

Association Analysis of Brain-Derived Neurotrophic Factor Gene Polymorphisms in Asthmatic Families

Aleksandra Szczepankiewicz^a Matthew J. Rose-Zerilli^{b, c} Sheila J. Barton^c
Stephen T. Holgate^c John W. Holloway^{b, c}

^aDepartment of Pediatric Pulmonology, Allergy and Clinical Immunology, Third Department of Pediatrics, Poznan University of Medical Sciences, Poznan, Poland; ^bDivisions of Human Genetics and ^cInfection, Inflammation and Repair, School of Medicine, University of Southampton, Southampton, UK

Key Words

Asthma • Brain-derived neurotrophic factor • Polymorphism, *BDNF*

Abstract

Background: A role for neuronal modulation of inflammation and airway hyper-responsiveness has been well described in asthma, and neurotrophins provide the link between inflammation and neuronal dysfunction. Brain-derived neurotrophic factor (BDNF) is an important mediator in this interaction. The aim of this study was to analyze the possible relationship between polymorphisms of the gene encoding BDNF and susceptibility to asthma. **Methods:** 341 families with at least 2 siblings with asthma were genotyped for 4 *BDNF* polymorphisms (rs6265, rs2030324, rs988748 and rs7124442). **Results:** Analysis by family-based association tests revealed no significant association between any polymorphisms analyzed and asthma susceptibility. Furthermore, *BDNF* polymorphism was not associated with asthma-related phenotypes such as FEV₁ % predicted, bronchial hyper-responsiveness, IgE level, asthma and atopy severity scores. **Conclusions:** Our results suggest that genetic variation in the *BDNF* gene does not contribute significantly to asthma susceptibility or severity.

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Introduction

Asthma is a chronic inflammatory disease characterized by airway remodeling, hyper-reactivity and recurrent attacks of wheezing and airflow obstruction caused by chronic eosinophilic inflammation in the airways. In consequence, this leads to pathological changes in the lung neuronal network that result in sensory hyper-reactivity and enhanced responsiveness to nonspecific stimuli.

Neurotrophins play an important role in mediating the interactions between immune and nerve cells in inflammatory conditions such as asthma. Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in the regulation of differentiation and survival of various types of neurons, including sensory ones. It is produced not only by the neurons themselves, but also by epithelial cells and inflammatory cells such as macrophages, T cells, B cells and mast cells that upon activation synthesize increased amounts of this neurotrophin [1]. BDNF exerts its effect through binding with a specific TrkB receptor belonging to the tyrosine kinase family of receptors [2]. This receptor is expressed on activated blood and bronchoalveolar lavage fluid eosinophils [3, 4] and stimulation with BDNF results in eosinophil activation.

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Correspondence to: Dr. Aleksandra Szczepankiewicz
Department of Pediatric Pneumology, Allergy and Clinical Immunology
Third Department of Pediatrics, Poznan University of Medical Sciences
27/33 Szpitalna St., PL-60-572 Poznan (Poland)
Tel. +48 61 849 1311, Fax +48 61 848 0111, E-Mail alszczep@amp.edu.pl

A number of animal models have provided evidence for the involvement of neurotrophins in airway hyper-responsiveness and allergic inflammation. Increased expression of neurotrophin-positive cells can be detected in the lung and enhanced levels of neurotrophins are detected in serum and bronchoalveolar lavage fluids in response to allergen challenge in animal models [5, 6]. Administration of exogenous neurotrophins results in increased Th2 mediated inflammation and airway hyper-responsiveness [5, 7], mice lacking the pan-neurotrophin receptor p75 (NTR) exhibit reduced airway inflammation [8], and transgenic mice over-expressing nerve growth factor (NGF) under control of a lung specific promoter exhibit increased airway innervation and hyper-responsiveness [9].

In humans, elevated BDNF and NGF levels have been found in bronchoalveolar lavage fluid after allergen challenge in allergic asthmatic patients [6, 10] and BDNF levels are significantly higher in untreated asthmatic patients in comparison to those treated with inhaled glucocorticoids and nonasthmatic controls [11]. It has also been shown that allergic inflammation increases local BDNF production and that enhanced platelet BDNF concentration correlates with clinical parameters of allergic airway dysfunction [12]. The specificity of BDNF to neuronal dysfunction in allergic inflammation is supported by the finding that the reduction of serum BDNF levels after inhaled glucocorticoid therapy is not correlated with changes in acetylcholine responsiveness of the airways [11]. Recently, it was also reported that dendritic cells may be activated by neurotrophins such as BDNF or NGF as they express functional high-affinity neurotrophin receptors. This in turn, may modulate allergen sensitization and regulation of allergic inflammation [13].

The *BDNF* gene is localized on the short arm of chromosome 11 [14], adjacent to a chromosomal region previously linked to asthma [15]. The gene consists of 4 short 5' exons with separate promoters and one 3' exon encoding the mature BDNF protein [16]. Several polymorphisms have been reported in the *BDNF* gene including a complex multi-allelic repeat polymorphism 1.0 kb upstream of the translation initiation site of the gene that has been shown to affect the transcriptional activity of the *BDNF* gene [17]. Other single nucleotide polymorphisms (SNPs) include a coding change in the pre-protein, Val66Met (196G/A) [18], and several promoter SNPs including -374A/T, -256G/A and -270C/T [19, 20].

We have recently demonstrated association of the -270C/T polymorphism with asthma susceptibility in a

Polish pediatric asthma case-control study [21]. Given these previous results, and the potential biological role of BDNF in allergic airway inflammation and neuronal dysfunction, further investigation of *BDNF* gene polymorphism is warranted. Therefore, the aim of this study was to provide further evidence for a role of BDNF in asthma susceptibility by genotyping a large UK family-based asthma cohort for 4 common polymorphisms in the *BDNF* gene and examining association with asthma and asthma related phenotypes.

Patients and Methods

Subjects

Analysis was performed in a cohort of 341 families previously recruited from the Southampton area of the UK. Family inclusion criteria required 2 siblings (aged 5–21 years) diagnosed with asthma and currently using asthma medication [22, 23]. The phenotypic characteristics of the cohort are shown in table 1. In brief, clinical phenotyping was based on a case report form and health survey questionnaire completed by each family member on the study day visit. Asthma was defined as 3 positive responses to the following questions: 'Have you ever had asthma?' and, 'Was this confirmed by a doctor?' and, 'Have you used any medicines to treat asthma, or any breathing problems, at any time in the last 12 months?'. The baseline forced expiratory volume in 1 s (FEV₁) was obtained from pulmonary function testing. Bronchial responsiveness was measured as the provocation concentration of inhaled methacholine required to reduce FEV₁ by 20% (PC₂₀ FEV₁). Methacholine dilutions (0–16 mg/ml) were given at 5-min intervals by DeVilbiss nebuliser with KoKo dosimeter (PDS Instruments Inc., Louisville, Calif., USA). Skin prick testing was carried out to the following 6 common aeroallergens: mixed grass, mixed trees, cat, dog, *Dermatophagoides pteronyssinus* and *Alternaria* (Bayer Corp., Spokane, Wash., USA), with a negative (saline) and positive (histamine) control. Atopy was defined as either a positive skin prick test result (3 mm increase in wheal diameter over negative control) or raised specific IgE (0.35 kU_A/l) to 1 or more common allergens. Total and specific IgE measurements were carried out by the IBT laboratory (Kansas, Mo., USA) using the ImmunoCAP System (Phadia, Uppsala, Sweden). Specific IgE levels were measured for the same allergens as used for skin prick testing. Total IgE was adjusted for age by using the number of SD away from the median for each age group. Severity scores for atopy and asthma were generated as previously described [22]. Ethical approval for this work was granted by the Southampton & South West Hampshire Joint Research Ethics Committee.

Genotyping

Four SNPs were chosen for analysis in this study. rs6265 (Val66Met), which has been previously shown to alter the intracellular trafficking and packaging of pro-BDNF [25], and rs2030324, rs988748 (intronic) and rs7124442, which are within the 3' UTR and which were identified utilizing HapMap Caucasian data (www.hapmap.org) as tag SNPs for the *BDNF* gene region.

Table 1. Phenotypic characteristics of the study group

	Pedigrees	Parents	Asthmatic parents	Nonasthmatic parents	Asthmatic sibling 1	Asthmatic sibling 2
Subjects, n	1,508	681	189	492	341	341
Mean age, years	24.6	40.5	40.2	40.7	13.0	9.9
Gender, % male	51.8	49.9	47.1	51.0	56.9	53.6
FEV ₁ , % predicted	98.1	100.8	94.1	103.4	94.7	95.6
BHR, % with PC ₂₀ <4 mg/ml ^a	32.4	15.3	34.3	7.9	43.7	56.0
Median total serum IgE, IU/ml	107	56	107	43	249	307

All of the asthmatic family members had a current physician's diagnosis of asthma. FEV₁ = Forced expiratory volume in 1 second; BHR = bronchial hyper-responsiveness; PC₂₀ = provocation concentration of inhaled methacholine required to reduce FEV₁ by 20%; Ig = immunoglobulin.

^a The percentage of subjects for whom PC₂₀ was <4 mg/ml methacholine.

Genotyping was performed utilizing assay-on-demand Taq-Man SNP genotyping assays C_11592758_10, C_27833027_10, C_120354688_20 and C_1197570_10 (Applied Biosystems, Foster City, Calif., USA). 5 µl reactions were performed containing 10 ng of genomic DNA (dried), 2.5 µl of 2× QPCR master mix (Eurogentec), 0.0625 µl of 40× SNP assay mix (final concentration 0.5×) and PCR-grade water to volume. Thermal cycling was performed in a PTC-225 DNA engine (MJ Research Inc., Cambridge, Mass., USA) as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, plates were transferred to an ABI Prism® 7900 ht sequence detection system and the fluorescence of each reaction was read. Data acquisition and analysis was performed using the allelic discrimination analysis module in SDS v.2.1 software (Applied Biosystems). For each reaction plate genomic control DNA samples and nontemplate controls (water) were included. Genotyping was repeated for 96 samples (6% of cohort) to check for genotyping accuracy.

Statistical Analysis

Genotype data from unrelated parents was assessed for concordance with the Hardy-Weinberg equilibrium law using a χ^2 test with 1 degree of freedom. Genetic association analysis was performed using Family-Based Association Tests (FBAT v.1.7.3. software; www.biostat.harvard.edu/~fbat/default.html) using the additive model and bi-allelic tests [25] to investigate association of *BDNF* genotypes with asthma and atopy affection status and with related intermediate variables [FEV₁ % predicted, bronchial hyper-responsiveness (using the least squares slope method as described in [22]), serum total IgE levels and atopy and asthma severity scores]. $p \leq 0.05$ was regarded as significant.

We also performed linkage disequilibrium and haplotype analysis of the *BDNF* genotypes using unrelated parents selected from the family cohort (518 healthy and 157 asthmatic subjects) using Haploview software (www.broad.mit.edu/haploview/haploview) [26]. TDT study power calculations were performed using a formula that assumes that the recombination fraction is 0 and there is no linkage disequilibrium [27].

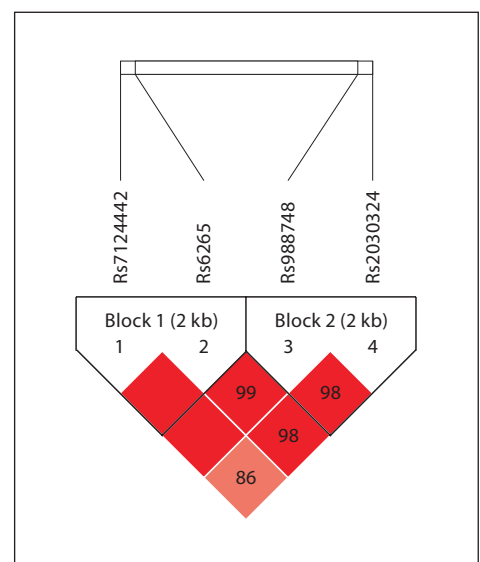


Fig. 1. Relative positions and LD estimates between 4 *BDNF* polymorphisms in the analyzed cohort. Colored squares correspond to D' values with numerical estimates given within the squares.

Results

Genotyping and Allele Frequencies

Minor allele frequencies for each *BDNF* polymorphism were as follows: rs2030324 C = 0.479, rs7124442 C = 0.331, rs988748 G = 0.203, and rs6265 A = 0.184. The genotyping success rates were between 98.5 and 99.1%. Genotyping error rates for all the assays were <1%, we detected only 1 Mendelian error in 341 families and this family was excluded from further analysis.

Table 2. FBAT results between *BDNF* SNPs and asthma and allergy phenotypes

dbSNP Reference	Gene Location	Allele	AF	Atopy n	Z-score	Asthma n	Z-score	+BHR n	Z-score	+FEV ₁ n	Z-score	+IgE n	Z-score	+Severity n	Z-score
rs2030324	Intron 1	T	0.52	222	0.39	238	0.32	220	-0.46	242	0.47	242	-0.04	234	0.25
		C	0.48		-0.39		-0.32		0.46		-0.47		0.04		-0.25
					p = 0.70		p = 0.75		p = 0.64		p = 0.64		p = 0.97		p = 0.80
rs988748	Intron 1	G	0.20	175	0.11	186	-0.05	178	0.36	189	0.11	189	0.03	181	-0.06
		C	0.80		-0.11		0.05		-0.36		-0.11		-0.03		0.06
					p = 0.92		p = 0.96		p = 0.72		p = 0.91		p = 0.97		p = 0.95
rs6265	Exon 2 (Val66Met)	G	0.82	161	0.06	167	0.10	157	-0.62	171	-0.14	171	0.07	165	0.26
		A	0.18		-0.06		-0.10		0.62		0.14		-0.07		-0.26
					p = 0.95		p = 0.91		p = 0.54		p = 0.89		p = 0.94		p = 0.80
rs7124442	Exon 2 (3' UTR)	C	0.33	184	0.61	198	0.89	183	-0.50	202	0.91	202	0.28	195	0.83
		T	0.67		-0.61		-0.89		0.50		-0.91		-0.28		-0.83
					p = 0.54		p = 0.37		p = 0.61		p = 0.36		p = 0.78		p = 0.41

AF = Allele frequency; Atopy = atopy severity; BHR = bronchial hyper-responsiveness; n = number of families analyzed; Severity = asthma severity; UTR = untranslated region. We have included the Z-score which indicates the direction of any potential association (+ means the MAF was over-transmitted, which equals risk for asthma affection; - means under-transmitted, which equals protection with respect to asthma affection; for continual traits a negative sign indicates that the presence of the allele con-

fers lower trait values, a positive sign indicates that the allele confers higher values). The Z-score is a measure of transmission equilibrium, i.e. the deviation from the number of times an allele was transmitted to affected offspring (statistic S; not shown) and the number of times it should be transmitted [expected statistic S, E(S); not shown] and the variance of the S statistic [Var(S); not shown] used to calculate the Z-score under the null hypothesis of no association and no linkage.

Linkage Disequilibrium in the *BDNF* Gene Region

Strong linkage disequilibrium (LD) was observed between all of the *BDNF* polymorphisms analyzed. Haplo-view analysis defined 2 LD blocks, namely rs7124442 and rs6265 in 1 block ($D' = 1.0$, $LOD = 24.75$, $r^2 = 0.112$) and rs988748 and rs2030324 ($D' = 0.98$, $LOD = 43.58$, $r^2 = 0.23$) in the other (fig. 1).

TDT Power Calculations

The study had between 85.6% (rs6265) and 98% power (rs2030324; rs988748 and rs7124442) to detect an association with a genotypic risk ratio 1.5 at the 5% significance level.

Genetic Association Analysis

The FBAT found none of the genotyped *BDNF* polymorphisms to be significantly associated with asthma affection status or with any of the asthma related phenotypes (atopy, FEV₁ % predicted, bronchial hyper-responsiveness, IgE level, and asthma severity score; table 2). Furthermore, case-control analysis using unrelated parental samples from the cohort also failed to show association with asthma affection status for any of the SNPs analyzed ($p = 0.654$ – 0.968).

Haplotype Analysis

We observed 6 common haplotypes (estimated population frequency >0.005). Haplotype analysis for relationship with affection status using FBAT found no significant association (table 3). Similarly, using the parents from the pedigrees as the unrelated cases and controls, no association was seen between either block 1 (rs7124442 and rs6265) or block 2 (rs988748 and rs2030324) haplotypes and affection status (table 4).

Discussion

In conclusion, this study has failed to identify an association between any of the 4 polymorphisms assessed in *BDNF* and asthma or asthma related phenotypes such as elevated IgE, atopy, FEV₁ % predicted, asthma severity or bronchial hyper-responsiveness. This contrasts with our previous study that identified association between -270C/T polymorphism and asthma [21]. The functional Val66Met substitution in the prodomain of *BDNF* has been reported to significantly alter the intracellular trafficking and packaging of pro-*BDNF*, [24, 28] with individuals with the homozygous Val/Val genotype having higher activity of *BDNF*. Given this, we hypothesized this would also lead to increased risk of asthma and enhanced

Table 3. Results of FBAT analysis between *BDNF* haplotypes and asthma

BDNF haplotypes	Alleles	EM estimated frequency	Asthma n	S	E(S)	Var(S)	Z-score	p value
1	CTCA	0.46	216	464	470.0	157.1	-0.479	0.632
2	TCCG	0.31	174	338	331.9	121.0	0.610	0.541
3	TTGA	0.18	154	220	218.7	98.0	0.130	0.896
4	CCCG	0.02	25	26	24.5	11.8	0.438	0.661
5	TTGG	0.02	25	27	28.5	13.3	-0.412	0.680
6	TTCG	0.01	11	7	10.0	5.0	-1.342	0.179

EM = Expectation maximization algorithm; n = number of families analyzed. We have included the Z-score which indicates the direction of the association (+ means the MAF was over transmitted, which equals risk for asthma affection; - means under-transmitted = protection with respect to asthma affection). The S statistic is a measure of transmission equilibrium, i.e. the deviation from the number of times an allele was transmitted to affected offspring and the number of times it should be transmitted [expected statistic S, E(S)] and the variance of the S statistic [Var(S)] used to calculate the Z-score under the null hypothesis of no association and no linkage.

Table 4. Results of Haploview haplotype analysis between asthmatic and healthy parents from the Caucasian cohort

Haplotype	Haplotype frequency	Case:control ratio	χ^2	p
Block 1 (rs7124442 and rs6265)				
TG	0.485	0.490:0.484	0.031	0.859
CG	0.332	0.327:0.333	0.046	0.830
TA	0.183	0.184:0.183	0.001	0.975
Block 2 (rs988748 and rs2030324)				
CC	0.476	0.474:0.477	0.011	0.918
CT	0.318	0.313:0.320	0.048	0.826
GT	0.204	0.212:0.201	0.175	0.675

airway responsiveness. However, in the present study we were not able to detect an association of this polymorphism with bronchial asthma or related phenotypes. Power analysis revealed that the study was well powered (>85%) for the detection of effects with OR ≥ 1.5 . The other polymorphisms analyzed in this study (rs2030324 C/T, rs988748 G/C and rs7124442 C/T) have not been studied previously in relation to asthma. However, those SNPs have been analyzed previously in several studies in relation to psychiatric disorders such as schizophrenia [29–31], major depressive disorder [30], bipolar disorder [30, 32, 33], and nicotine dependence [34] yielding conflicting results both in single marker and haplotype analyses.

LD spanning the whole 68 kb genomic region of the *BDNF* gene was detected by Vepsäläinen et al. [35]. This was further confirmed by the results of the HapMap Cau-

casian data, which show that the *BDNF* gene is located within a single 102-kb haplotype block which is defined by 7 haplotype tagging SNPs [30]. The 4 markers tested in our study, all in LD, are located within this block, and the *BDNF* gene does not contain other haplotype blocks that would not be covered by these markers. However, we cannot exclude the possibility that alterations exist in regulatory regions more distal to *BDNF*, not in LD with any of the genotyped SNPs that affect asthma susceptibility.

In conclusion, considering the size of the group analyzed in this study as well as results of power calculations, our data would indicate that polymorphisms in the *BDNF* gene are unlikely to play a major role in determining susceptibility to asthma. However, this does not imply that *BDNF* itself is not an important mediator in asthma pathogenesis. In this regard, Joachim et al. [36] have re-

cently reported that BDNF serum level is significantly correlated with stress perception and lung function in asthmatic patients but not in control subjects, suggesting that a neuro-immunological interaction mediated by BDNF may explain the relationship between stress and airway function in asthma.

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