susceptibility to asthma[8,~~16~~ 15]. Recently, Chan IH and his colleagues showed a significant interaction between I50V in the ~~**IL4RA**~~ ***IL4RA*** and R130Q in the ~~**IL13**~~ ***IL13*** for asthma which were also included in the present study using MDR analysis[8]. However, like the present study, they failed to demonstrate an interaction between ~~**IL4R**~~ ***IL4RA*** and ~~**IL13**~~ ***IL13*** for total serum IgE concentration, another important

intermediate phenotype of atopy. Taken together, these findings implied that, although atopy is known to be an important risk factor, additional or different genetic factors are needed for the development of asthma. The present study showed a new insight in the genetic basis for atopy, that is, a significant interaction between V297I in **the** ~~***VEGFR2***~~ ***KDR*** and -308G>A in **the** ~~***TNFα***~~ ***TNF***. ~~**Vascular endothelial growth factor (**~~VEGF~~**)**~~ was originally described as a vascular permeability factor ~~**(VPF)**~~ because of its ability to generate tissue edema and ~~**S**~~**s**ubsequently, it was appreciated to be a multifunctional angiogenic regulator that stimulates epithelial cell proliferation, blood vessel formation, and endothelial cell survival.[~~29~~ 33] ~~**VEGFR2**~~ **VEGF receptor 2** is known to be a major VEGF signaling receptor.[~~29~~ 33] The ability of cockroach antigen to directly stimulate epithelial VEGF elaboration [~~30~~ 34] may account for the impressive levels of sensitization that are caused by even low-level exposure to this antigen.[~~31~~ 35] Moreover, our previous study demonstrated that V297I causing amino acid change in regions in **the** ~~***VEGFR2***~~ ***KDR*** which is known to be essential for maintaining the high association rate with VEGF and retention of the VEGF on the receptor, was significantly associated with the prevalence of atopy.[~~32~~ 26] It is plausible that VEGF contributes to the proclivity of individuals to become sensitized to respiratory antigens and thus genetic variation in **the** ~~***VEGFR2***~~ ***KDR*** may have effects on the development of atopy. Tumo~~u~~r necrosis factor

α (TNFα) is a potent proinflammatory cytokine that is ~~proven~~ **thought** to be associated with predisposition to atopy.[~~33~~ 36] During early maturation of the infant's immune system, TNFα might be produced by antigen-presenting cells, such as monocytes-macrophages, dendritic cells[~~33~~ 37] and mast cells,[~~34~~ 38] playing an important role in the interactions between innate and adaptive immunity. Innate immune cytokines, such as TNFα, are likely involved in priming the adaptive immune-humoral responses. Notably, recent evidence has linked TNFα to the development of allergic rhinitis in mice.[~~34~~ 39] Interestingly, it has been demonstrated that TNFα and VEGF react upon each other in inflammatory site; TNFα induces VEGF[~~35~~ 40] and *vice versa*.[~~36~~ 41] Collectively, ~~*TNFα*~~ ***TNF*** and ~~*VEGFR2*~~ ***KDR*** may synergistically influence on the development of atopy through gene-gene interaction.

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~~**Table 1 Candidate genes and SNPs analyzed in this study**~~

~~**Gene**~~	~~**SNP**~~	~~**Association**~~
~~***FcεR1B****~~	~~**E237G**~~	~~**atopy[10], IgE[10,11]**~~
~~***IL4R***~~	~~**-3223T>C, Q576R, I75V**~~	~~**atopy[12-14], IgE[15,16]**~~
~~***IL13***~~	~~**-1510A>C, -1111C>T, R130Q**~~	~~**atopy[14,17], IgE[14,18]**~~
~~***TNFα***~~	~~**-308G>A**~~	~~**atopy[19,20], IgE[21]**~~
~~***VEGFR2***[†]~~	~~**I297V, H472Q**~~	~~**atopy[22]**~~
~~***IL12B***~~	~~**S226N**~~	~~**atopy[23]**~~
~~***IL12R1B***~~	~~**M356T**~~	~~**atopy[24]**~~

~~***FcεR1B: high affinity IgE receptor beta subunit**~~

~~[†]**VEGFR2: Vascular growth factor receptor 2**~~

Table 1 Candidate genes and SNPs analyzed in this study

Gene	SNP	rs number	Association
IL4R	-3223T>C	rs2057768	atopy[11-13], IgE[14]
	Q576R	rs1801275	
	I75V	rs1805010	
IL13	-1510A>C	rs1881457	atopy[16,17], IgE[15,18]
	-1111C>T	rs1800925	
	Q144R	rs20541	
TNF	-308G>A	rs1800629	atopy[19,20], IgE[21]
IL12B	S226N	-	atopy[22,23]
IL12RB1	M365T	rs375947	atopy[24,25]
*KDR**	V297I	rs2305948	atopy[26]
	H472Q	rs1870377	
MS4A2±	E237G	rs569108	atopy[27,28], IgE[27,28]

* *KDR*: gene coding vascular growth factor receptor 2

±*MS4A2*: gene coding high affinity IgE receptor beta subunit

Table 2. The characteristics of study population

Characteristics	Number of positivity (%)
Age, mean (range)	14.6 (10-18)
Male	1004 (48.9%)
Atopy*	767 (37.3%)
History of passive smoking	1342 (65.3%)
Family history of allergic disease	376 (18.3%)
Vaccination history	1552 (75.5%)

* Positive skin test responses to one or more common aeroallergens.

Table ~~2~~ 3. Genetic effects of individual SNP on atopy

	Genotype frequency			*P* value*		
				Dominant†	Recessive†	Co-dominant†
IL4R						
	-3223T>C					
	TT	TC	CC			
Atopy	278 (38.0%)	346 (47.3%)	108 (14.7%)	0.130	0.413	0.301
Control	515 (41.4%)	561 (45.1%)	167 (13.5%)			
~~***IL4R***~~	**Q576R**					
	QQ	QR	RR			
Atopy	529 (69.2%)	214 (28.0%)	21 (2.8%)	0.831	0.426	0.728
Control	895 (68.8%)	362 (27.8%)	44 (3.4%)			
~~***IL4R***~~	**V75I**					
	VV	VI	II			
Atopy	244 (31.9%)	378 (49.5%)	142 (18.6%)	0.789	0.283	0.214
Control	461 (35.5%)	619 (47.7%)	217 (16.8%)			
IL13						
	-1510A>C					
	AA	AC	CC			
Atopy	381 (53.4%)	267 (36.5%)	65 (10.1%)	0.630	0.170	0.248
Control	646 (52.3%)	498 (40.3%)	91 (7.4%)			
~~***IL13***~~	**-1111C>T**					
	CC	CT	TT			
Atopy	478 (67.3%)	203 (28.6%)	29 (4.1%)	0.614	0.907	0.880

Control	805 (66.2%)	360 (29.6%)	51 (4.2%)			
~~*IL13*~~	**R130Q**					
	RR	RQ	QQ			
Atopy	364 (47.5%)	334 (43.5%)	69 (9.0%)	0.631	0.891	0.853
Control	628 (48.7%)	546 (42.4%)	114 (8.9%)			
~~*TNFα*~~ *TNF*						
	-308G>A					
	GG	GA	AA			
Atopy	606 (82.6%)	126 (17.2%)	2 (0.2%)	**0.031**	0.857	**0.043**
Control	1083 (86.2%)	170 (13.5%)	4 (0.3%)			
~~*VEGFR2*~~ *KDR*						
	V297I					
	VV	VI	II			
Atopy	543 (71.3%)	206 (27.1%)	12 (1.6%)	**0.048**	0.212	**0.029**
Control	976 (75.3%)	289 (22.3%)	31 (2.4%)			
~~*VEGFR2*~~**H472Q**						
	HH	HQ	QQ			
Atopy	256 (33.7%)	355 (46.8%)	148 (19.5%)	0.559	0.698	0.827
Control	451 (35.1%)	597 (46.4%)	240 (18.5%)			
IL12B						
	S226N					
	SS	SN	NN			
Atopy	724 (94.3%)	43 (5.7%)	0 (0%)	0.744	-	0.748
Control	1230 (95.5%)	58 (4.5%)	0 (0%)			

IL12R~~1~~B1

	~~M356T~~ M365T					
	MM	MT	TT			
Atopy	247 (32.8%)	351 (46.6%)	155 (20.6%)	0.845	0.555	0.767
Control	420 (32.4%)	624 (48.1%)	253 (19.5%)			

~~FceR1B~~ MS4A2

	E237G					
	EE	EG	GG			
Atopy	568 (80.8%)	120 (17.7%)	15 (1.5%)	0.076	0.129	**0.028**
Control	936 (77.4%)	259 (21.4%)	15 (1.2%)			

*P values for logistic analyses controlling age, sex, a family history of allergic diseases, passive smoking history, and vaccination history

†Dominant model (AA *vs*. AB+BB), Recessive model (AA+AB *vs*. BB), and Co-dominant model (AA *vs*. AB *vs*. BB), where A is the major frequency allele and B is the minor frequency allele

Table ~~3~~ 4. Summary of multiloci interaction for atopy by MDR analysis

No. of loci	Combination of SNPs	CV consistency	Prediction error (%)
2	-308G>A (~~*TNFα*~~ ***TNF***), V297I (~~*VEGFR2*~~ ***KDR***)	10	35.93
3	-308G>A (~~*TNFα*~~ ***TNF***), V297I (~~*VEGFR2*~~ ***KDR***), Q576R (*IL4R*)	3	35.44
4	E237G (~~*FcεR1B*~~ ***MS4A2***), V297I (~~*VEGFR2*~~ ***KDR***), I75V (*IL4R*), -1510A>C (*IL13*),	4	39.39
5	I75V (*IL4R*), R130Q (*IL13*), ~~M356T~~ **M365T** (*IL12R~~1~~B1*), H472Q (~~*VEGFR2*~~ ***KDR***), S226N (*IL12B*)	8	39.41

Table 5. Interactions between *TNF* and *KDR* genotypes for atopy.

Genotype		Phenotype		P value*	OR (95%CI)
TNF (-308G>A)	*KDR* (V297I)	atopy	control		
GG	VV	434 (35.0%)	806 (65.0%)		1
GG	VI or II	81 (37.5%)	135 (62.5%)	0.53	1.11 (0.83-1.50)
GA or AA	VV	166 (38.5%)	265 (61.5%)	0.21	1.16 (0.93-1.46)
GA or AA	VI or II	47 (55.3%)	38 (44.7%)	< 0.001	2.30 (1.48-3.57)

*P values for logistic analyses controlling age, sex, a family history of allergic diseases, passive smoking history, and vaccination history

Table S1 The minor allele frequencies of SNPs compared to those previously reported

SNP	Minor allele frequency	
	Present study	Previous reports (population and reference)
IL4R		
-3223T>C	37.2 %	10.7 % (Swedish healthy adults, ref. 29)
Q576R	16.8 %	22.0 % (Japanese healthy adults, ref. 13)
		30.5 % (American asthmatics, ref. 12)
I75V	41.6 %	40.4 % (Japanese healthy adults, ref. 14)
		42.0 % (American asthmatics, ref. 12)
IL13		
-1510A>C	28.0 %	22.0 % (American healthy adults, ref. 15)
-1111C>T	18.9 %	23.0 % (American healthy adults, ref. 15)
Q144R	30.8 %	40.8 % (Chinese healthy children, ref. 16)
		26.7 % (British healthy adults, ref. 13)
TNF		
-308G>A	7.7 %	7.0 % (Taiwanese healthy children, ref. 20)
		12.9 % (Czech healthy adults, ref. 19)

IL12B		
S226N	3.6 %	4.0 % (Japanese asthmatics‡)
IL12RB1		
M365T	33.0 %	36.2 % (Japanese healthy adults, ref. 31)
KDR*		
V297I	14.2 %	-
H472Q	42.6 %	-
MS4A2±		
E237G	11.7 %	5.3 % (Australian healthy adults, ref. 27)
		11.5 % (Japanese healthy adults¶)

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* ***KDR***: gene coding vascular growth factor receptor 2

±***MS4A2***: gene coding high affinity IgE receptor beta subunit

‡ Noguchi E, Yokouchi Y, Shibasaki M, et al. Identification of missense mutation in the IL12B gene: lack of association between IL12B polymorphisms and asthma and allergic rhinitis in the Japanese population. Genes Immun 2001; 2:401-3

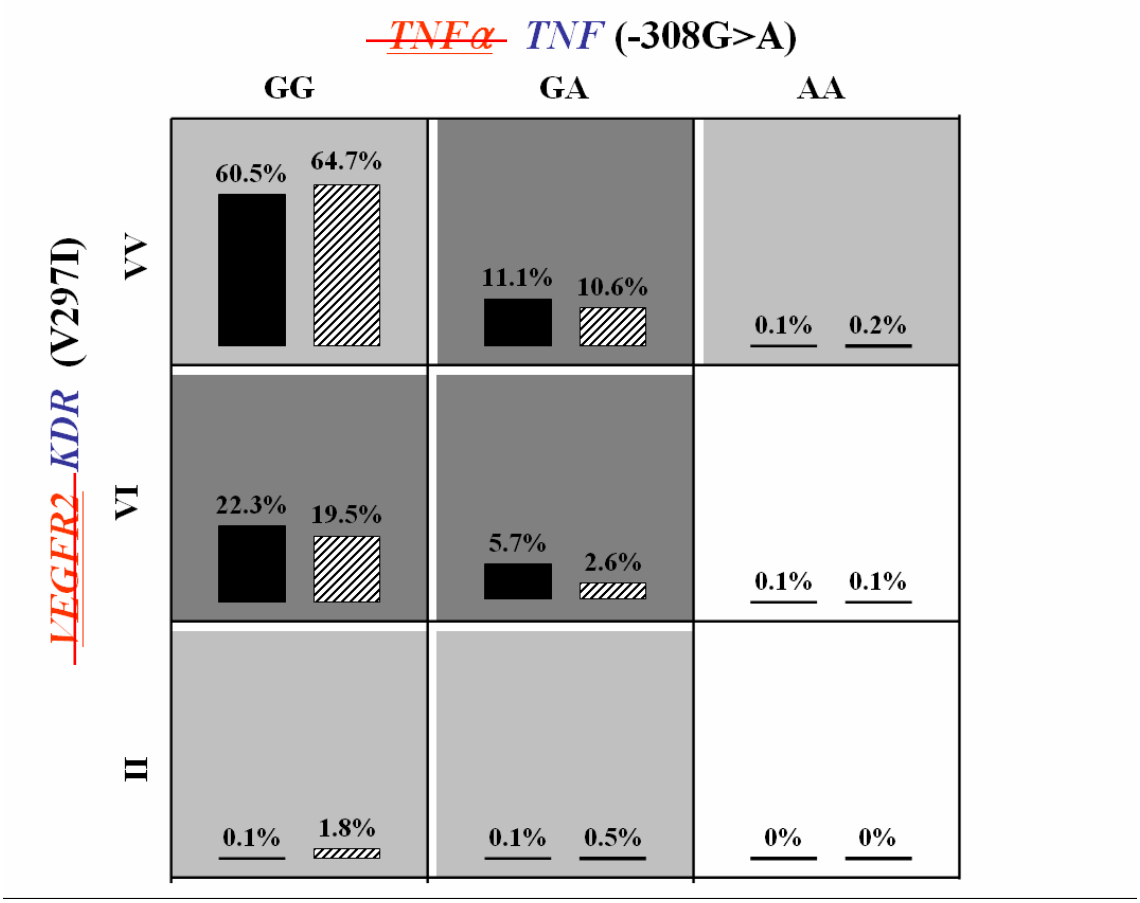
¶ Nagata H, Mutoh H, Kumahara K, et al. Association between nasal allergy and a coding variant of the Fc epsilon RI beta gene Glu237Gly in a Japanese population. Hum Genet 2001; 109:262-6.

Figure legends

Figure 1. Distribution of high-risk and low-risk genotypes in the best two-locus model. This summary of the distribution shows high-risk (dark shading) and low-risk (light shading) genotypes associated with atopy in the two-locus interaction detected by MDR analysis. The percentage of atopic subjects (left black bar in boxes) and control subjects (right hatched bar in boxes) is shown for each two-locus genotype combination. The white boxes are unclassified.

~~**Figure 2. Interactions between *TNFα* and *VEGFR2* genotypes for atopy. Bars indicate the ORs between the different combinations of genotypes for *TNFα* (-308G>A) and *VEGFR2* (V297I). The non-risk genotype for each gene was used as the reference OR.**~~

Figure 1.


TNFα TNF (-308G>A)
GG
GA
AA
VEGFR2 KDR (V297I)
VV
VI
II
60.5%
64.7%
11.1%
10.6%
0.1%
0.2%
22.3%
19.5%
5.7%
2.6%
0.1%
0.1%
0.1%
1.8%
0.1%
0.5%
0%
0%