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Relationship between neurokinin 2 receptor gene polymorphisms and serum vascular endothelial growth factor levels in patients with toluene diisocyanate-induced asthma

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Summary

Background Among the various pathogenic mechanisms of toluene diisocyanate (TDI)-induced asthma, a contribution from neurogenic inflammation has been suggested.

Objective To evaluate neurokinin 2 receptor (NK2R) gene polymorphisms in association with the clinical phenotype of TDI-induced asthma, 70 TDI-induced occupational asthma (TDI-OA) patients, 59 asymptomatic exposed controls (AEC), and 93 unexposed healthy controls (NC) were enrolled in the study.

Methods Two single-nucleotide polymorphisms (SNPs) of NK2R, 7853G > A (Gly231Glu) and 11 424G > A (Arg375His), were genotyped using a single base extension method. The levels of PC₂₀ methacholine, specific IgE and IgG to TDI-human serum albumin conjugate, and serum vascular endothelial growth factor (VEGF), matrix metalloproteinase-9, and TGF-β1 were compared according to the NK2R genotypes of the subjects with TDI-OA and AEC.

Results No significant differences in allele, genotype, or haplotype frequencies of these two SNPs were noted among the three groups ($P > 0.05$, respectively). Moreover, subjects with the NK2R 7853GG genotype had higher serum VEGF levels than those with GA or AA among the TDI-exposed workers ($P = 0.040$).

Conclusion The NK2R 7853GG genotype may contribute to increased serum VEGF levels, which result in airway inflammation after TDI exposure.

Keywords asthma, genetic susceptibility, isocyanate, neurokinin 2 receptor

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Introduction

Although toluene diisocyanate (TDI) is one of the most common causes of occupational asthma [1, 2], the pathogenesis of isocyanate-induced asthma remains unclear. The immunological mechanism underlying TDI-induced asthma has been suggested to involve activated CD4⁺ T cells, elevated T-helper type 2 (Th2) cytokine levels produced, and the presence of specific antibodies [3–10]. Previous studies have indicated that the prevalence of specific IgE antibodies is < 50%, which suggests that these antibodies are not the only major mechanism of TDI-induced asthma [6–8]. However, our recent investigation comparing vapour TDI conjugate vs. conventional

liquid-type TDI-human serum albumin (HSA) conjugate [7] has shown that vapour TDI conjugate is superior for the detection of specific IgE and IgG antibodies in the sera of TDI-induced occupational asthma (TDI-OA) patients, which suggests that vapour-type conjugates are more physiologically relevant than those produced previously.

It has long been recognized that neurogenic inflammation plays an important role in TDI-induced airway hyper-responsiveness (AHR) [11–13]. Several studies have demonstrated that TDI exposure stimulates the release of neuropeptides from sensory nerves in the airways and inhibits neural endopeptidase, which is a glycosylated zinc metalloproteinase that cleaves and inactivates tachykinins [14–16]. The tachykinin substance P (SP) and

neurokinin (NK) A are potent bronchoconstrictors and are vasoactive and pro-inflammatory substances that have been widely studied in animal and human airways. Tachykinins exert their effects via three specific receptors: NK1, NK2, and NK3. These receptors belong to the G protein-coupled superfamily of receptors and have their own preferred endogenous ligand: NK1 receptors are preferentially activated by SP; NK2 receptors (NK2R) by NKA; and NK3 receptors by NKB [17, 18]. NK2R mRNA expression is fourfold higher in asthmatics than in non-smoking controls [18, 19], and the modulation of tachykinin receptor gene expression may augment or diminish the inflammatory, secretory, or bronchoconstrictive effects of released tachykinins.

As only 5–15% of the TDI-exposed individuals develop asthma, genetic factors may modify individual susceptibility. Glutathione S-transferases [20], *N*-acetyl transferases [21], and HLA class II genes [22, 23] have been suggested to confer susceptibility to or protection against TDI exposure-associated asthma risk. Although recent studies have shown that a single mutation of the NK2R gene impacts agonist-induced desensitization in *Xenopus* oocytes [24] and has a significant association with capsaicin-sensitive coughing in patients with chronic cough [25], no study to date has evaluated NK2R polymorphisms as potential disease modifiers in TDI-induced asthma.

The first aim of this study was to identify NK2R polymorphisms and evaluate their associations with the clinical characteristics of TDI-induced asthma. Two candidate single-nucleotide polymorphisms (SNPs), G231E and R375H, of the NK2R gene were determined according to a previous study in which these two non-synonymous SNPs were identified after direct sequencing of the NK2R exons of healthy Korean subjects [25].

More than 50% of the patients have persistent asthmatic symptoms despite complete avoidance of TDI exposure [6]. Vascular endothelial growth factor (VEGF) is an endothelial, cell-specific, mitogenic peptide that plays a critical role in the initiation and maintenance of asthma, enhances antigen sensitization, and increases vascular permeability, which allows inflammatory mediators and cells to leak into the extracellular space [26, 27]. Matrix metalloproteinase-9 (MMP-9) is the predominant MMP in the asthmatic airway. MMPs may contribute to the release and activation of growth factors, such as VEGF and TGF- β 1, and facilitate the migration of inflammatory cells [28]. Furthermore, pharmacological MMP inhibitors and VEGF receptor inhibitors have been found to reduce airway inflammation induced by TDI [26, 29, 30]. The second aim of this study was to investigate the potential associations between NK2R polymorphisms and the serum levels of VEGF, TGF- β 1, and MMP-9, which have been proposed as key mediators in the airway inflammation associated with TDI-induced asthma [2, 26–31].

Materials and methods

Subjects

Seventy patients with TDI-induced asthma, which was confirmed mainly by positive responses to TDI broncho-provocation tests, were enrolled into the TDI-OA group. Fifty-nine TDI-exposed asymptomatic workers aged 27–61 years, with similar exposure intensities to TDI and from the same working environments as the TDI-OA group members, which included the spray-painting and polishing departments of furniture and musical instrument industries, were classified as the asymptomatic-exposed controls (AEC). Ninety-three unexposed healthy control subjects (NC), aged 16–77 years, were recruited from a volunteer population. The demographic data of the three study groups are summarized in Table 1. Atopy was determined by a positive skin test to at least one common inhalant allergen, such as house dust mite, tree and pollen mixtures, mugwort, ragweed pollens, and *Alternaria* (Bencard, Bradford, UK). Sera from the TDI-OA group were collected at initial diagnosis; all of these subjects stopped taking inhaled or oral steroids 4 weeks before the study. All of the subjects underwent an interview, chest radiography, and skin prick test with common inhalant allergens, while lung function measurements and inhalation challenges with methacholine were conducted for all the TDI-OA subjects. All of the subjects gave informed consent, which was overseen by the Institutional Review Board of Ajou Medical Center, Suwon, Korea.

Bronchial challenge test with methacholine and TDI

The methacholine bronchial challenge test was performed on patients in the TDI-OA group according to the method described previously [6]. Briefly, aerosols were generated by a DeVilbiss 646 nebulizer connected to a DeVilbiss dosimeter, which was driven by compressed air (Devilbiss Co., Doylestown, PA, USA). Five inhalations of normal saline were administered at 5-min intervals, followed by a series of successively doubling doses of methacholine (0.075–25 mg/mL), until a 20% decline in forced expiratory volume in 1 s (FEV₁) was observed or the maximum dose given. FEV₁ was measured 5 min after the beginning of each set of inhalations of aerosolized methacholine. The methacholine PC₂₀ level was determined by interpolation from the dose–response curve. The TDI bronchial challenge test was performed according to the protocol described previously [6].

Preparation of vapour TDI–HSA conjugates

Vapour TDI–HSA conjugates were prepared by Wisniewski and colleagues using their recently described isocyanate vapour phase exposure system [9]. Briefly, TDI vapour

Table 1. Clinical characteristics of the three study groups

	TDI-OA, <i>n</i> = 70 (%)	AEC, <i>n</i> = 59 (%)	NC, <i>n</i> = 93 (%)
Age (years)	41.8 ± 9.4	41.9 ± 7.8	40.7 ± 14.9
Sex (male)	47 (67.1)	44 (74.6)	60 (64.5)
Atopy (presence/total)*	34/66 (51.5)	NA	19/69 (27.5)
Smoking status (NS/Ex/S) [†]	47/10/13	17/5/37	57/15/21
Exposure duration (years) [§]	8.3 ± 6.6	11.1 ± 6.7	NA
Baseline FEV ₁ (%)	86.6 ± 22.6	90.2 ± 20.2	NA
PC ₂₀ methacholine (mg/mL)	5.10 ± 7.7	NA	NA
Serum total IgE (IU/mL)	308.8 ± 434.5	261.2 ± 580.4	166.0 ± 217.5
Specific IgG to TDI-HSA [†]	16/47 (34%)	6/51 (11.8%)	NA
Specific IgE to TDI-HSA [†]	18/47 (38.3%)	3/51 (5.9%)	NA

Results are expressed as the mean ± SD. FEV₁ and PC₂₀ methacholine were measured at initial diagnosis.

**P* < 0.01, for TDI vs. NC.

[†]*P* < 0.01, for TDI vs. AEC.

[§]*P* = 0.05, for TDI vs. NC.

TDI-OA, TDI-induced asthma; AEC, asymptomatic-exposed controls; NC, normal controls; NS, non-smoker; Ex, ex-smoker; S, smoker; NA, not applicable; FEV₁, forced expiratory volume in 1 s.

concentrations in the range of 20–200 p.p.b. were passively generated in a closed-circuit system and monitored with the Autostep monitor (GMD, Pittsburgh, PA, USA). Low-endotoxin human albumin in PBS (pH 7.2) at a concentration of 0.5% (w/v) was exposed in open 60 mm Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA). The exposure unit was sterilized with 70% ethanol, and the protein solutions were passed through a 0.2 µm filter. The vapour TDI–albumin conjugates were prepared with an 80/20 mixture of 2,4-TDI/2,6-TDI.

ELISA for specific IgG and IgE antibodies to TDI-HSA conjugate

The serum-specific IgE and IgG levels were detected by ELISA as described previously [7]. Briefly, 1 µg of TDI–albumin conjugate or mock conjugate was dissolved in normal saline and used to coat ELISA plates (Corning, New York, NY, USA) at 37 °C for 2 h, followed by incubation at 4 °C overnight. After washing with PBS–Tween-20 (PBS-T), the plates were blocked with 350 µL of blocking buffer (PBS that contained 5% BSA and 0.05% Tween-20). Diluted serum samples (50 µL) from patients or control subjects at ratios of 1:5 for specific IgG and 1:3 for specific IgE in the preliminary experiments were incubated for 1 h at 25 °C in TDI–albumin and mock albumin-coated wells and washed four times. Alkaline phosphatase-conjugated anti-IgG (1:20 000, 100 µL; Sigma Chemical Co., St. Louis, MO, USA) or goat anti-IgE antibody (1:1000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), followed by alkaline phosphatase-conjugated rabbit anti-goat IgG antibody (1:200, Kirkegaard & Perry Laboratories), were added to each well and incubated for 1 h at room temperature (RT). The wells were washed, and 100 µL of substrate solution (Sigma) was

added. After 30 min at RT, the absorbances at 405 and 450 nm were read in an ELISA reader. To quantify the antibody binding specific for TDI, the optical density (OD) value for the mock-conjugated albumin wells was subtracted from the value obtained for the TDI–albumin coated wells. The positive cut-off values (71 for IgG, 24 for IgE) were based on the mean plus threefold SD of the absorbance values obtained for 80 unexposed healthy controls.

Genotyping

The sequences of the amplifying and extension primers for NK2R Gly231Glu (G231E) and NK2R Arg375His (R375H) were as follows: for NK2R G231E, forward 5'-tagcctacagcgtcatcg-3', reverse 5'-cttctcatggcctgcag-3', and extension 5'-caggttgccacgtgcgcctgatgt-3'; and for NK2R R375H, forward 5'-gcaagcaaaccataccca-3', reverse 5'-taaggagacttggctcatggc-3', and extension 5'-gaggctacagtgggggaggcggggc-3'. A single-base extension method was used for SNP genotyping according to the previously described methodology [32]. Primer extension reactions were performed with the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA).

ELISAs for VEGF, TGF-β1, and MMP-9 in sera

VEGF, MMP-9, and TGF-β1 levels in sera were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). The assays were performed according to the manufacturer's instructions.

Statistical analysis

A significant departure of genotype frequency from the Hardy–Weinberg equilibrium at each SNP was tested

using the χ^2 test with one degree of freedom (df). Differences in genotype frequency between the case and control subjects were tested by the χ^2 test. Logistic regression models were used for analyses of SNPs and haplotypes controlling for age, sex, and smoking status as covariables, with three alternative models (co-dominant, dominant, and recessive). The NK2R haplotypes were analysed using the Haploview version 2.05 software based on an estimation-maximization (EM) algorithm. Linkage disequilibrium between loci was measured using the absolute value of Lewontin's $|D'|$ [33]. Student's *t*-test was used to compare continuous variables (expressed as means $\pm$ SD), while categorical variables were compared using the χ^2 or Fisher's exact test. Continuous variables that did not show a normal distribution were log-transformed and presented in their original scale. The effects of NK2R+7853G > A (G231E) and NK2R+11424G > A (R375H) polymorphisms on the serum levels of VEGF, TGF- β 1, and MMP-9 (log-transformed) were analysed by univariate analysis of covariance using generalized linear models in which G231E and R375H were designated as fixed factors, respectively, and age, sex, and smoking status were designated as covariates. The statistical analysis was performed using the SPSS version 11.0 software (SPSS, Chicago, IL, USA). A *P*-value equal to or less than 0.05 was regarded as being statistically significant.

Results

Demographics of the study population

Table 1 shows the demographic data on the 70 patients in the TDI-OA group and the two control groups, 59 asymptomatic subjects in the AEC group, and 93 NC group subjects. There were no significant differences in mean

age or sex distribution among the three groups. The exposure duration was significantly longer and the ratio of current smokers was higher in the AEC group than in the TDI-OA group. Methacholine challenge testing was not performed on the asymptomatic TDI-exposed subjects or unexposed controls because these healthy individuals reported no respiratory symptoms.

Allele, genotype, and haplotype frequencies of the NK2R gene

We performed a genetic association study of two genetic polymorphisms of NK2R (NK2R G231E, NK2R R375H) in three groups of study subjects (Table 2). There were no significant differences in the allele, genotype, or haplotype frequencies of two polymorphisms of NK2R among the three groups. The two NK2R polymorphisms were in linkage disequilibrium ($|D'| = 1$), and three common haplotypes were constructed utilizing the EM algorithm. The analysis of genotype distribution of three haplotypes of NK2R did not show any significant differences among the three groups (Table 3).

Effects of NK2R polymorphisms on clinical features of patients with TDI-induced asthma

To assess whether two genetic polymorphisms (G231E and R375H) were related to the phenotype of TDI-induced asthma, we compared the clinical characteristics according to the genotypes (Table 4). There were no significant differences in asthma duration, baseline FEV₁, PC₂₀ methacholine dose, or serum total IgE levels according to the genotypes of these two polymorphisms (G231E and R375H) in the TDI-OA group.

Table 2. Genotype, allele, and haplotype frequencies of the NK2 receptor gene (NK2R)

Loci	Genotype/haplotype	TDI-OA, <i>n</i> = 70 (%)	AEC, <i>n</i> = 59 (%)	NC, <i>n</i> = 93 (%)	<i>P</i> -value*	
					TDI-OA vs. AEC	TDI-OA vs. NC
Gly231Glu	AA	17 (24.3)	9 (15.3)	25 (26.9)	0.69	0.81
	GA	35 (50.0)	31 (52.5)	46 (49.5)	0.37	0.82
	GG	18 (25.7)	19 (32.2)	22 (23.7)	0.10	0.99
	q.	0.51	0.58	0.49	0.25	0.98
Arg375His	GG	47 (75.8)	40 (69.0)	74 (85.1)	0.87	0.59
	GA	12 (19.4)	14 (24.1)	11 (12.6)	0.43	0.42
	AA	3 (4.8)	4 (6.9)	2 (2.3)	0.12	0.41
	q.	0.14	0.19	0.09	0.09	0.13
	ht1[AG]	0.51	0.41	0.51	0.10	0.38
	ht2[GG]	0.35	0.40	0.40	0.76	0.17
	ht3[GA]	0.14	0.19	0.09	0.10	0.40

*Each *P*-value was calculated using the co-dominant, dominant, and recessive models. Logistic regression analysis was applied for controlling age, sex, and smoking status as covariables. Haplotypes with frequency > 1% are listed.

TDI-OA, toluene diisocyanate-induced occupational asthma; AEC, asymptomatic-exposed controls; NC, unexposed controls; *n*, number of subjects; q, minor allele frequency.

Table 3. Genotype distribution of NK2 receptor haplotypes

	TDI-OA, <i>n</i> = 62 (%)	AEC, <i>n</i> = 58 (%)	NC, <i>n</i> = 87 (%)	<i>P</i> -value*	
				TDI-OA vs. AEC	TDI-OA vs. NC
ht1[AG]					
ht1/ht1	15 (24.2)	9 (15.5)	24 (27.6)	0.09	0.91
ht1/–	33 (53.2)	30 (51.7)	42 (48.3)	0.22	0.72
–/–	14 (22.6)	19 (32.8)	21 (16.1)	0.09	0.78
ht2[GG]					
ht2/ht2	7 (11.3)	9 (15.5)	14 (16.1)	0.68	0.50
ht2/–	29 (46.8)	28 (48.3)	41 (47.1)	0.87	0.65
–/–	26 (41.9)	21 (36.2)	32 (36.8)	0.56	0.50
ht3[GA]					
ht3/ht3	3 (4.8)	4 (6.9)	2 (2.3)	0.12	0.19
ht3/–	13 (21.0)	16 (27.6)	11 (12.6)	0.09	0.21
–/–	46 (74.2)	38 (65.5)	74 (85.1)	0.42	0.41

*Each *P*-value was calculated using the co-dominant, dominant, and recessive models. Logistic regression analysis was applied for controlling age, sex, and smoking status as covariables. Haplotypes with frequency > 1% are listed.

TDI-OA, toluene diisocyanate-induced occupational asthma; AEC, asymptomatic-exposed controls; NC, unexposed controls; *n*, number of subjects; q, minor allele frequency; NK, neurokinin.

Table 4. Clinical features of TDI-induced asthma patients according to NK2R polymorphisms (*N* = 70)

Loci	Genotype	FEV ₁ (%)	PC ₂₀ methacholine (mg/mL)
Gly231Glu	GA or AA (<i>n</i> = 52)	87.2 ± 20.8	4.89 ± 7.4
	GG (<i>n</i> = 18)	84.7 ± 28.5	5.65 ± 8.5
	<i>P</i> -value	0.466	0.747
Arg375His	GG or GA (<i>n</i> = 59)	84.0 ± 21.5	5.2 ± 8.0
	AA (<i>n</i> = 3)	106.9 ± 18.0	2.7 ± 2.2
	<i>P</i> -value	0.135	0.890

Data shown are the means ± SD. PC₂₀ methacholine, inhaled methacholine dose causing a 20% decline in FEV₁.

FEV₁, forced expiratory volume in 1 s; TDI, toluene diisocyanate; NK2R, neurokinin 2 receptor.

Associations between NK2R polymorphisms and inflammatory mediators in TDI-exposed workers (TDI-OA and AEC groups)

We examined the association of both NK2R polymorphisms with inflammatory mediators in the sera of TDI-exposed workers (Fig. 1). Carriers of the GG genotype of the G231E polymorphism showed significantly higher serum VEGF levels than carriers of the GA or AA genotype among the TDI-exposed workers after adjustment for age, sex, and smoking status (345.3 ± 224 pg/mL for GG; 239.2 ± 161 pg/mL for GA+AA; *P* = 0.040). Although no statistical significance was noted, TDI-exposed workers with the GG or GA genotype of the R375H polymorphism showed higher serum VEGF levels than those with the AA genotype. In addition, carriers with the haplotype ht2[GG] had significantly higher serum VEGF levels than those with other haplotypes (333.6 ± 217 pg/mL for [GG]; 240.0 ± 165 pg/mL for other haplotypes; *P* = 0.014). Furthermore, the significant association of two genetic

polymorphisms with serum VEGF levels was reproduced in the haplotypes of these two SNPs; the subjects who had the homozygous ht2[GG] haplotype tended to have significantly higher serum VEGF levels than those who carried other diploids (479.1 ± 206 pg/mL for ht2[GG]/ht2[GG]; 245.7 ± 172 pg/mL for ht2[GG]/ht2[GG] or ht2[GG]/–; *P* < 0.001). However, no significant correlations were found between the two SNPs and the levels of TGF-β1 and MMP-9 and the prevalence of specific antibodies to TDI-HSA conjugates in TDI-exposed workers (Table 5).

Discussion

In this study, we investigated the involvement of two genetic polymorphisms of NK2R, +7853G > A (Gly231-Glu or G231E) and +11424G > A (Arg375His or R375H), in the pathogenesis of TDI-induced asthma in the Korean population. There is abundant evidence to suggest a possible role for tachykinins in the pathophysiology of

asthma and bronchial hyper-responsiveness. Stimulation of a non-adrenergic non-cholinergic nerve by TDI as well as by irritants, cigarette smoke, capsaicin, and histamine leads to the release of neuropeptides, which exert their effects via interactions with NK receptors. Although Kim *et al.* [25] have previously reported that the G231E polymorphism of NK2R has a significant association with capsaicin-sensitive cough in patients with chronic cough, to date, no data have been published to confirm any association between NK2R polymorphisms and bronchial asthma, including TDI-induced asthma.

We did not identify any statistical significance in the allele or genotype distributions between TDI-OA and AEC

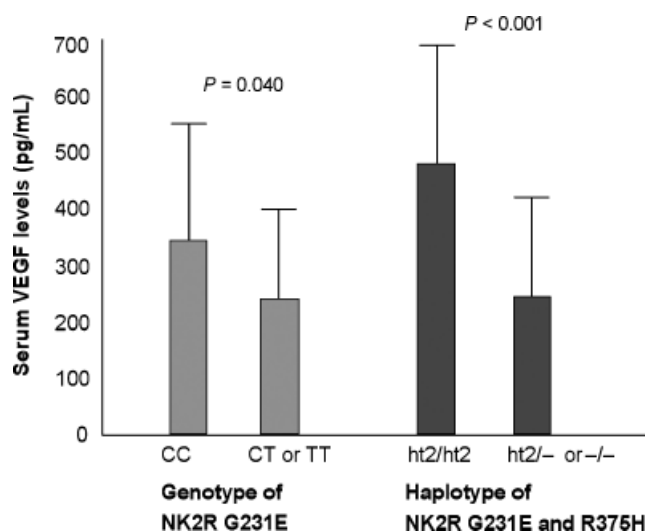


Fig. 1. Amplification of the significance of the association between serum vascular endothelial growth factor (VEGF) levels and two neurokinin 2 receptor (NK2R) gene polymorphisms analysed from the genotypes of diploid cells. Three haplotypes had a frequency > 1%: ht1[AG], ht2[GG], and ht3[GA]. Results are shown as the mean $\pm$ SD of VEGF levels from the sera of 129 toluene diisocyanate (TDI)-exposed workers.

or healthy unexposed controls in our population of Korean subjects. Our sample size may have been too small to allow reliable statistical evaluations, although this study comprises the largest cohort of TDI-OA and two control groups, asymptomatic-exposed workers and unexposed healthy subjects, from a single ethnic group.

A recent study has demonstrated using an immunohistochemical method that NK2R are present within inflammatory cells, which include macrophages, mast cells, and T lymphocytes, as well as smooth muscles, glands, and vessels of the human central airways [34]. NK2R clearly mediate a wide array of immunomodulatory effects, which include enhancing the plasma extravasation, infiltration, and activation of inflammatory cells, such as eosinophils, mast cells, neutrophils, monocytes, and lymphocytes, and the activation of mesenchymal cells [18]. Moreover, NK2R have been suggested to play a role in antigen-induced airway inflammation in various animal studies [18, 35, 36]. In analogy with the influence of β_2 -adrenergic receptor polymorphisms on the ligand binding and functional properties of the receptor [37], genetic polymorphisms for the NK2R, which has the same G protein-coupled receptor, may alter receptor down-regulation and agonist-binding affinities or coupling efficiency to adenylate cyclase. Indeed, direct comparison of the wild-type and mutant (F248S) forms of the NK2R in *Xenopus* oocytes has indicated that the mutant prevents agonist-induced desensitization but is as active as the wild-type receptor in stimulating secondary messenger pathways [24].

In the present study, we demonstrate a possible association of the NK2R SNPs G231E and R375H with airway inflammation in TDI-exposed workers by showing that subjects with the GG genotype of NK2R G231E have higher serum VEGF levels and that the G allele of G231E and the G allele of R375H improve the significance of the

Table 5. Comparison of the clinical markers according to the genotypes of NK2R polymorphisms in TDI-exposed workers, including both TDI-induced asthma patients and asymptomatic-exposed controls ($N = 129$)

Loci	Genotype	VEGF* (pg/mL)	TGF- β * (ng/mL)	MMP-9* (ng/mL)	Total IgE* (IU/mL)	IgE to TDI† <i>n</i> (%)	IgG to TDI† <i>n</i> (%)
Gly231Glu	GA or AA ($n = 92$)	239.2 $\pm$ 161.0	33.6 $\pm$ 17.4	149.0 $\pm$ 158.0	330.0 $\pm$ 580.9	15/67 (22.4)	15/67 (22.4)
	GG ($n = 37$)	345.3 $\pm$ 224.0	32.5 $\pm$ 17.5	180.4 $\pm$ 170.0	171.3 $\pm$ 179.3	6/31 (19.3)	7/31 (22.6)
	<i>P</i> -value	0.040 (0.538)‡	0.565	0.091	0.270	0.963	0.957
Arg375His	GG or GA ($n = 113$)	275.8 $\pm$ 192.0	33.6 $\pm$ 17.4	164.1 $\pm$ 167.0	279.5 $\pm$ 512.7	19/88 (21.6)	20/88 (22.7)
	AA ($n = 7$)	209.7 $\pm$ 148.0	32.5 $\pm$ 17.5	171.2 $\pm$ 79.0	60.8 $\pm$ 69.7	1/5 (20)	1/5 (20)
	<i>P</i> -value	0.560	0.440	0.620	0.791	0.789	0.859

Data shown are the means $\pm$ SD.

*The *P*-values for univariate analysis of covariance were generated using generalized linear models analyses for controlling age, sex, and smoking status as covariables.

†The *P*-values for univariate analysis of covariance were generated using logistic regression analyses for controlling age, sex, and smoking status as covariables.

‡Observed power computed using $\alpha = 0.05$.

NK2R, neurokinin 2 receptor; VEGF, vascular endothelial growth factor; TDI, toluene diisocyanate.

elevated serum VEGF levels. These results suggest that NK2R polymorphisms augment the airway inflammation conferred by VEGF in TDI-exposed workers.

TDI-induced asthma is a chronic inflammatory process of the airways that is associated with the accumulation of activated inflammatory cells, structural changes, and remodelling of extracellular matrix (ECM) components [2, 3]. A role for VEGF in TDI-induced asthma has recently been suggested. In a murine model of TDI-induced asthma [26], the increased VEGF level was significantly correlated with the numbers of neutrophils and eosinophils in the bronchoalveolar lavage fluids after challenge. Furthermore, VEGF inhibitors were dramatically effective at reversing all the pathophysiological findings, which included AHR, increased vascular permeability, and presence of inflammatory mediators. VEGF is a multi-functional cytokine with potent actions on epithelial cell proliferation, blood vessel formation, and endothelial permeability. VEGF can be produced by a variety of human cells, including vascular smooth muscle cells, keratinocytes, fibroblasts, mesangial cells, epithelial cells, and airway smooth muscle cells. Considering the relationship between NK2R polymorphism and airway inflammation, airway smooth muscle cells, in which NK2R are mainly found, are not only passive partners in airway inflammation but also contribute to the formation of the ECM and act as a rich source of many cytokines, chemokines, adhesion molecules, and growth factors [38]. Changes in NK2R expression and function may modulate airway inflammation via the activation and proliferation of airway smooth muscle cells mediated by neuropeptides. The marked difference in serum VEGF levels stratified by the genetic polymorphisms of NK2R observed in this study may be linked to recent studies that suggest that VEGF plays a key role in airway inflammation in asthma patients [26, 27]. Taken together, our results suggest that the GG genotype of NK2R G231E potentiates increases in the levels of neuropeptides as a result of TDI exposure and activates more inflammatory cells to release inflammatory mediators, such as VEGF. Accordingly, NK2R G231E may be a determinant that augments chronic airway inflammation in TDI-exposed workers. This study does have some limitations. First, the study populations were too small to document statistical significance. This was partly due to the difficulties associated with recruiting TDI-exposed workers and asthma patients. Second, the relationship between G231E and the serum VEGF levels was based on indirect results, and further investigations should be conducted.

While TGF- β 1 has been found to increase the production of metalloproteinases and the expression of TGF- β 1 is known to be increased in the airways of TDI-induced asthmatic patients [2, 28–31], apart from the serum VEGF levels, we did not find any significant associations between the NK2R polymorphisms and the serum levels of MMP-9 and TGF- β 1.

In conclusion, we have shown that the NK2R G231E polymorphism results in increased serum levels of VEGF, which may contribute to perpetuating airway inflammation in TDI-exposed workers. Clarification of the functional differences of NK2R polymorphisms and their associations with serum VEGF levels will undoubtedly lead to a better understanding of the pathogenic mechanism of TDI-induced asthma and may offer new targets for preventive or therapeutic approaches.

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References

- 1 Perters JM, Wegman DH. Epidemiology of toluene diisocyanate (TDI)-induced respiratory disease. *Environ Health Perspect* 1975; 11:97–100.
- 2 Park HS, Cho SH, Hong CS *et al.* Isocyanate-induced occupational asthma in far-east Asia: pathogenesis to prognosis. *Clin Exp Allergy* 2002; 32:198–204.
- 3 Redlich CA, Karol MH. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol* 2002; 2:213–24.
- 4 Bentley AM, Maestrelli P, Saetta M *et al.* Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. *J Allergy Clin Immunol* 1992; 89:821–9.
- 5 Maestrelli P, Occari P, Turato G *et al.* Expression of interleukin (IL)-4 and IL-5 proteins in asthma induced by toluene diisocyanate (TDI). *Clin Exp Allergy* 1997; 27:1292–8.
- 6 Park HS, Lee SK, Kim HY *et al.* Specific immunoglobulin E and immunoglobulin G antibodies to toluene diisocyanate-human serum albumin conjugate: useful markers for predicting long-term prognosis in toluene diisocyanate-induced asthma. *Clin Exp Allergy* 2002; 32:551–5.
- 7 Ye YM, Kim SH, Lee SK *et al.* Immunologic changes in human albumin exposed to toluene diisocyanate (TDI) vapor: differences compared with liquid TDI exposure and association with IgE/IgG binding and TDI-induced asthma. *J Allergy Clin Immunol* 2006; 117:S267.
- 8 Pezzini A, Riviera A, Paggiaro P *et al.* Specific IgE antibodies in twenty-eight workers with diisocyanate-induced bronchial asthma. *Clin Allergy* 1984; 14:453–61.
- 9 Wass U, Belin L. Immunologic specificity of isocyanate-induced IgE antibodies in serum from 10 sensitized workers. *J Allergy Clin Immunol* 1989; 83:126–35.
- 10 Wisnewski AV, Stowe MH, Cartier A *et al.* Isocyanate vapor-induced antigenicity of human albumin. *J Allergy Clin Immunol* 2004; 113:1178–84.
- 11 Chitano P, Blasi PD, Lucchini RE *et al.* The effects of toluene diisocyanate and of capsaicin on human bronchial smooth muscle in vitro. *Eur J Pharmacol* 1994; 270:167–73.
- 12 Thompson JE, Svypinski LS, Gordon T *et al.* Tachykinins mediate the acute increase in airway responsiveness caused by toluene diisocyanate in guinea pigs. *Am Rev Respir Dis* 1987; 136:43–9.

- 13 Mapp CE, Lucchini RE, Miotto D *et al*. Immunization and challenge with toluene diisocyanate decrease tachykinin and calcitonin gene-related peptide immunoreactivity in guinea pig central airways. *Am J Res Crit Care Med* 1998; 158:263–9.
- 14 Marck W, Potthast JJW, Marczynski B *et al*. Role of substance P and neurokinin A in toluene diisocyanate-induced increased airway responsiveness in rabbits. *Lung* 1996; 174:83–97.
- 15 Scheerens H, Buckley TL, Mutis T *et al*. The involvement of sensory neuropeptides in toluene diisocyanate-induced tracheal induced hyperreactivity in the mouse airways. *Br J Pharmacol* 1995; 119:1665–71.
- 16 Nadel JA, Borson DB. Modulation of neurogenic inflammation by neutral endopeptidase. *Am Rev Respir Dis* 1991; 143:S33–6.
- 17 Maggi CA, Patacchini R, Rovero P *et al*. Tachykinin receptors and tachykinin receptor antagonists. *J Auton Pharmacol* 1993; 13:23–93.
- 18 Joos GF, Germonpre PR, Pauwels RA. Role of tachykinins in asthma. *Allergy* 2000; 55:321–37.
- 19 Bai TR, Zhou D, Weir T *et al*. Substance P (NK1)- and neurokinin A (NK2)-receptor gene expression in inflammatory airway diseases. *Am J Physiol* 1995; 269:L309–17.
- 20 Mapp CE, Fryer AA, Marzo ND *et al*. Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. *J Allergy Clin Immunol* 2002; 109:867–72.
- 21 Wikman H, Piirilä P, Rosenberg C *et al*. N-acetyl transferase genotypes as modifiers of diisocyanate exposure-associated asthma risk. *Pharmacogenetics* 2002; 12:227–33.
- 22 Mapp CE, Beghe B, Zamorani BG *et al*. Association between HLA genes and susceptibility to toluene diisocyanate-induced asthma. *Clin Exp Allergy* 2000; 30:651–6.
- 23 Kim SH, Oh HB, Lee KW *et al*. HLA DRB1*15-DPB1*05 Haplotype: a susceptible gene marker for isocyanate-induced occupational asthma. *Allergy* 2006; 61:819–4.
- 24 Nemeth K, Chollet A. A single mutation of the neurokinin-2 (NK2) receptor prevents agonist-induced desensitization: divergent conformational requirements for NK2 receptor signaling and agonist-induced desensitization in *Xenopus* oocytes. *J Biol Chem* 1995; 270:27601–5.
- 25 Kim TB, Park HK, Park W *et al*. Association between capsaicin sensitive cough and nonsynonymous single nucleotide polymorphism of neurokinin receptor-2 gene in chronic cough patients. *Eur Respir J* 2004; 24:710S, (abstract).
- 26 Lee YC, Kwak YG, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2002; 168:3595–600.
- 27 Lee CG, Link H, Baulk P *et al*. Vascular endothelial growth factor (VEGF) induces remodeling and enhances T_H2-mediated sensitization and inflammation in the lung. *Nat Med* 2004; 10:1095–103.
- 28 Kelly EA, Jarjour NN. Role of matrix metalloproteinase in asthma. *Curr Opin Pulm Med* 2003; 9:28–33.
- 29 Lee KS, Jin SM, Lee H *et al*. Imbalance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in toluene diisocyanate-induced asthma. *Clin Exp Allergy* 2004; 34:276–84.
- 30 Lee YC, Song CH, Lee HB *et al*. A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor. *J Allergy Clin Immunol* 2001; 108:1021–6.
- 31 Park HS, Kim HA, Jung JW *et al*. MMP-9 is increased after toluene diisocyanate (TDI) exposure in the induced sputum from patients with TDI-induced asthma. *Clin Exp Allergy* 2003; 33:113–8.
- 32 Shin HD, Park BL, Jung JH *et al*. Association of thromboxane A2 receptor (TBXA2R) with atopy and asthma. *J Allergy Clin Immunol* 2003; 112:454–7.
- 33 Hedrick PW. Gametic disequilibrium measures: proceed with caution. *Genetics* 1987; 117:331–41.
- 34 Mapp CE, Miotto D, Braccioni F *et al*. The distribution of neurokinin-1 and neurokinin-2 receptors in human central airways. *Am J Respir Crit Care Med* 2000; 161:207–15.
- 35 Kraneveld AD, Nukamp FP, Van Oosterhout AJM. Role of neurokinin-2 receptor in interleukin-5-induced airway hyperresponsiveness but not eosinophilia in guinea pigs. *Am J Respir Crit Care Med* 1997; 156:367–74.
- 36 Schuiling M, Zuidhof A, Zaagsma J *et al*. Role of tachykinin NK2-receptor activation in the allergen-induced late asthmatic reaction, airway hyperreactivity and airway inflammatory cell influx in conscious, unrestrained guinea-pigs. *Br J Pharmacol* 1999; 127:1030–8.
- 37 Green SA, Cole G, Jacinto M *et al*. A polymorphism of the human β 2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J Biol Chem* 1993; 268:23116–21.
- 38 Howarth PH, Knox AJ, Amrani Y *et al*. Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 2004; 114:S32–50.