

Nitric oxide synthase polymorphisms and asthma phenotypes in Chinese children

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Summary

Background Nitric oxide (NO) is a key factor for balancing T-helper type 1/T-helper type 2 immunity. Single nucleotide polymorphisms (SNPs) in nitric oxide synthase (NOS) genes have been associated with atopy and exhaled NO concentrations in Caucasians. We investigated the association between asthma traits and genetic polymorphisms in neuronal NO synthase (NOS1) and endothelial NO synthase (NOS3) in Chinese children.

Methods Asthmatic children between 5 and 18 years of age and non-allergic controls were recruited. Plasma total IgE was measured by microparticle immunoassay, whereas allergen-specific IgEs were measured by fluorescent enzyme immunoassay. Fractional exhaled NO concentration (FeNO) was measured by a chemiluminescence analyser. NOS1 C5266T and NOS3 G894T were genotyped by restriction fragment length polymorphism, and (AAT)*n* polymorphism in intron 20 of NOS1 was determined by GeneScan analysis.

Results The mean (SD) ages of 295 asthmatics and 174 controls were 11.1 (3.8) years and 11.6 (4.0) years, respectively ($P = 0.162$). NOS1 C5266T and NOS3 G894T were not associated with asthma, atopy or FeNO. However, significantly more subjects with T/T in NOS1 C5266T had increased plasma total IgE as compared with those with C/T or C/C ($P = 0.017$). This SNP was also associated with sensitization to *Dermatophagoides pteronyssinus* ($P = 0.049$). Among asthmatic patients, log-transformed plasma total IgE levels were significantly higher among those homozygous for 5266T of NOS1 [mean (SD): 2.84 (0.44) for T/T, 2.68 (0.42) for C/T, 2.59 (0.69) for C/C; $P = 0.021$]. This study found a significant inter-ethnic difference in the allele frequencies of AAT repeats, and this polymorphism was associated with high plasma total IgE levels ($P = 0.044$) but not FeNO ($P = 0.158$). NOS3 G894T was not associated with any asthma or atopy phenotype.

Conclusions NOS1 C5266T and AAT repeats affect plasma IgE concentrations in Chinese children. On the other hand, neither NOS1 nor NOS3 SNP was associated with FeNO or the risk of having asthma.

Keywords asthma, immunoglobulin E, NOS, polymorphism

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Introduction

Asthma is a chronic inflammatory disease of the airway, and is characterized by reversible airway obstruction, bronchial hyper-responsiveness and atopy. Many studies also reported increased exhaled nitric oxide (NO) in these patients [1–4]. NO is synthesized from L-arginine by NO synthase (NOS). Three isoforms of NOS are known. Both NOS1 and NOS3 are constitutively expressed in the human airway, whereas NOS2 is inducible by inflammatory stimuli [5]. These three human NOS genes are located at 12q24 (NOS1) [6], 17q12 (NOS2) [7] and 7q35–36 (NOS3) [8], where genome-wide searches have identified candidate loci for asthma and atopy [9–13]. Genetic association studies further confirmed that a

CA-repeat polymorphism as well as another single nucleotide polymorphism (SNP) C5266T in exon 29 of NOS1 were linked to asthma in Caucasians [14, 15]. Hollá et al. [16] showed that this SNP was also associated with increased serum total IgE levels in Czech populations. Another study suggested that an intragenic microsatellite variant in NOS1 was associated with asthma but not total IgE [17]. Moreover, these studies did not examine the effects of NOS1 polymorphisms on sensitization to individual allergens. Other investigators genotyped the number of AAT repeats in intron 20 of NOS1 in Caucasian asthmatic patients. The mean exhaled NO and its variability were significantly lower in patients with a high number (≥ 12) of AAT repeats at this locus than in those with fewer repeats [18]. The allele frequencies of this repeats were markedly different between American Caucasians and African Americans [19].

As for NOS3, the common genotype for a 27-base pair tandem repeat polymorphism in intron 4 was associated with

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asthma development in Koreans [20] but not Caucasians [21]. On the other hand, the latter study found this polymorphism to be linked to total IgE levels, specific IgE levels for feathers and a positive skin prick test for hay. Another study that genotyped the functional *NOS3* missense variant G894T found this polymorphism to be associated with exhaled NO but not asthma or lung functions in Caucasians [22]. The possible association between asthma-related phenotypes and *NOS1* and *NOS3* polymorphisms has not been reported in Chinese participants. In the present case-control study, we investigated whether AAT repeats of *NOS1* and the SNPs C5266T of *NOS1* and G894T of *NOS3* are associated with asthma and atopy phenotypes in Chinese children.

Methods

Study population

This study recruited unrelated Chinese patients aged 5–18 years with asthma, diagnosed according to the American Thoracic Society guideline [23], from the paediatric outpatient clinics of a university teaching hospital in Hong Kong. Age- and sex-matched control subjects were selected among children attending the hospital for minor non-allergic and non-immunologic complaints. All subjects were free from symptoms of infection for 4 weeks before study. Subjects' parents gave written consent, and the Clinical Research Ethics Committee of our University approved this study.

Spirometry

Asthmatic patients underwent spirometric assessment (Compact II, Vitalograph, Buckingham, UK) to measure their forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC), which were compared with the local reference values [24].

Plasma total and allergen-specific IgE concentrations

Plasma total IgE concentration was measured by micro-particle immunoassay (IMx analyser, Abbott Laboratories, Abbott Park, IL, USA) and specific IgE to *Dermatophagoides pteronyssinus*, cat and cockroaches were measured by fluorescent enzyme immunoassay (AutoCAP analyser, Pharmacia Diagnostics AB, Uppsala, Sweden). Total IgE concentrations were expressed following logarithmic transformation, and also compared with our in-house reference

values. A specific IgE concentration ≥ 0.35 kIU/L was considered to be positive, and atopy was defined as the presence of at least one type of allergen-specific IgE.

Exhaled nitric oxide measurement

Fractional exhaled nitric oxide concentration (FeNO) was measured at an expiratory flow rate of 50 mL/s using a chemiluminescence analyser (NOA280i, Sievers Instruments, Boulder, CO, USA) according to the ERS/ATS standard [25]. Repeated exhalations were performed without nose clip until three NO plateau values agreed at the 10% level. The mean FeNO value was then recorded.

Genotyping for nitric oxide synthase 1 and nitric oxide synthase 3 polymorphisms

Genomic DNA was extracted from peripheral blood leucocytes using a High Pure Viral Nucleic Acid Kit (Boehringer Mannheim GmbH, Mannheim, Germany). Polymorphisms at *NOS1* C5266T and *NOS3* G894T were genotyped by PCR and restriction fragment length polymorphism (RFLP), and AAT repeats at intron 20 of *NOS1* were determined by direct sequencing with an ABI-310 autosequencer using GeneScan software (Applied Biosystems, Foster City, CA, USA). The primers, PCR conditions, restriction enzymes and digestion products are described in Table 1. For the two SNPs, PCR reactions were carried out in a total volume of 30 μ L containing 60 ng DNA and 1 U AmpliTaq Gold (Roche Molecular Biochemicals, Mannheim, Germany) for *NOS1* C5266T. Similarly, the 30 μ L reaction mix for *NOS3* G894T contained 60 ng DNA and 1 unit AmpliTaq Gold. The PCR products were digested overnight at 37 °C using *Pml*I and *Dpn*II, respectively, and visualized on 3.5% agarose gel. Eight DNA samples of known genotypes were included in every PCR reaction in addition to restriction enzyme digestion as a positive control together with 88 samples in 96-well plates. Finally, RFLP results for both polymorphisms were confirmed in 30 randomly selected samples by direct sequencing using BigDye Terminator Cycle sequencing kits with an ABI-310 autosequencer. For AAT repeats in *NOS1*, the reactions were carried out in a 15 μ L reaction mix containing 115 ng DNA, 0.375 U AmpliTaq Gold and forward primer labelled with phosphoramidite reagents (6-FAM and HEX) as fluorescent dyes (Table 1). The numbers of AAT repeats in the PCR products were then identified by their fluorescence signals relative to the size standard (GeneScan-400HD) using GeneScan software (Fig. 1).

Table 1. Summary of primers and protocols for PCR, RFLP and gene sequencing

Polymorphism	Primers	PCR conditions	Restriction enzyme (units)	RFLP products*
<i>NOS1</i> C5266T	F: 5'-ACT CCT TGA GTT TTC CTG CTG CGA TG-3' R: 5'-CCA TGT TCC AGT GGT TTC ATG CAC AC-3'	61 °C/30 cycles	<i>Pml</i> I (5)	T allele: 128 bp C allele: 100, 28 bp
<i>NOS3</i> G894T	F: 5'-AAG GCA GGA GAC AGT GGA TG-3' R: 5'-CAG TCA ATC CCT TTG GTG CT-3'	58 °C/30 cycles	<i>Dpn</i> II (3)	G allele: 246 bp T allele: 158, 88 bp
<i>NOS1</i> (AAT) <i>n</i>	F†: 5'-GCA ATG GTG TGT TGG TGA ACC A-3' R: 5'-GAA ACT GGC AAA CAT GAA AAT CAA-3'	60 °C/30 cycles	NA	NA

NA, not applicable; RFLP, restriction fragment length polymorphism.

*All digested products were visualized on 3.5% (w/v) agarose gel containing ethidium bromide.

†Forward primer was labelled with fluorescent dyes (6-FAM or HEX) in order to provide a signal for GeneScan analysis.

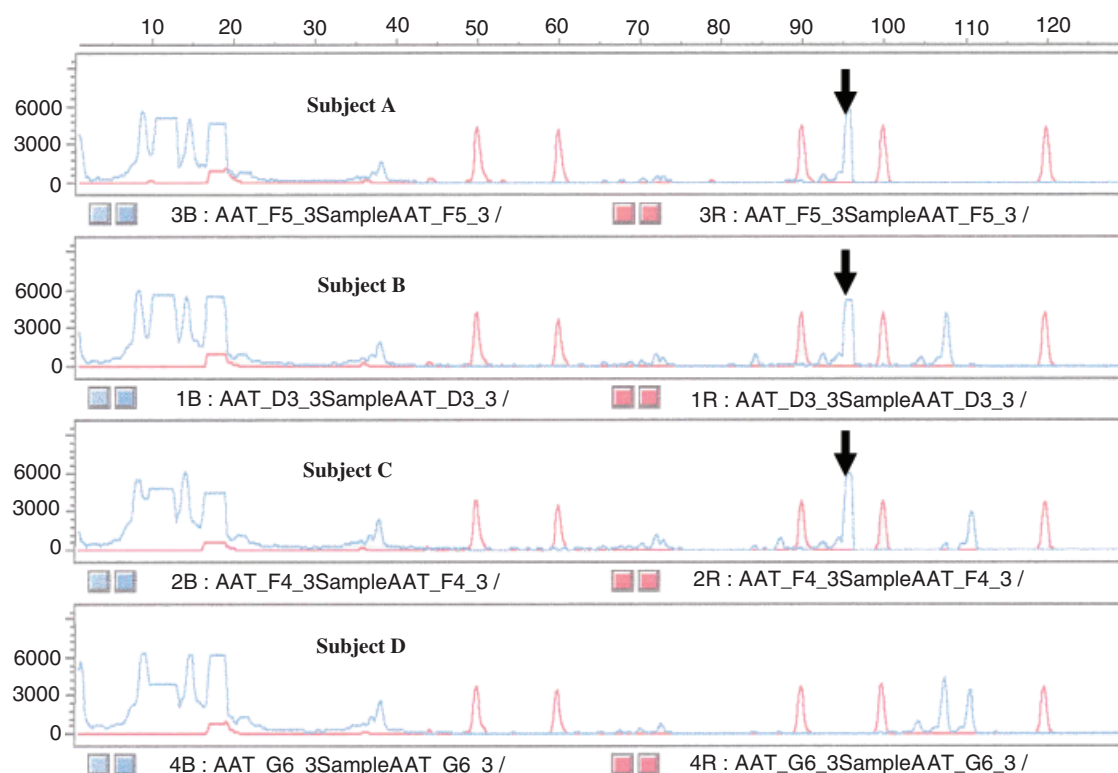


Fig. 1. GeneScan analysis of the number of AAT repeats in intron 20 of *NOS1*. Red tracings represent size standards (GeneScan-400HD) with peaks at 50, 60, 90, 100 and 120 bp, and blue tracings show PCR products amplified from 6-FAM-labelled primers. The results were bi-allelic for (a) 10-repeat (arrows) homozygosity for subject A; (b) 10- and 14-repeat for subject B; (c) 10- and 15-repeat for subject C and (d) 14- and 15-repeat for subject D. The PCR products as amplified using HEX-labelled primers would appear green on GeneScan analysis.

Statistical analysis

Results were expressed as proportions or mean and standard deviation (SD). Patient characteristics were compared using the Student's *t*-test or ANOVA for numerical data with a normal distribution and the Mann-Whitney *U*-test or the Kruskal-Wallis test for those with non-normal distribution. χ^2 or Fisher's exact test was used to analyse categorical variables. The associations between genotypes in *NOS1* C5266T and AAT repeats and *NOS3* G894T and dichotomous asthma and related phenotypes (e.g. atopy, sensitization to individual allergens, increased plasma total IgE) were analysed using logistic regression. Two alternative models (recessive and co-dominant) were fitted for each outcome, adjusting for age and gender as covariates for asthma and for age, gender and disease status (i.e. asthma or control) for other traits. Quantitative traits were analysed with a co-dominant model using linear regression. The genotype for (AAT)*n* of *NOS1* was dichotomized into low (<12) and high number (≥ 12) of repeats for each allele [18]. FeNO and total IgE were compared among subjects with different AAT-repeat genotypes using the Kruskal-Wallis test. *P*-values <0.05 were considered significant.

Results

Subjects

Two hundred ninety-five asthmatic children and 174 controls were recruited. The mean (SD) ages of patients and controls were 11.1 (3.8) years and 11.6 (4.0) years, respectively

Table 2. Clinical and laboratory characteristics of the subjects

Characteristics	Asthmatics (<i>n</i> = 295)	Controls (<i>n</i> = 174)
Mean (SD) age, years	11.1 (3.8)	11.6 (4.0)
Male gender (%)	190 (64)	100 (57)
Mean (SD) log-transformed plasma total IgE concentration	2.61 $\pm$ 0.61 [†]	1.78 $\pm$ 0.72
Number of subjects with allergen-specific IgE in plasma*		
<i>Dermatophagoides pteronyssinus</i> (%)	250 (90) [†]	72 (48)
Cockroaches (%)	83 (30) [†]	18 (12)
Cat (%)	48 (17) [†]	6 (4)
Atopy (i.e. with $\geq$ one specific IgE) (%)	253 (91) [†]	74 (49)
Spirometric variables		
FEV ₁ /FVC (%)	80 (11)	83 (10)
FEV ₁ predicted (%)	92 (20)	83 (11)

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; SD, standard deviation.

*Samples available in 278 patients and 151 controls for analysis.

[†]*P* < 0.0001.

(*P* = 0.162). Table 2 summarizes the demographics and results of laboratory investigations in these subjects. The total IgE levels were increased in 76% patients and 28% controls (*P* < 0.0001).

Fractional exhaled nitric oxide and nitric oxide synthase polymorphisms

The frequencies for minor alleles 5266T for *NOS1* and 894T for *NOS3* in our controls were 0.36 and 0.09, respectively.

Table 3. Relation between *NOS1* genotypes and quantitative traits in 295 asthma patients

Phenotype	<i>NOS1</i> C5266T*			<i>P</i> -values†
	C/C	C/T	T/T	
IgE _{log}	2.59 (0.69)	2.68 (0.42)	2.84 (0.44)	0.021
FeNO (p.p.b.)	72 (59)	81 (48)	102 (91)	0.224
FEV ₁ percent (%)	90 (16)	89 (17)	84 (20)	0.120
FEV ₁ /FVC (%)	78 (11)	78 (10)	78 (10)	0.668

FeNO, fractional exhaled nitric oxide concentration; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; IgE_{log}, log-transformed plasma total IgE concentration.

*Results expressed in means (standard deviations).

†Analysed by linear regression using the co-dominant model, with age and gender being covariates.

Table 4. Allele distribution of AAT repeats in intron 20 of *NOS1* in our Chinese children, and comparison with published results for other ethnic groups [19]

Number of AAT repeat	Allele frequency			<i>P</i> -values†*	<i>P</i> -values‡*
	Our controls (n = 171)	Caucasians (n = 305)	African Americans (n = 105)		
7	0	0.003	0.019	0.539	0.021
8	0	0.003	0.010	0.539	0.144
9	0	0.053	0.033	<0.001	0.001
10	0.398	0.438	0.110	0.258	<0.001
11	0.015	0.010	0.038	0.537	0.089
12	0	0.010	0.110	0.093	<0.001
13	0.190	0.267	0.214	0.009	0.561
14	0.193	0.161	0.338	0.239	<0.001
15	0.178	0.053	0.114	<0.001	0.057
16	0.023	0.003	0.014	0.006	0.545
17	0.003	0	0	0.359	1.000

*Analysed between different ethnic groups by χ^2 or Fisher's exact test in 2×2 contingency tables of the counts of a given allele (e.g. 13 number of AAT repeat) vs. all other alleles (e.g. non-13 repeat).

†Comparing our Chinese subjects with Caucasian subjects.

‡Comparing our Chinese subjects with African-American subjects.

The genotype distributions of these polymorphisms followed the Hardy–Weinberg equilibrium. FeNO was measured in 149 patients and 59 controls, with the respective mean (SD) results being 78 (59) p.p.b. and 40 (43) p.p.b. ($P < 0.0001$). FeNO did not differ among patients with different *NOS1* ($P = 0.131$) or *NOS3* ($P = 0.508$) genotypes. The same findings were obtained when analyses were repeated for either the asthma (Table 3) or the control subgroup. For *NOS1* AAT repeats, DNA was not available from four subjects (one patient and three controls) for genotyping. Table 4 summarizes the allele distribution of AAT-repeat numbers, and its relation to asthma diagnosis in our 465 genotyped subjects. A bi-modal distribution of this trinucleotide repeat was observed, with two peaks at 10 and 13–15 numbers of ATT repeats. This polymorphism was not associated with FeNO ($P = 0.158$ by the Kruskal–Wallis test).

Asthma traits and nitric oxide synthase polymorphisms

Table 5 summarizes the associations between dichotomous asthma and related phenotypes and the two *NOS* poly-

morphisms. For increased plasma total IgE and the presence of *D. pteronyssinus*-specific IgE, we found a significant excess percentage of the homozygous mutants. The frequencies of T/T genotypes in *NOS1* C5266T among subjects with increased and normal plasma total IgE were 14% and 9% ($P = 0.017$ by the recessive model). *NOS1* 5266T/T was found in 13% of *D. pteronyssinus*-specific IgE-positive subjects and 10% of those without this IgE ($P = 0.049$ by the co-dominant model). *NOS1* C5266T and *NOS3* G894T were not associated with physician-diagnosed asthma or atopy. Table 6 summarizes the association between asthma and intermediate phenotypes and AAT repeats in this study. Similar to the results for *NOS1* C5266T, this trinucleotide polymorphism was also associated with increased plasma total IgE in our Chinese children ($P = 0.044$ by the recessive model).

Linear regression models were used to analyse continuous outcome variables, namely the logarithm of plasma total IgE and spirometric indices (i.e. FEV₁ percentage and FEV₁/FVC). No significant relation was found between these quantitative traits and *NOS1* or *NOS3* genotypes. Table 3 summarizes the results on similar analyses for subgroups of asthma patients. Among the asthmatic patients, log-transformed plasma total IgE levels were significantly associated with *NOS1* C5266T [mean (SD): 2.84 (0.44) for T/T, 2.68 (0.42) for C/T, 2.59 (0.69) for C/C; $P = 0.021$]. None of the results was significant in the controls. When AAT alleles in *NOS1* were dichotomized, plasma total IgE concentrations in our subjects were significantly associated with this polymorphism ($P = 0.006$ by the Kruskal–Wallis test; Fig. 2). On the other hand, FeNO ($P = 0.158$), FEV₁ ($P = 0.693$) and FEV₁/FVC ($P = 0.805$) did not differ among subjects with different AAT repeats.

Discussion

Two SNPs in *NOS1* and *NOS3* as well as AAT repeats in *NOS1* were genotyped in southern Chinese children in this case–control genetic association study. The allele frequencies for 5266T of *NOS1* and 894T of *NOS3* in our controls were 36% and 9%, respectively. Significant inter-ethnic differences in the allele frequencies of AAT repeats were observed when our results were compared with those of Caucasians and African Americans. FeNO was significantly higher in asthmatics than in controls, but did not differ among subjects with different *NOS1* or *NOS3* genotypes. The two *NOS1* polymorphisms were not associated with asthma or atopy. On the other hand, significantly more subjects homozygous for 5266T in *NOS1* had elevated plasma total IgE and sensitization to *D. pteronyssinus* as compared with those with C/T or C/C. Within the asthma subgroup, log-transformed plasma total IgE levels were highest among those homozygous for 5266T of *NOS1*, followed by patients with C/T, then C/C genotypes. The number of AAT repeats in *NOS1* was also associated with plasma total IgE concentrations in our Chinese children. On the other hand, *NOS3* G894T was not associated with any asthma phenotype in our Chinese children. This finding is consistent with that found in Caucasians [17, 21, 22].

A number of genome-wide searches have suggested the importance of chromosomal region 12q14–24.2 in asthma

Table 5. Association between dichotomous asthma phenotypes and single-nucleotide polymorphisms in *NOS1* and *NOS3*

Phenotype	<i>NOS1</i> C5266T			<i>P</i> -values*		<i>NOS3</i> G894T			<i>P</i> -values*	
	C/C (%)	C/T (%)	T/T (%)	RM	CDM	G/G (%)	G/T (%)	T/T (%)	RM	CDM
Asthma	45	44	11			77	23	1		
vs. control	42	44	14	0.267	0.655	80	19	1	0.607	0.487
Subjects with atopy†	42	45	13			78	21	1		
vs. subjects without atopy	48	42	11	0.094	0.209	78	22	0	0.726	0.714
Subjects with increased plasma total IgE‡	42	43	14			77	22	1		
vs. subjects with normal total IgE	46	45	9	0.017	0.650	79	20	1	0.408	0.430
Sensitization to aeroallergens										
DP-IgE positive	41	46	13			78	21	1		
vs. DP-IgE negative	50	40	10	0.061	0.049	79	22	0	0.626	0.609
Cockroaches-IgE positive	49	35	16			80	19	1		
vs. cockroaches-IgE negative	42	47	12	0.096	0.096	78	21	1	0.977	0.836
Cat-IgE positive	43	47	9			79	21	0		
vs. cat-IgE negative	43	44	13	0.647	0.573	78	21	1	0.758	0.765

CDM, co-dominant model; DP, *Dermatophagoides pteronyssinus*; RM, recessive model.

*Analysed by logistic regression analyses with recessive and co-dominant models, controlling for age and gender as covariates (for asthma vs. controls) and age, gender and disease status (for other traits) in order to adjust for possible biases that these characteristics might exert on the outcomes.

†Defined as having ≥ 1 allergen-specific IgE.

‡Compared with upper normal limits for local reference established in our laboratory (120 kIU/L for 5–6 years old; 160 kIU/L for 6–8 years old and 180 kIU/L for ≥ 8 years old).

Table 6. Relation between asthma and related phenotypes and AAT repeat genotypes

Phenotype	<i>NOS1</i> AAT repeats			<i>P</i> -values*	
	HS (%)	HE (%)	HL (%)	RM	CDM
Asthma	15	49	37		
vs. control	20	42	38	0.109	0.201
Subjects with atopy†	15	47	38		
vs. subjects without atopy	22	36	42	0.562	0.507
Subjects with increased total IgE‡	14	50	37		
vs. normal total IgE	22	39	40	0.044	0.129
DP-IgE positive	15	47	38		
vs. DP-IgE negative	21	37	41	0.682	0.614
Cockroaches-IgE positive	15	50	35		
vs. cockroaches-IgE negative	17	43	40	0.342	0.713
Cat-IgE positive	13	48	39		
vs. cat-IgE negative	17	44	39	0.810	0.898

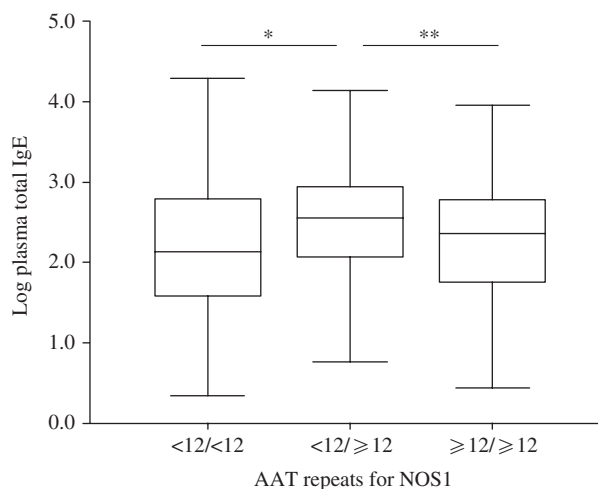
CDM, co-dominant model; DP, *Dermatophagoides pteronyssinus*; HE, heterozygous with one allele for each of ≥ 12 -repeat and <12 -repeat; HL, homozygous for ≥ 12 -repeat; HS, homozygous for <12 -repeat; RM, recessive model.

*Analysed by logistic regression analyses of recessive and co-dominant models, controlling for age and gender as covariates (for asthma vs. controls) and age, gender and disease status (for other traits).

†Defined as having ≥ 1 allergen-specific IgE.

‡Refer to footnote in Table 5 for the definition of increased total IgE concentration.

susceptibility. Evidence of a linkage between this region and asthma diagnosis has been established in Afro-Caribbeans [9], Hispanics [10], Japanese [26] and Caucasians [10–13]. This region has also been linked to total IgE in various ethnic groups [9, 27]. Many asthma candidate genes such as *NOS1*, *IFN- γ* (*IFNG*) and insulin-like growth factor-1 (*IGF1*) were located in chromosome 12q14–24.2. The present study, as well as a number of recent reports, highlights a consistent association between *NOS1* polymorphisms and the susceptibility of developing asthma and other IgE manifestations in

**Fig. 2.** Log-transformed plasma total IgE concentrations among subjects homozygous for <12 -repeat ($<12/<12$), heterozygous ($<12/\geq 12$) and homozygous for ≥ 12 -repeat ($\geq 12/\geq 12$) for *NOS1* AAT repeats (*P* for trend = 0.006 by the Kruskal–Wallis test). **P* < 0.05, ***P* < 0.01 (two-group comparison by the Mann–Whitney *U*-test).

different ethnic groups [14–16, 26, 28]. In particular, our results regarding significant associations between plasma total IgE levels and the two *NOS1* variants were in keeping with the same finding of a significant linkage between *NOS1* C5266T and high IgE levels in German patients [28]. These observations suggest *NOS1* to be an important candidate gene for asthma and atopy.

Several lines of evidence support a crucial role for *NOS1* in the pathogenesis of asthma and atopy. NO is a ubiquitous intercellular messenger being synthesized from L-arginine by NO synthases. Among the three distinct NOS isoforms, *NOS1* and *NOS3* are constitutively active, exhibiting rapid responses to a small amount of NO, while *NOS2* is constitutively expressed in the human airway epithelium and inflammatory cells (including macrophages) and is

further up-regulated many times by inflammatory agents [5, 29, 30]. Neurally derived NO, produced by *NOS1*, is a neurotransmitter for bronchodilator non-adrenergic non-cholinergic nerves. This mediator was physiologically linked to asthma [31]. In a murine model of allergic asthma, *NOS1* gene deletion resulted in airway hyporesponsiveness to methacholine inhalation. This effect was not observed in mice with defective *NOS2* or *NOS3* expression [32].

The genotype frequencies of two *NOS1* and *NOS3* SNPs in our Chinese children are very much different from the figures published in other populations. In the present study, the minor allele 5266T for *NOS1* was detected in 36% of the controls. This *NOS1* polymorphism was found in 27–30% of Caucasian controls [15, 16], and the corresponding frequency for Japanese participants was 51% [33]. Our result is closer to that published for the Caucasians. Ten repeat (44%) was the most common allele for AAT in *NOS1*, followed by 13 repeat (27%) and 14 repeat (16%), in Caucasians. African Americans in this study had higher numbers of AAT repeats (Table 4) [19]. The present study found a significant inter-ethnic variation in the allele distribution of AAT repeats ($P < 0.0001$ for Chinese children vs. both Caucasians and African Americans). Our Chinese children showed similar frequencies for 10-repeat, less 13-repeat but more 15-repeat as compared with Caucasians (Table 4). On the other hand, the frequency of *NOS3* 894T was 9% in our southern Chinese controls. This figure is much lower than the value (26–27%) reported in Caucasian adults [17, 22], and is the same as in Japanese populations [34]. These differences in allele frequencies of SNPs likely influence the relative contribution of different candidate genes on asthma susceptibility at the population level, and highlight the need to delineate the molecular epidemiology of asthma- and atopy-related SNPs in different ethnic groups.

Exhaled NO levels were much higher in Chinese subjects as compared with similar reports in Caucasians [1–4]. The mean FeNO in our asthmatics and controls was 78 and 40 p.p.b., respectively. Differences in *NOS* polymorphisms between the two ethnic groups may partly explain this finding. Storm van's Gravesande et al. identified a strong association between *NOS3* G894T and FeNO level in Caucasian asthmatics. In their study, age- and sex-adjusted FeNO levels were lowest in asthmatics with the T/T genotype [22]. In the present study, the low NO-producing 894T allele in *NOS3* was found only in 9% of controls in contrast to the high reported frequency of 26–27% in Caucasians [17, 22]. Homozygosity for *NOS3* 894T was very rare (0–1%) in our subjects. For polymorphisms in *NOS1*, two Caucasian studies showed AAT repeats in intron 20 of this gene to be associated with FeNO in patients with asthma [18] and cystic fibrosis [35]. Those with a high number (≥ 12) of repeats had lower FeNO. This trinucleotide repeat polymorphism, on the other hand, was not associated with FeNO in our Chinese children.

This study has not investigated the interactions between our *NOS1* and *NOS3* SNPs and polymorphisms either in the same *NOS* genes or in other asthma candidate genes. Our group reported that C-431T in the gene encoding thymus and activation-regulated chemokine (TARC) was linked to plasma TARC levels, atopy, total IgE and peripheral eosinophilia in Chinese children [36]. We recently conducted multifactor-dimensionality reduction, an emerging statistical

method for studying gene–gene interactions in studies involving a relatively small sample size [37], on SNPs in four atopy candidate genes [*TARC*, *IL13*, *CD14* and cytotoxic T lymphocyte antigen-4 gene (*CTLA4*)] [36, 38, 39]. Although asthma was not independently linked to *TARC* or *CTLA4* polymorphism, we found a significant interaction between these two SNPs on asthma susceptibility. Among subjects homozygous for the A allele of *CTLA4*+49A/G, those carrying at least one at-risk –431T for *TARC* had 4.44 times the risk of developing asthma as compared with those homozygous for –431C ($P_c = 0.039$) [40]. As NOS is also intimately involved in bronchial inflammation, it would be important to investigate whether our three polymorphisms interact with other candidate genes in the Chinese population to determine atopic manifestations. Another possibility is that significant interactions may exist between polymorphic variants (i.e. haplotype combinations) within these *NOS* genes in modulating plasma IgE levels.

Another limitation at the start of this study was related to our inability to estimate the required sample size accurately because all the three genetic markers had not been well studied in the Chinese population. As it turned out, the frequencies of our three polymorphisms were significantly different in Chinese participants as compared with the published results for other ethnic groups. Retrospectively and given the allele frequencies in our controls, this study had 80% power to detect within 95% confidence the odds ratios of 1.75 and 2.35 for any association between physician-diagnosed asthma and *NOS1* 5266T and *NOS3* 894T, respectively. For the *NOS1* (AAT) $_n$, the present sample size was able to detect an odds ratio of 0.55 for any inverse relation between asthma and alleles with ≥ 12 repeats. Thus, this study should be reasonably sized for the two *NOS1* polymorphisms, but might be underpowered to detect any effect for *NOS3* G894T in our Chinese children. The present study managed to find significant associations between plasma total IgE levels and the two *NOS1* variants in our Chinese children, as also described in the literature [16, 28].

In conclusion, *NOS1* C5266T and *NOS3* G894T as well as AAT repeat in *NOS1* are not associated with asthma or FeNO in Chinese children. However, the two genetic variants in *NOS1* are linked to plasma total and specific IgE concentrations. These findings support the fact that *NOS1* is a candidate gene for IgE-mediated diseases.

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