

ORIGINAL PAPER

A polymorphism in the *NPPA* gene associates with asthmaJ. J. Lima^{*†}, S. Mohapatra^{‡†}, H. Feng[§], R. Lockey^{††}, P. K. Jena[‡], M. Castro^{††}, C. Irvin^{||}, J. A. Johnson[§], J. Wang^{*} and J. E. Sylvester^{*}^{*}Pharmacogenetics Center, Nemours Children's Clinic, Jacksonville, FL, USA, [†]American Lung Association Asthma Clinical Research Centers, [‡]Department of Internal Medicine, College of Medicine, University of South Florida, Tampa, FL, USA, [§]Center for Pharmacogenomics, University of Florida, Gainesville, FL, USA, ^{††}Department of Internal Medicine, Washington University School of Medicine, St Louis, MO, USA and ^{||}Health Science Research Facility, University of Vermont, Burlington, VT, USAClinical and
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Allergy

Summary

Background Atrial natriuretic peptide (ANP) plays an important role in the lung and in augmenting allergic inflammation in asthma. The gene encoding ANP, *NPPA*, is located on chromosome 1p36, a region that has been linked to asthma.**Objectives** Determine associations between asthma and four common SNPs on the *NPPA* gene: C/G (rs13305986) in the promoter; G/A (rs5063) in Exon 1 resulting in *NPPA*Met32 → Val substitution; T/C (rs5065) in Exon 3 resulting in an Arg152 → Ter substitution; and T/C in the 3'UT region (rs5067).**Methods** A case-control design was used in White participants. The screening cohort consisted of 336 asthmatic cases who participated in a large clinical trial and 154, non-asthmatic controls. The replicate cohort consisted of 172 asthmatic cases from a second clinical trial and 115 healthy controls. Demographic characteristics were well matched for cases and controls in the screening cohort. Adjusted (age, gender, body mass index) odds ratio (OR) were calculated by χ^2 and logistic regression; a *P*-value of 0.0167 defined the threshold of significance.**Results** The C allele of rs5067 was associated with asthma in the screening and replicate cohorts: adjusted ORs (95% confidence intervals) 0.5 (0.29–0.84; *P* = 0.009) and 0.24 (0.11–0.53; *P* < 0.0001), respectively. The C allele of rs5065 was associated with asthma in the screening cohort but not in the replicate. The population-attributable risk for asthma in carriers of the C allele for rs5067 was 23.3%.**Conclusions** For rs5067, the risks of asthma in carriers of the C allele in the screening and replicate cohorts were reduced by 50% and 76%, respectively. *NPPA* may be an important susceptibility gene for asthma.**Keywords** asthma, atrial natriuretic peptide, genetics, polymorphisms, replicate cohort*Submitted 25 October 2007; revised 14 December 2007; accepted 14 January 2008*

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Introduction

Asthma is a chronic complex disease characterized by inflammation, constriction of the airways and bronchial hyper-responsiveness to external stimuli. Asthma affects an estimated 20 million people in the US and 300 million people world-wide. It is the most common disease of childhood and is the third leading cause of hospitalization among individuals <18 years old. Numerous candidate genes have been linked to asthma, and susceptibility loci for asthma have been mapped to regions in most chromosomes [1–7]. Moreover, a region on chromosome 1p36 has been identified as an asthma candidate locus [3].

Natriuretic peptides (NPs) consist of a family of peptides that are synthesized mainly in the atria and ventricles [8] and are distributed in most tissues of the body [9]. NP regulate renal haemodynamics, vascular cell function, cell growth and proliferation, cardiovascular function and reproductive cell function. The NP family includes three homologous peptides: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), which are products of three different genes [9]. The gene encoding the synthesis of ANP preprohormone, *NPPA*, consists of three exons separated by two introns, and is located on the short arm of chromosome 1 (p36.21). Exon 3 encodes ANP, which consists of 28 amino acids [10].

ANP has been localized in various tissues of the lung, suggesting that the lung synthesizes the peptide [11]. Moreover, the ANP system plays an important role in the biological activities of the lung including vasodilation, bronchorelaxation, pulmonary permeability and surfactant production and action [12, 13]. ANP is also thought to play an important role in augmenting allergic inflammation and in the genesis of asthma [11]. Given the important role of ANP in the lung and in the genesis of asthma, and that a region of chromosome 1p36 in which *NPPA* gene is located, has been linked to asthma [3], we hypothesized that polymorphisms in the *NPPA* gene are associated with asthma. To address this hypothesis, we determined associations between *NPPA* SNPs and asthma using a case-control approach.

Methods

Selection of cases and controls

Individuals with well-characterized asthma, who participated in two different clinical trials that were sponsored by the American Lung Association Asthma Clinical Research Centers, were selected. The first trial, Safety of Inactivated Influenza Vaccination in Asthma (SIIVA), studied over 2000 children and adults with asthma [14]; the second trial, Effectiveness of Low Dose Theophylline as Add-On Therapy in the Treatment of Asthma (LODO), studied 489 individuals with poorly controlled asthma [15]. A portion of participants from each study agreed to participate in genetic substudies. The screening cohort consisted of 336 White participants from SIIVA [16], and 154 White non-asthmatic controls who participated in a clinical trial of obesity [17]. The replicate cohort consisted of 172 White participants from LODO [15] and 115 healthy controls who were recruited as a genetic control population. Control subjects were healthy by physical examination, denied having asthma, a history of asthma or asthma symptoms and were not taking any asthma drugs or other drugs chronically. All participants were between the ages of 3 and 75 years. Representatives of other ethnic groups including African Americans were not studied owing to the small number of subjects for genetic analyses. The Institutional Review Boards at each institution approved this work.

Genotyping and determination of haplotypes

Four SNPs in the *NPPA* gene were typed: C/G (rs13305986) in the promoter; G/A (rs5063) in Exon 1 resulting in Met32 → Val substitution; T/C (rs5065) in Exon 3 resulting in an Arg152 → Ter substitution; and T/C in the 3'UT region (rs5067). These markers were selected because in previous studies they were associated with cardiovascular diseases or with diabetes [18–24]. Rs5065

SNP in Exon 3 has been associated with diabetic nephropathy [23], stroke [19] and insulin resistance [24]. The promoter polymorphism rs13305986 has been associated with increased left ventricular mass index [19] and hypertension [18]. Rs5067 is one of five known SNPs in the 3'UT that is common with a heterozygosity of 0.23. Other SNPs in the 3'UT are infrequent (heterozygosity < 0.1) or their frequencies are undetermined.

The SNP genotypes of *NPPA* were determined by analysing the melting curves of fluorescent-labelled oligo probes bound to PCR-generated targets from individual DNA samples on a LightTyper instrument (Roche Applied Science, Indianapolis, IN, USA). The sequences of the primers (Operon Technologies, Alameda, CA, USA) and probes (Roche) are listed in the table.

SNP ID	Forward primer	Reverse primer	Probe
rs13305986	cacgaagag-tctgagca	ggtcagg-cgaaagga	ttgCtatttc-tatttgggggttg-gattcctgg-SPC-Fluorescein
rs5063	AGACGTAG-GCCAAGAG	ggagtgag-cacagcat	TggacaCggcattgta-catgggat-SPC-Fluorescein
rs5065	cactcatacat-gaagctgac	tctacagttag-cataagatgtg	Atgtctgtgttctctttg-cagtacCgaag-SPC-Fluorescein
rs5067	cactcatacat-gaagctgac	tctacagttag-cataagatgtg	AcacAaatgcagca-gagaccccagg-SPC-Fluorescein

PCR cocktail consists of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 0.2 mM dNTPs, 1 µM probe-targeted strand primer, 0.1 µM non-targeted strand primer, 0.2 µM Probe, 0.5 U JumpStart Taq DNA Polymerase (Sigma, St Louis, MO, USA) and 50 ng DNA in a total volume of 20 µL overlaid with 20 µL mineral oil. The PCR conditions are 60 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 20 s using a DNA Thermal 9600 (Perkin Elmer Cetus, Oak Brook, IL, USA), followed by 5 min at 95 °C and 10 min at 50 °C. The 96-well PCR plate is then transferred to the LightTyper (Roche Applied Science) instrument for melting curve analysis following the manufacturer's instruction.

Statistical analysis

Violation of the assumption of Hardy-Weinberg Equilibrium (HWE) among the SNPs was examined using a classical χ^2 goodness-of-fit test by SAS JMP 6. Linkage disequilibrium analyses were performed by Haploview 3.32 (<http://www.broad.mit.edu/mpg/haploview/>). Haplotypes were constructed using HAP [25]. Descriptive statistics were utilized to assess subjects' demographic

information and distributions of allele, genotype and haplotype. Genetic additive and dominant effects were considered in the genotypic model. Fisher's exact test was used to examine the significance of the association between two variables in a 2×2 contingency table with small sample size. If any genetic association with asthma was found in the preliminary χ^2 analysis, Cochran's and Mantel-Haenszel statistics were further estimated including crude odds ratio (OR) and association hypothesis test. Because possible confounding factors including age, gender and body mass index (BMI) could bias the results, adjusted OR were also calculated by logistic regression, implementing these factors as covariates into the model [26]. The population-attributable risk (PAR) was determined using standard techniques (<http://www.bmj.com/epidem/epid.3.html>). A Bonferroni's factor was used to define the threshold of significance, which was 0.0167 (0.05/3 SNPs). One SNP was not in HWE and therefore was not studied further.

Results

Baseline characteristics

The baseline characteristics of the cases and controls in the screening and replicate cohorts are shown in Table 1. The mean ages and BMIs, percent female and percent obese for the screening cohort are in reasonable agreement. The ages, gender, BMI and percent obesity were higher in LODO participants compared with controls in the replicate cohort. The scores for asthma symptom utility index (ASUI) indicate that SIIVA participants had mild asthma while LODO participants had mild to moderate

asthma [27]. Values for peak flow, forced expiratory volume in 1 s (FEV₁) and percent predicted FEV₁ were lower in LODO participants compared with SIIVA participants. Asthma drug use among cases in the screening and replicate cohorts was evenly distributed for long-acting β agonist use. Inhaled steroid use was higher in LODO participants compared with SIIVA, which is consistent with more severe asthma in LODO compared with SIIVA participants. The difference in leukotriene modifier use between the two trials is related to differences in study design. In LODO, participants were required to discontinue the leukotriene modifier at baseline [15].

Genotyping

A high rate of genotyping was achieved for the four SNPs (> 91%). For rs13305986 HWE was not achieved in controls ($P=0.0003$). The minor allele frequency for rs13305986 was 2.4%, which was in excellent agreement with the frequency of 2.6% in a European population of hypertensives reported by Rubattu et al. [20], who reported HWE for this SNP. The reason for this difference is not clear. The frequencies of CC homozygotes in cases and controls were similar, 94.2% and 95.2%, respectively, ruling out asthma as a factor contributing to not achieving HWE for this SNP. Therefore, rs13305986 was no longer considered in the analyses. The remaining SNPs were in HWE for controls (data not shown). The minor allele frequencies for rs5063 (A), rs5065 (C) and rs5067 (C) were 0.045, 0.160 and 0.100, respectively, which were in excellent agreement with the frequencies listed in Hap Map for these SNPs (www.ncbi.nlm.nih.gov/sites/entrez).

Table 1. Comparison of demographic and other characteristics in White asthmatic cases and healthy controls of the screening and replicate cohorts

Characteristic, mean $\pm$ SE or Percent	Cohort			
	Screening		Replicate	
	SIIVA	Control	LODO	Control
Number	336	154	172	116
Age, years	35 $\pm$ 1	29.6 $\pm$ 0.65	43 $\pm$ 1.1	30 $\pm$ 1.2
Female, (%)	64.1	73.4	73.8	60.9
Obese, (%)	30.9	34.4	36.6	7.0
Body mass index (BMI) kg/m ²	27 $\pm$ 0.42	29 $\pm$ 0.61	29 $\pm$ 0.55	24 $\pm$ 0.37
Current smokers (%)	3.3	0	0	0
Former smokers (%)	21.1	0	22.4	0
Daily inhaled steroid (%)	51.8	0	82.0	0
Daily long-acting β agonist (%)	28.1	0	21.7	0
Daily leukotriene modifier (%)	33.9	0	4.35	0
ASUI	0.85 $\pm$ 0.01	NA	0.68 $\pm$ 0.01	NA
Peak flow (L/min)	397 $\pm$ 7.0	NA	371 $\pm$ 8.1	NA
FEV ₁ (L)	2.69 $\pm$ 0.05	NA	2.55 $\pm$ 0.06	NA
% predicted FEV ₁	87.1 $\pm$ 1.1	NA	78.3 $\pm$ 1.3	NA

FEV₁, forced expiratory volume in 1 s; ASUI, asthma symptom utility index; SIIVA, Safety of Inactivated Influenza Vaccination in Asthma; LODO, Effectiveness of Low Dose Theophylline as Add-On Therapy in the Treatment of Asthma.

Determination of linkage disequilibrium and haplotypes

Pair-wise linkage disequilibrium (LD) was observed between rs5065 and rs5067 (D' was 0.76; $r^2 = 0.33$ $P < 0.0001$). Six haplotypes were identified (Table 2), with GTT, GCC and GCT being the most common ($> 5\%$). No differences in haplotype frequencies were observed between cases in the screening cohort and in the replicate cohort.

Association analyses of genotypes and haplotypes

Owing to the small number of minor homozygotes and heterozygotes for rs5063, rs5065 and rs5067, we assumed a dominant model. Significant associations were found between asthma and rs5065 and rs5067 in the screening cohort, with a trend for rs5063 (Table 3). The adjusted OR [95% confidence interval (CI)] in carriers of the C allele for rs5065 was 0.45 (0.29–0.70; $P < 0.0001$), indicating that the risk of asthma was reduced by 55% compared with TT homozygotes in the screening cohort. The adjusted OR (95% CI) for carriers of the C allele for rs5067 was 0.5 (0.29–0.84; $P = 0.009$), indicating that the risk of asthma was reduced by 50% compared with TT homozygotes.

To validate our findings in the screening cohort, we analysed associations between asthma and rs5063, rs5065

Table 2. Distribution of common haplotypes of *NPPA* SNPs rs5063, rs5065 and rs5067 in 508 cases and 269 controls

Haplotype number	Haplotype	Frequency (%)	Cumulative frequency (%)
1	GTT	79.2	79.2
2	GCC	7.7	86.9
3	GCT	7.2	94.1
4	ATT	4.1	98.2
5	GTC	1.7	99.9
6	ACT	0.1	100

Table 3. Influence of *NPPA* SNPs on the risk of asthma risk for White participants in the screening cohort

SNP	Genotype	Participants		Adjusted odds ratio (95% CI)*	P-value
		Cases N (%)	Controls N (%)		
rs5063	AG	18 (6.1)	19 (12.8)	0.43 (0.21–0.88)	0.02
	GG	275 (93.9)	129 (87.2)	1	
rs5065	CC+TC	70 (24.1)	60 (41.4)	0.45 (0.29–0.70)	< 0.0001
	TT	220 (75.9)	85 (58.6)	1	
rs5067	CC+TC	40 (13.6)	37 (25.0)	0.50 (0.29–0.84)	0.009
	TT	254 (86.4)	111 (75.0)	1	

*Adjusted for age, gender and body mass index.

CI, confidence interval.

Table 4. Influence of genotype for *NPPA* SNPs on the risk of asthma for participants in the replicate cohort

SNP	Genotype	Participants		Adjusted Odds ratio (95% CI)*	P-value
		Cases N (%)	Control N (%)		
rs5063	AA+AG	17 (9.9)	9 (8.1)	1.02 (0.36–2.83)	0.98
	GG	155 (90.1)	102 (91.9)	1	
rs5065	CC+TC	53 (30.8)	24 (22.4)	1.16 (0.60–2.24)	0.66
	TT	119 (69.2)	83 (77.6)	1	
rs5067	CC+TC	23 (13.4)	28 (25.0)	0.24 (0.11–0.53)	< 0.0001
	TT	149 (86.6)	84 (75.0)	1	

*Adjusted for age, gender and body mass index.

CI, confidence interval.

and rs5067 SNPs in our replicate cohort (Table 4). Twenty-five percent of controls carried the C allele for rs5067 compared with 13.4% of cases, resulting in an adjusted OR (95% CI) of 0.24 (0.11–0.53), indicating that the risk of asthma was reduced by 75% in carriers of the C allele for rs5067, thereby replicating the results for this SNP in the screening cohort. In contrast, we did not replicate our findings for rs5065 SNP associations with asthma in the screening cohort while rs5063 genotypes were similarly distributed among cases and controls in the replicate cohort.

To control for the possible confounding effect of the clinical trials, a conditional association test was performed to estimate the effect of the rs5067 SNP over the screening and replicate cohorts using the Cochran–Mantel–Haenszel statistic with the Breslow–Day test for homogeneity of the ORs (<http://v8doc.sas.com/sashtml/stat/chap28/sect39.htm>).

After adjusting for the trial, we found an association between asthma and rs5067 ($P < 0.0001$). Carriers of the C allele had a decreased risk of asthma compared with controls (OR = 0.47; 95% CI 0.31–0.60). The large P -value for the Breslow–Day test (0.96) indicates no significant trial difference in the ORs between the two trials.

The PAR for carriers of the C allele for rs5067 was 23.3%, which suggests that in this population, if all participants carried the C allele, one would estimate a reduction of 23% in the prevalence of asthma.

In the screening cohort, 11% of asthmatic cases carried one or two copies of the GCC haplotype compared with 20.8% in controls: adjusted OR (95% CI) = 0.52 (0.30–0.87); $P = 0.013$. For the GCT haplotype, 10.4% of the cases carried one or two copies compared with 18.8% in controls: adjusted OR (95% CI) = 0.47 (0.27–0.82); $P = 0.008$. We were not able to replicate these associations. It should be noted that our sample size is underpowered to evaluate comprehensively the associations between haplotype of the *NPPA* gene and asthma.

Discussion

NP are a family of peptides that include ANP, BNP and CNP, and are distributed in most tissues of the body [9], including the lung [11, 28]. ANP relaxes human airway epithelium and smooth muscle, induces bronchodilation and is bronchoprotective against several different stimuli in subjects with asthma [29–34]. ANP is also thought to play an important role in augmenting allergic inflammation and in the genesis of asthma [11]. ANP evokes its effects in the lung by activating the NP receptor A, a particulate transmembrane guanylyl cyclase, which catalyses the conversion of GTP to cGMP [9, 28].

The gene encoding the synthesis of ANP preprohormone, *NPPA*, consists of three exons separated by two introns, and is located on the short arm of chromosome 1 (p36.21). Exon 3 encodes the synthesis ANP, which consists of 28 amino acids [10]. Several studies have reported associations between polymorphisms in the *NPPA* gene with cardiovascular disease or with diabetes [18–24]. A region of chromosome 1p36, which includes the *NPPA* gene, has been linked to asthma [3]. However, to our knowledge, there are no studies exploring associations between *NPPA* polymorphisms and asthma.

In the present study, two SNPs in the *NPPA* gene were associated with asthma in the screening cohort: T/C (rs5065) in Exon 3 resulting in an Arg152 → Ter substitution; and T/C in the 3'UT region (rs5067). The C allele for rs5065 and the C allele for rs5067 were associated with a reduced risk of asthma by 50–55%. The association between SNP rs5067 and asthma was validated in the replicate cohort. Carriers of the C allele in cases and controls for the screening cohorts (0.136 vs. 0.25) were very similar to the frequencies we observed in the replicate cohort (0.134 vs. 0.25), suggesting that the association between rs5067 and asthma is real. For participants in the screening cohort and in the replicate cohort, the C allele reduced the risk of asthma 50 (95% CI 0.29–0.84) to 76% (95% CI 0.11–0.53) (Tables 3 and 4). To our knowledge, ours is the first report of an association between asthma and a genetic variant in the *NPPA* gene. Our data complement data in studies of human alveolar and bronchial epithelial cells, and in murine models that ANPs play an important role in lung physiology and in asthma [11–13, 35, 36]. Our results are also consistent with a genetic study reporting that a region on chromosome 1p36, which harbours the *NPPA* gene, has been identified as an asthma candidate locus [3]. Taken together, these data suggest that the *NPPA* may be an important susceptibility gene for asthma.

We found that the rs5065 and rs5067 are in LD and that the GTT was the most prevalent haplotype in Whites, followed by the GCC and GCT haplotypes. The GCC and GCT haplotypes were significantly associated with a reduced risk of asthma in the screening cohort. However,

these findings were not replicated, indicating that the 3-SNP haplotype is no more informative than genotype of the rs5067 SNP.

It is not clear how the rs5067 SNP affects the function of the *NPPA* gene and the encoded protein, and ultimately the mechanism underlying its role in the pathogenesis of asthma. A survey by the MicroInspector program <http://www.imbb.forth.gr/microinspector> computationally reveals that there are many miRNA target sites in *NPPA* mRNA (NM-006172). SNP rs5067 in the 3'-UTR of *NPPA* mRNA locates within the target sites of microRNAs hsa-miR-205 and hsa-miR-328. Change of nucleotide T to C of rs5067 results in the loss of hsa-miR-205 binding and in the creation of hsa-miR-328 binding. Thus, it is possible that the highly frequent SNP rs5067 may play a major role in regulating ANP activity. It is also possible that the rs5067 SNP is in LD with one or more SNPs that regulate transcription or translation of message encoding the ANP and/or the function of the protein. For example, rs5067 is in tight LD with several SNPs in the *CLCN6* (location 1p36) gene and less so in the *MTHFR* (location 1p36.22) gene, which are adjacent to *NPPA*. Future association studies should include SNPs that are in tight LD with rs5067.

Our results show that the C allele of rs5067 is associated with an adjusted OR of 0.5–0.25 in Whites, which represents a modest reduction in the risk of asthma. However, given that the frequency of rs5067 is common, the PAR was 23%, which estimates the fraction of cases of disease that would be avoided if exposure (genotype) were removed [37]. Thus, we conclude that the NP system requires further study to determine the contribution that genetic variants in genes encoding the NP system make to asthma.

Our study has several limitations. The number of cases and controls in the screening cohort was modest, while the number of participants in the replicate cohort was small. Thus, our study may have been underpowered to detect true associations between asthma and other *NPPA* SNPs that may contribute to asthma susceptibility. We did not study African Americans or other ethnic groups because of insufficient numbers. This is a weakness in this study because of ethnic disparities in asthma. Future studies should include a broader representation of ethnic groups. Another weakness is that we determined associations between asthma and only three *NPPA* SNPs and yet >30 SNPs have been identified in this gene, some of which may contribute to asthma susceptibility. Determining associations between asthma and other SNPs in *NPPA* and other candidate genes using a haplotype tagging SNP approach is probably the most efficient in determining the contribution of NP genetic variants to asthma susceptibility. This, however, underscores the need for larger more diverse populations.

In summary, the ANP system plays an important role in the biological activities of the lung, and is thought to play

an important role in augmenting allergic inflammation and in the genesis of asthma. The gene encoding ANP is located on chromosome 1p36, a region that has been linked to asthma. In the present study, we identified one SNP that associates with asthma in two different populations of cases and controls. Ours is the first to report a link between asthma and *NPPA* genetic variants, and suggests that *NPPA* may be an important susceptibility gene in asthma. Future association studies between asthma susceptibility and variants in NP candidate genes are warranted.

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There are no competing interests.

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