



Neurotrophin serum concentrations and polymorphisms of neurotrophins and their receptors in children with asthma

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Received 9 February 2012; accepted 26 September 2012

Available online 26 November 2012

KEYWORDS

Asthma;
Neurotrophin;
Gene;
Polymorphism;
Serum level

Summary

Background: In the recent years numerous studies have analysed the effects of neurotrophins on allergic inflammation in airway diseases reporting increased neurotrophin levels locally in the airways as well as in serum of asthmatic patients. We aimed to investigate if levels of neurotrophins in serum of asthmatic children are influenced by the genotype of functional variants within genes encoding analysed neurotrophins and their specific receptors.

Methods: In the study we included 98 children diagnosed with asthma. Genotyping of 9 polymorphisms located in neurotrophins genes and their receptors genes was done with use of TaqMan SNP genotyping assays or PCR-RFLP. The serum levels of four neurotrophins (BDNF, NGF, NTF3, NTF4) were analysed during exacerbation of asthma symptoms with use of DuoSet ELISA Development Kit (R&D).

Results: The two patients with the genetic variant A/A of *NTRK1* (rs6334) showed significantly higher NGF serum concentrations (113.4 and 218.1 pg/mL) as compared to the mean NGF serum concentrations in the total group of patients (34.8 pg/mL). We also observed a significant epistatic interactions between variants of *NGF* rs6330 and *NTRK1* rs6334 that influenced NGF serum level ($P = 0.0004$). Analysis of four neurotrophins serum levels in relation to different genotypes of analysed neurotrophins genes showed no significant differences among analysed asthmatic children.

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Conclusions: Our results suggest that, among analysed neurotrophins, NGF serum levels may be influenced by the genotype of *NTRK1* gene individually as well as in the interaction with *NGF* functional genetic variant suggesting their involvement in allergic inflammation in asthma. Serum levels of the other neurotrophins do not seem to be affected by the variants in the analysed genes.

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Introduction

Neurotrophins are neurotrophic growth factors that stimulate neurodevelopment in the nervous system. They are also mediators of the interactions between immune and neuronal cells, integrating the neuroimmune crosstalk in the pathogenesis of allergic diseases including asthma. Apart from neurons, neurotrophins are potent to activate also structural (airway epithelium) and immune cells (eosinophils, lymphocytes, mast cells). It was reported that structural and inflammatory cells express neuronal receptors and release mediators which directly communicate with nerve endings in the airways and skin.¹ It has been also observed that allergic asthma is associated with changes in neuronal control in the airways.² This, in turn, may modulate allergen sensitization and allergic inflammation.³

Neurotrophins are polypeptides that support growth, differentiation, and survival of neurons in developing and adult nervous systems. The prototypical neurotrophin is nerve growth factor (NGF) and this family also includes brain-derived neurotrophic factor (BDNF), neurotrophin 3 and neurotrophin 4. Neurotrophins act *via* a family of receptor tyrosine kinases first identified as tropomyosin-related kinases (Trks) initiating cell proliferation and survival. NGF preferentially activates TrkA receptors, BDNF and NTF3 are selective for TrkB receptors, and NTF4 is selective for TrkC receptors. Neurotrophins also act *via* an additional receptor (p75NTR), which has no tyrosine kinase activity and, unlike Trk receptors, binds neurotrophins with low affinity and specificity,⁴ however effectively binds pro-neurotrophins inducing cell death.⁵

In the recent years numerous studies have analysed the effects of neurotrophins on allergic inflammation in airway diseases. Elevated BDNF, NGF and NTF3 levels have been found in bronchoalveolar lavage fluid (BALF) after allergen challenge in allergic asthmatic patients.^{2,6} Asthmatic patients demonstrated elevated levels of neurotrophins (NGF and BDNF) in serum and locally in the airways^{7–9} and significant increase in neurotrophins levels in the airways was observed following allergen provocation.^{6,10} Increased neurotrophins concentration (BDNF) also correlated with clinical parameters of allergic airway dysfunction such as airflow limitation and airway hyperresponsiveness in asthmatic patients,¹¹ whereas its level normalized after anti-inflammatory treatment with inhaled corticosteroids.⁹ All neurotrophins analysed in this study were also found to upregulate the neurotrophin receptors on eosinophils from BALF following allergen challenge in asthmatic patients, resulting in increased viability of eosinophils *in vitro* after incubation with all four neurotrophins.¹² These results indicated that neurotrophin mediated activation of

bronchial eosinophils might play a role in the regulation of eosinophilic inflammation in allergic asthma.

Based on the previous studies indicating that functional polymorphisms within neurotrophins and their receptors genes may alter gene expression and protein secretion, we hypothesized that neurotrophins protein levels are affected by functional polymorphisms within neurotrophins genes and their receptors altering circulating neurotrophin concentrations in asthma. Therefore, in the present study we aimed to investigate the correlation between functional variants of four neurotrophin genes and their specific receptors genes and the serum levels in asthmatic children during symptoms exacerbation.

Methods

Study design

Children diagnosed with asthma were included in this study. The neurotrophins levels were analysed during exacerbation of symptoms in the course of disease. Exacerbation was defined as presence of asthma symptoms (daytime symptoms, nocturnal symptoms, limitation of daily activities, the need for reliever treatment, reduced lung function). All participants as well as their parents have given written informed consent. Local ethics committee accepted the project. Study was performed in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Patients

The study was performed on Polish sample of 98 asthmatic patients of Caucasian origin in age from 6 to 18 years old. Patients were recruited from inpatients treated in the Department of Pediatric Pulmonology, Allergy and Clinical Immunology of Poznan University of Medical Sciences and from the Outpatient Clinic of Allergology in Rzeszow. Asthma diagnosis was made according to GINA recommendation, based on clinical asthma symptoms and lung function test. Spirometry was performed on Lung Test 1000 (MES) according to ERS/ATS guidelines.¹³

Asthma severity was based on GINA guidelines into mild, moderate and severe at least 6 months before inclusion in the study.¹⁴

Allergic predisposition was suggested by current or past symptoms of atopic dermatitis, allergic rhinoconjunctivitis (seasonal or perennial) or food allergy. Atopy was confirmed when children fulfilled the following criteria: total IgE level higher than the upper normal limits for age;

positive skin prick test to at least one aero-allergen (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, *Alternaria alternata*, *Cladosporium herbarum*; pollen: grass mix, rye, birch pollen, alder, hazel – Allergopharma, Germany). Any reaction with mean wheal diameter at least 3 mm greater than negative control was regarded positive. Total serum IgE level was measured by a fluoroimmunoassay with Pharmacia UniCap 100 System® (Pharmacia, Uppsala, Sweden) following manufacturer's instruction.

Genotyping

We have genotyped 9 SNPs from 7 genes involved in neurotrophic signalling cascade and encoding neurotrophins (*BDNF*, *NGF*, *NTF-3*, *NTF-4*) and their specific receptors (*NTRK1*, *NTRK2*, *NTRK3*). The SNP selection was based on at least two of the following criteria: functionality confirmed in previous experimental studies, high frequency (MAF > 0.15), indication as tagSNP in HapMap Caucasian database (www.hapmap.org/) or previously reported papers with serum protein level (both positive and negative findings). The chosen SNPs cover both coding regions as well as non-coding regions (promoters, introns, UTRs). The list of polymorphisms included in this study was presented in Table 1.

The DNA was extracted from 10 ml of EDTA anti-coagulated whole blood using the salting out method Miller 1988.¹⁵ Genotyping of 9 polymorphisms was done with use of PCR-RFLP or TaqMan SNP genotyping assays (Applied Biosystems). The sequences of the primers and conditions for the variants analysed with PCR-RFLP method and the assays ID numbers for polymorphisms analysed with use of 5'nuclease method (TaqMan assays) were shown in Table 1. The amplification of DNA samples with use of TaqMan SNP genotyping assay plates was done in ABI Prism® 7900HT Sequence Detection System. Data acquisition and analysis was performed using the allelic discrimination analysis module in SDS v2.1 software (Applied Biosystems). Reaction components and amplification parameters were based on manufacturer's instructions.

For each reaction plate genomic control DNA samples and non-template controls (water) were included. The control of RFLP analysis and TaqMan SNP genotyping assay was also performed (25% of randomly chosen samples from

both groups for each SNP) to check for genotyping accuracy and identical genotypes were identified in all repeated samples. The genotyping was performed without knowing the clinical status of the subjects.

Analysis of neurotrophins concentration

Blood samples for serum were taken for tubes without anticoagulant between 10 and 12 a.m. and kept at room temperature for 1 h. After that, blood was centrifuged to obtain serum and samples were immediately frozen at –80 °C for further analysis. Concentrations of neurotrophins was measured using DuoSet ELISA Development Kit (R&D). For BDNF serum samples were diluted 1:100 in Reagent Diluent (1% BSA in PBS), for other neurotrophins, due to low serum levels, undiluted samples were used. The absorbance was read on a plate reader at 450 nm wavelength (Asys UVM 340). Neurotrophin concentration was quantified against a standard curve calibrated with known amounts of protein. Assays were run in duplicates and intrassay variability coefficient was assessed to be below 5%.

Statistical analysis

Differences in neurotrophins levels between patients with different genotypes were analysed with use of ANOVA test. *P* value lower than 0.05 was considered significant. All significance levels were two-tailed. To investigate if neurotrophin levels are influenced by the genotype of both neurotrophin gene and neurotrophin receptor gene, we performed two-way ANCOVA analysis with neurotrophin levels as dependent variables and genotypes of appropriate polymorphisms as independent predictors. Calculations were performed using the Statistica version 9.0 software.

Results

Subjects characteristics

Ninety eight asthmatic children were included into the study. The demographic characteristics and clinical description of the studied population was given in Table 2.

Table 1 Description of candidate gene polymorphisms included in this study.

Gene	SNP	Location in the gene	MAF (Caucasian)	Genotyping method	Enzyme/TaqMan assay
<i>BDNF</i>	rs6265	Val74Met, exon 1	0.19	RFLP	Eco72I
<i>NGF</i>	rs6330	Ala35Val, exon 3	0.40	TaqMan	C_2525309_10
	Rs11102930	Promoter region	0.27	TaqMan	C_26680904_10
<i>NTF3</i>	Rs1805149	Glu89Gly, exon 1	0.14	RFLP	HaeIII
	Rs6332	Pro84Pro, exon 1	0.45	TaqMan	C_170592_10
<i>NTF4</i>	Rs11669977	Promoter region	0.20	TaqMan	C_2884550_10
<i>NTRK1</i>	Rs6334	Gln558Gln, exon 4	0.22	RFLP	Tail
<i>NTRK2</i>	Rs1212171	Promoter region	0.40	TaqMan	C_7424025_10
<i>NTRK3</i>	Rs11073763	Intron 12, tagSNP	0.43	TaqMan	C_3041241_10

Table 2 Characteristics of the asthmatic patients.

	Patients group
Gender, male, <i>N</i> (%)	81 (82.6%)
Age, years (mean $\pm$ SD)	11.1 $\pm$ 3.8
Asthma severity	
Mild, <i>N</i> (%)	19 (19.4)
Moderate, <i>N</i> (%)	55 (56.1)
Severe, <i>N</i> (%)	24 (24.5)
Positive SPT (%)	73.2
GC inhaled treatment (%)	97.6
LABA treatment (%)	66.7
Antileukotrienes treatment (%)	45.3
FEV ₁ % pred exacerbation (mean $\pm$ SD)	81.6 $\pm$ 14.44
FEV ₁ % pred asymptomatic (mean $\pm$ SD)	103.8 $\pm$ 87.7
FEV ₁ /FVC pred exacerbation (mean $\pm$ SD)	85.8 $\pm$ 14.5
FEV ₁ /FVC pred asymptomatic (mean $\pm$ SD)	96.4 $\pm$ 12.5
exNO (ppb) (mean $\pm$ SD)	33 $\pm$ 43.4
IgE (IU/mL) (mean $\pm$ SD)	303.1 $\pm$ 411.5

Asthmatic boys were more prevalent in our group of patients (82.6% of the patients' group).

Analysis of neurotrophin serum levels and neurotrophin gene variants

First, we examined the hypothesis that serum neurotrophin levels are influenced by functional polymorphisms localized within the four neurotrophin genes (*BDNF*, *NGF*, *NTF3*, *NTF4*). We found that none of the variants analysed in the present study affected significantly serum level of the appropriate protein (rs6265 variant for *BDNF*; rs6330 and rs11102930 for *NGF*; rs6332 and rs1805149 for *NTF3* and rs11669977 for *NTF4*) (Table 3). We observed that patients with Met/Met genotype of rs6265 *BDNF* gene polymorphism demonstrated lower *BDNF* serum levels in comparison to patients with other *BDNF* genotypes and that patients carrying GG genotype of rs6332 *NTF3* gene presented higher *NTF3* serum levels, however those differences were not statistically significant.

Analysis of neurotrophin serum levels and neurotrophin receptors gene variants

Next, we investigated if polymorphic variants located within the genes encoding specific neurotrophin receptors may influence the serum level of unbound/active protein. We found that in two patients with AA genotype of *NTRK1* rs6334 variant was associated with significantly higher NGF serum level (Table 3). The other analysed variants of receptors genes in relation to neurotrophin serum levels did not differed significantly between patients with different genotypes (Table 3).

Analysis of epistatic effects of neurotrophins and neurotrophins receptors variants on serum level

Finally, we tested the interactive effects of the genetic variants in four neurotrophins and polymorphisms in the

corresponding specific receptors on neurotrophins serum level in asthmatic children. We observed a significant epistatic interactions between *NGF* rs6330 variant and *NTRK1* rs6334 polymorphism that influenced NGF serum level ($F = 6.715$, $P = 0.0004$) (Fig. 1).

Discussion

This is the first study analysing a relationship between neurotrophins serum levels (*BDNF*, *NGF*, *NT-3* and *NT-4*) and genotypes of the variants within neurotrophins genes and their receptors genes (*NTRK1* encoding the high-affinity NGF receptor TrkA; *NTRK2* encoding the high-affinity *BDNF* and *NT-4* receptor TrkB; and *NTRK3* encoding the high-affinity *NT-3* receptor TrkC) that are possibly involved in the gene expression and protein secretion. The main finding of this study is a plausible impact of *NTRK1* rs6334 genotype and interaction between variants of *NGF* rs6330 and *NTRK1* rs6334 on NGF serum level in asthmatic patients.

The most extensively studied neurotrophin in regard to asthma was *BDNF*. It was found that serum *BDNF* level was increased in adult patients with asthma^{9,11} and its level was normalized after glucocorticoid treatment.⁹ However, this finding was not confirmed by the other study.¹⁶ Recently, the first paper in a paediatric population of asthmatic patients was published, where the authors observed that *BDNF* plasma levels were significantly higher in paediatric patients with moderate and severe asthma in comparison to controls and mild asthmatic patients.¹⁷ The studies reporting the association of the other neurotrophins levels with asthma are less abundant. In respect to *NGF*, a study by Bonini et al.⁷ demonstrated increased serum *NGF* level in patients with allergic asthma as compared to healthy controls. The other studies reporting on serum levels of neurotrophins in asthma included analysis by Noga et al.⁹ where the authors investigated three neurotrophins (*BDNF*, *NGF* and *NTF3*) in allergic asthmatic patients treated and untreated with inhaled steroids and control subjects. They reported that *BDNF* and *NGF* levels were significantly higher in untreated patients with allergic asthma as compared to healthy controls and asthmatic patients treated with inhaled corticosteroids, whereas the highest level of *NTF3* was observed in the asthmatic patients treated with ICS. The more recent study performed by Koskela et al.¹⁸ in relation to chronic cough reported that *NGF* level in both serum and sputum was significantly increased in a subgroup of patients with asthma, whereas *BDNF* showed no difference between the analysed groups. In our previous study,¹⁹ we analysed the serum levels of four neurotrophins (*BDNF*, *NGF*, *NTF3*, *NTF4*) in the same group of asthmatic children and we found that *NTF3* and *NTF4* levels were associated with asthma severity.

In respect to analysis of possible influence of genotypes of functional variants on neurotrophin levels, there are to date only few reports. The previous studies in this regard analysed *BDNF* level in relation to *BDNF* rs6265 variant that results in abnormal cellular trafficking and packaging of pro-*BDNF* leading to reduced secretion of mature *BDNF* protein in case of Met allele.²⁰ The influence of this variant on *BDNF* serum or plasma level was confirmed in Met allele carriers as they had lower *BDNF* serum levels in comparison

Table 3 Analysis of serum neurotrophin levels stratified by genotype of neurotrophins and neurotrophin receptors genes variants (* indicates significance).

Gene	SNP ID	Genotype	N	Mean	SD	F	P
BDNF serum level (pg/mL)							
BDNF	Rs6265	Met/Met	5	28,073.22	10,601.95	1.144	0.323
		Met/Val	37	41,082.74	23,260.50		
		Val/Val	43	36,964.22	16,763.81		
NTRK2	Rs1212171	T/T	25	31,022.61	12,585.33	0.976	0.382
		C/T	34	36,258.55	17,622.30		
		C/C	8	37,999.26	18,950.99		
NGF serum level (pg/mL)							
NGF	Rs6330	A/A	9	32.80	69.06	0.300	0.742
		A/G	22	22.57	47.91		
		G/G	14	16.91	28.81		
	Rs11102930	A/A	27	23.09	56.85	0.210	0.810
		A/G	25	22.46	33.32		
		G/G	7	11.18	9.91		
NTRK1	Rs6334	G/G	23	31.50	52.30	5.993	0.005*
		G/A	14	46.97	51.50		
		A/A	2	113.44	—		
				218.13			
NT3 serum level (pg/mL)							
NTF3	Rs6332	G/G	16	298.40	386.97	1.376	0.259
		G/A	34	163.18	323.33		
		A/A	20	129.40	252.47		
	Rs1805149	A/A	2	21.73	—	0.399	0.671
		A/G	1	48.38	0.00		
		G/G	78	195.14	315.66		
NTRK3	Rs11073763	A/A	14	118.44	298.21	0.448	0.640
		A/G	33	210.56	346.04		
		G/G	40	180.30	270.08		
NT4 serum level (pg/mL)							
NTF4	Rs11669977	A/A	71	52.63	88.66	0.184	0.831
		A/G	25	66.33	121.01		
		G/G	4	48.08	39.78		
NTRK2	Rs1212171	T/T	38	68.68	107.13	1.714	0.185
		C/T	40	36.04	50.80		
		C/C	14	81.14	145.78		

to carriers of Val allele,²¹ and the same trend was observed in our study (carriers of Met/Met genotype had lower BDNF serum levels than carriers of other genotypes, but this difference was not significant). BDNF protein level reduction in Met carriers (Met/Met or Val/Met) in a peripheral system (amniotic fluid) was also confirmed by Cattaneo et al.²² However, other studies did not confirm that observation.^{23–25} So far, the other neurotrophins levels have not been analysed in regard to serum level.

Our main observation that NGF serum level may be influenced by variation in *NTRK1* gene as well as by the interaction between this variant and *NGF* rs6330 polymorphism which indicate that this signalling pathway may be one of the determinants of *NGF* protein level. The biological relevance of rs6334 polymorphism in *NTRK1* gene may be explained by the fact that this silent substitution (Q558Q) is located in tyrosine kinase domain and neighbouring mutations (G516R and G571R) in this region lead to diminished NGF-stimulated autophosphorylation of the receptor²⁶ suggesting the possible role in the altered

intracellular signalling in response to NGF stimulation.²⁷ In regard to rs6330 variant in *NGF* gene, this substitution results in alanine to valine change and is located in the pro-NGF sequence possibly affecting trafficking of the protein. The increase in amino acid size (Val) could modify the tertiary structure of pro-NGF, leading to altered interaction and signalling via the p75 neurotrophin receptor, as C allele (corresponding to Ala) was suggested to enhance interaction with the p75 neurotrophin receptor that mediate cell death.²⁸ We observed that the T allele was associated with increased NGF serum level, and that increase could be explained by diminished interaction with p75 receptor and therefore, impaired intracellular signalling.

This study for the first time presents the comprehensive analysis of neurotrophins levels in relation to genotypes of functional variants of neurotrophins genes and their receptors. However, this report has several limitations such as relatively small sample size and also a detection limit for NGF in serum, as it is on relatively low level, so in some children we were not able to detect it with ELISA method or

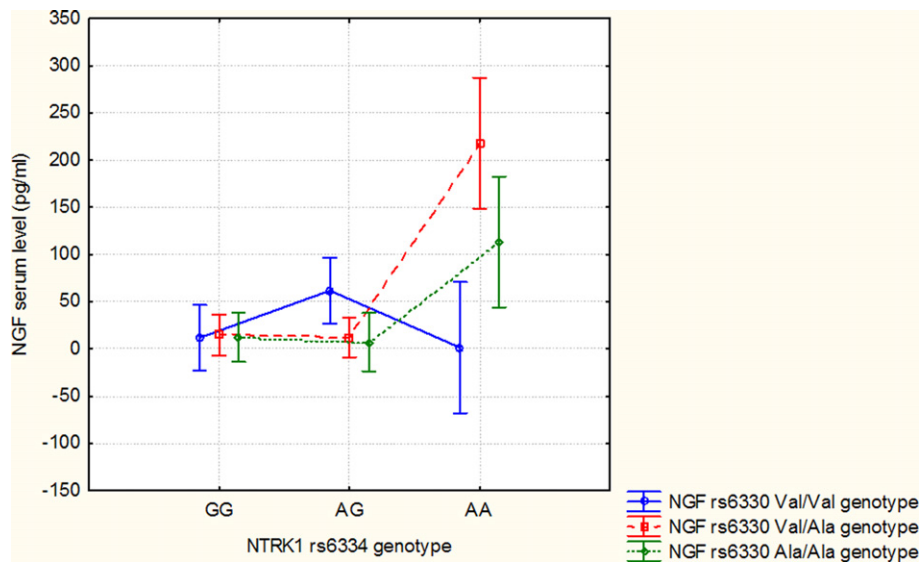


Figure 1 The interaction between rs6330 of *NGF* gene and rs6334 of *NTRK1* gene significantly influences NGF serum level in asthmatic patients ($F = 6.715$, $P = 0.0004$; vertical lines are 0.95 confidence intervals).

its level was below the detection limit of the method (8 pg/mL), therefore those measurements were excluded from further analysis. However, positive association between NGF serum level and rs6334 polymorphism remained significant when we analysed all samples, including those with undetectable serum NGF. Another limitation of this paper is the cause of asthma exacerbation – in about 80% of patients it was due to allergen exposure, however in the remaining patients 20% exacerbations were triggered by acute respiratory infection which could also influence our results (at least for BDNF) based on the previous report showing that acute bacterial infection may affect BDNF level.²⁹ Among other limitations, we should also mention the fact that neurotrophin serum levels could be influenced by combined treatment with inhaled steroids and LABA present in 66.7% of patients, as such combination was reported previously to influence BDNF level.³⁰ However, our priority was to adjust the best treatment schedule for the paediatric patients with asthma.

In conclusion, we found that *NTRK1* variant (rs6334) and epistatic interaction between *NGF* and *NTRK1* variants are possibly influencing NGF serum level in asthmatic patients. The other genetic variants in neurotrophins genes and their receptors do not affect neurotrophins serum levels in the analysed population. However, our preliminary study involved a limited number of patients and further studies are necessary to confirm our observations and draw definite conclusions.

Acknowledgements

This study was supported by the Polish National Science Centre grant no. 2011/01/D/NZ5/02771.

Conflict of interest statement

There are no potential conflicts of interest for any of the authors to the subject of the report.

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