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Association of A/T polymorphism of the *CHRM2* gene with bronchodilator response to ipratropium bromide in asthmatic children

Abstract

Introduction: The aim of this study was to analyze the possible association of A/T polymorphism of the *CHRM2* gene with asthma, and pharmacogenetic analysis of the polymorphism with bronchodilator response to ipratropium bromide, an anticholinergic drug used in asthma.

Material and methods: Analysis was performed in a group of 113 children diagnosed with bronchial asthma, and in a group of 123 healthy children from a control group. Moreover, in the group of 32 asthmatic children without concurrent treatment with long-acting β_2-agonists, bronchodilator response to ipratropium bromide was evaluated by the spirometric lung function test. Genetic analysis was performed for A/T polymorphism (rs6962027) of the *CHRM2* gene. Genotyping was done with the PCR-RFLP method. Statistical analysis was performed using Statistica v.7.1 software.

Results: No association of A/T polymorphism was found with asthma ($p = 0.865$ for genotypes and $p = 0.782$ for alleles). In the pharmacogenetic analysis, it was observed that patients carrying TT genotype of *CHRM2* gene polymorphism demonstrated significantly poorer response to anticholinergic drug as compared to the patients with other genotypes for this polymorphism ($p = 0.035$).

Conclusions: We found that TT genotype in the *CHRM2* gene was associated with poor bronchodilator response in asthmatic patients. The results should be analyzed carefully considering the small sample size and should be confirmed by other research groups.

Key words: ipratropium bromide, asthma, muscarinic type 2 receptor gene (*CHRM2*), polymorphism, bronchodilator response

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Introduction

Anticholinergic agents are bronchodilators used as auxiliary drugs in the treatment of asthma. Chemically, these drugs are derivatives of atropine and are used to treat patients that show no tolerance for β_2-agonists, demonstrate no satisfactory bronchodilation after β_2-agonist administration, and have nocturnal asthma attacks (dyspnea) or severe asthma. They act by blocking the cholinergic component of bronchoconstriction, inhibiting all muscarinic receptor subtypes.

Based on their pharmacological properties, 5 muscarinic receptor subtypes have been identified, 3 of which (M_1, M_2 i M_3) exert their physiological effect in the airways. A large amount of these receptor subtypes have been observed in the airway smooth muscles [1, 2], as well as in the airway

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epithelium and submucosal glands stimulating, through acetylcholine, mucus secretion [1].

Muscarinic receptors are G_i protein-coupled receptors and, upon their activation, several biochemical pathways are triggered leading to contraction. Although M_2 receptors (autoreceptors, inhibiting receptors) represent the majority of muscarinic receptors in the airway smooth muscles, they do not participate directly in the contraction; however, their activation inhibits the bronchodilator action of β_2-adrenergic receptors [3]. In the asthmatic airways, an increased basal tone was observed [4, 5], which was confirmed further on the animal model, where, after allergen exposure, immediate bronchoconstriction with subsequent hyperreactivity for a number of stimuli was reported, including vagal nerve stimulation in the presence of electrical impulses or histamine [6]. Anticholinergic agents act by decreasing this basal tone and airway hyperreactivity [7].

Control of airway smooth muscle contraction is disturbed in asthma, and the observed increased bronchoconstriction and mucus secretion result from an increased expression and activity of signalling molecules that are crucial for muscarinic receptor-mediated contraction, as well as from increased acetylcholine secretion due to neural mechanism dysfunction associated with inflammation [8]. Therefore, anticholinergic drugs used as bronchodilators in asthma restore normal airway reactivity by blocking the cholinergic component of bronchoconstriction [9]. However, in the case of these drugs chronic exposure may also lead to a gradual decrease in the receptor amount on the cells, which results from enhanced internalization and degradation or decreased translation levels.

Muscarinic receptor type 2 gene is localized on the long arm of chromosome 7 (7q31–q35), approximately 20 cM from the site associated with PEFR value in the study by Wjst et al. [10] and bronchial hyperreactivity in the study by Daniels et al. [11]. The gene size of about 1.4 kb contains a single, intronless open reading frame (one exon) encoding 466-amino acid receptor protein and a large 5'UTR region that is alternatively spliced. In the study by Fenech et al. [12] performed on airway smooth muscle cells they showed numerous initiation transcription sites with sequences recognized by Sp1 and AP-2 transcription factors. In this region, $(CA)_n$ repeat was identified and localized 96 bp downstream of the initiation transcription site, which may influence gene transcription in airway smooth muscles and common C/A polymorphism located upstream of this site. The coding gene sequence is highly conservative and no studies analyzing polymorphisms in this gene region were found.

In the study by Minnette et al. [13] they found that asthmatic patients have different degree of M_2 receptor dysfunction that may lead to increased acetylcholine secretion and, as an effect, increased activation of M_3 receptors responsible for contraction. Therefore, polymorphisms in the receptor gene may possibly affect this disturbance. However, up to now, there have only been a few studies analyzing the association of *CHRM2* gene polymorphisms with asthma.

In the study of $(CA)_n$ repeat it was found that the variant with 14 copies (wild type) was associated with an increased expression level in comparison to the variant with 6 copies, in transfected airway smooth muscle cells [12]. This may indicate that this polymorphism influences gene activity and the differences in airway hyperreactivity observed in asthmatic patients. However, due to its localization in the promoter region of low activity, its significant influence on the total amount of M_2 receptor seems unlikely. The polymorphism analyzed in this study (rs6962027) is a single nucleotide polymorphism (SNP) leading to thymine (T) to adenine (A) change and is localized within the last exon, in the 3' untranslated region. Its possible influence on receptor function has not yet been elucidated.

The aim of this study was the association analysis of *CHRM2* A/T gene polymorphism with the presence of asthma and bronchodilator response to ipratropium bromide in patients stratified by *CHRM2* genotype.

Material and methods

Patients

The study was performed on a Polish sample of 113 asthmatic patients of Caucasian origin ranging from 6 to 18 years in age (68 boys with a mean age of 11.8 years, SD = 2.9; 45 girls with a mean age of 12.4 years, SD = 3.8). The patients were recruited from inpatients from the Wielkopolska region, considered as ethnically homogenous, and were treated for asthma in the Department of Paediatric Pulmonology, Allergy and Clinical Immunology of Poznan University of Medical Sciences. Asthma diagnosis was made according to GINA 2002 recommendations (http://www.ginasthma.com), based on clinical asthma symptoms and lung function tests (bronchodilator responsiveness, exercise-induced hyperresponsiveness).

Atopy was confirmed when children fulfilled one of the following criteria: total IgE level higher than the upper normal limits for age; positive skin prick test to at least one aero-allergen (Dermato-

phagoides pteronyssinus, Dermatophagoides farinae, cat, dog, feathers, 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 and defined atopy. Total serum IgE level was measured by a fluoroimmunoassay with a Pharmacia UniCap 100 System® (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. The upper limits of normal range for total IgE was age-dependent (70 KU/l for 6-year-old children; 79 KU/L for 7-year-old children, 89 KU/L for 8-year-old children, 98 KU/L for 9-year-old children, and 107.0 KU/L for children of 10 years and older).

We analyzed separately a subgroup of children with severe asthma (n = 54). Severe asthma was defined as follows: symptoms requiring daily therapy with high-dose inhaled corticosteroids (> 800 budesonide or > 500 fluticasone), despite regular long-acting β_2-agonists and/or leukotriene antagonist and/or theophylline (slow releasing), 1 or more emergency care visit or oral steroids burst per year.

Control group

Control group consisted of 123 healthy subjects of Caucasian origin (59 boys with a mean age of 10 years, SD = 2.2; 64 girls with a mean age of 9.6 years, SD = 1.8). Control subjects were also recruited from the Wielkopolska region from a group of carefully chosen volunteers without asthma or allergy symptoms. Any allergic diseases or asthma were excluded based on clinical examination, spirometry, and exhaled NO measurement.

All participants as well as their parents gave written informed consent. The local ethics committee accepted the project. This study was performed in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Genetic analysis

The DNA was extracted from 10 ml of EDTA anticoagulated whole blood using the salting out method [14] in the group of patients and from saliva in the control group with an OraGene kit according to the manufacturer's protocol. *CHRM2* polymorphism was assessed by PCR-RFLP method. PCR reaction was performed in the total volume of 15 μl. Mixture reaction was prepared in one tube and then 10 μl of mixture reaction was distributed to the reaction tubes containing 5 μl of DNA working stock (concentration 50 ng/μl) of the patients and control subjects. The mixture reaction contained: 250 ng of genomic DNA, 0.26 μM of each primer (with the following sequences: *forward*: 5'-TTTCTTCTTGTTATGCCACT-3' and *reverse*: 5'-CTTTAATAAACTTGGTCC-3'), 2.6 mM dNTP, 22.5 mM $MgCl_2$, 75 mM Tris-HCl (pH = 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween, and 0.5 U Taq polymerase (Fermentas). The reaction was performed in a PTC-200 thermal cycler MJ Research. The following conditions were used: initial denaturation 95°C for 2 min; 30 cycles of subsequent denaturation 94°C for 30 s; annealing for 30 s (50°C); elongation 72°C for 40 s, and final elongation for 10 min. at 72°C. The primer sequences were designed by the authors using "Primer3" software available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The PCR product (356 bp) was then digested overnight with use of HpyCH4IV restriction endonuclease (New England Biolabs), and the digested products were resolved in 2% agarose gel. The following alleles were observed: T allele for the DNA fragments of 250 and 106 bp, and A allele for DNA fragments of 178, 106, and 72 bp. Control of the RFLP analysis was also performed (25% of randomly chosen samples from both groups). The genotyping was performed without knowing the clinical outcome of the patient.

Pharmacogenetic analysis

In the pharmacogenetic analysis, 32 children with controlled asthma were included. The bronchodilator response to anticholinergic agent (ipratropium bromide) was evaluated prospectively and was assessed at least 2 weeks after long-acting β_2-agonist (salmeterol) withdrawal. All patients included in the study continued treatment with inhaled corticosteroids; therefore, their influence on the outcome of the pharmacogenetic analysis was considered equal, and the relationship between *CHRM2* genotype and bronchodilator response was attributed only to anticholinergic agent.

Evaluation of response to anticholinergic agent (ipratropium bromide) was assessed by spirometry (flow-volume curve) on LungTest 1000 apparatus according to ATS (American Thoracic Society) guidelines [15, 16]. The spirometry was performed in the Lung Function Laboratory in the Department of Paediatric Pulmonology, Allergy and clinical Immunology. FEV1 values were analyzed before and after drug administration. A ≥ 15% increase in FEV1 was diagnostic. Change of FEV1 value after drug administration was evaluated according to the following formula:

$$\%\ \text{reversibility} = \frac{\text{max. FEV1 after drug} - \text{max. FEV1 before drug}}{\text{max. FEV1 before drug}} \times 100$$

FEV1 value after drug administration was shown as a percentage of the predicted value.

Response to anticholinergic agent was measured in the bronchodilator response (reversibility of airflow obstruction) with ipratropium bromide (40 μg) applied through Volumatic holding chamber. Spirometry was performed before and 30 min after drug application.

Statistical analysis

Pearson's chi-square (χ^2) test and Fisher's exact test were used to test differences in the genotypic and allelic (respectively) distribution between the groups of patients and control subjects. Calculations were performed using the STATISTICA version 7.1 software. For polymorphisms containing < 5 observations per cell, we performed the Fisher-Freeman-Halton exact test using Cytel Studio Version 8 StatXact-8. Odds ratios were calculated using a demo of GraphPad InStat 3 software. Concordance with the Hardy-Weinberg law was performed using "Utility Programs For Analysis Of Genetic Linkage" application (Copyright © 1988 J. Ott).

In pharmacogenetic analysis of *CHRM2* polymorphisms with treatment response to ipratropium bromide, mean values of spirometric measures were compared (FEV1%, FVC%, PEF). We also analyzed bronchodilator response to ipratropium bromide stratified by patient genotype using one-way variance analysis ANOVA if the data distribution was concordant with normal distribution (Shapiro-Wilk test) and after checking variance homogeneity (Levene test). Means were compared with post-hoc Newman-Keuls test. If the data did not meet the criteria mentioned above, the non-parametric Kruskal-Wallis test was applied.

For analyses, a level (p) of 0.05 was considered statistically significant.

Results

The distribution of genotypes for the analyzed polymorphism was in concordance with the Hardy-Weinberg law in the group of patients ($p = 0.780$) and in the control group ($p = 0.784$). The number of subjects with each genotype (TT, AT, and AA) was 28, 55, and 30 in the group of patients; and 31, 63, and 29 in the control group, respectively. The distribution of genotypes and alleles of *CHRM2* polymorphism did not differ significantly between the group of patients and the control group ($p = 0.865$ for genotypes and $p = 0.782$ for alleles). In the subgroup of patients with severe asthma, we did not observe any significant differences in comparison to the control group ($p = 0.726$ for genotypes and $p = 0.488$ for alleles).

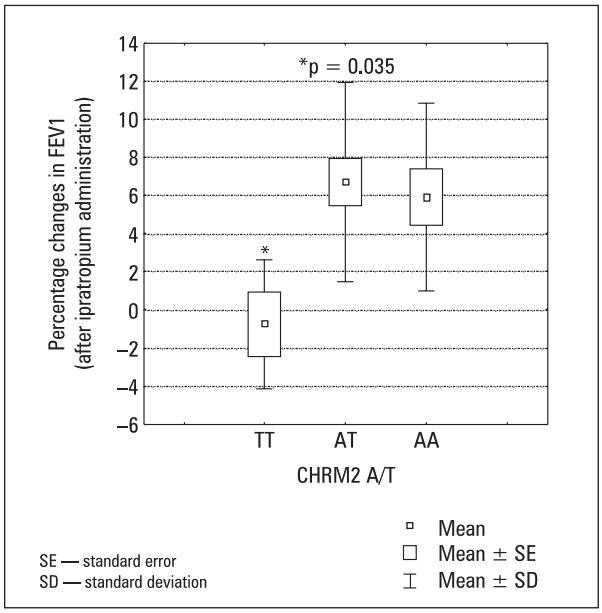

Figure 1. Comparison of CHRM2 A/T genotypes with bronchodilator response to ipratropium bromide in asthmatic patients

In the pharmacogenetic analysis of *CHRM2* gene polymorphism in the group of patients, we observed the following genotype frequencies: TT = 4 children; AT = 17 children; and AA = 11 children. We found significant differences between the mean FEV1 ($p = 0.021$), FVC ($p = 0.022$), and PEF (0.010) values in patients depending on the *CHRM2* genotype. In the analysis of mean percentage values of the bronchodilator response to the drug in patients stratified by genotypes, we observed significantly poorer responses in patients with TT genotype (ANOVA: $df = 2$, $F = 3.737$, $p = 0.035$) (fig. 1).

Discussion

It was found that M_2 receptors demonstrate impaired function and, subsequently, decreased ability to inhibit bronchodilator action [13]. In the present study, we performed association analysis for one of the polymorphisms localized within the gene region considering the gene function and its localization in the chromosomal region linked, among others, to airway hyperreactivity [11].

The following allele frequencies of *CHRM2* gene were reported for the Caucasian population: 44% for T allele and 56% for A allele, whereas allele frequencies observed in this study were equally represented among patients and control subjects (50% each). As a consequence, a lack of association was observed for the analyzed polymorphism with the presence of asthma. This *CHRM2* gene polymorphism has not been analyzed previo-

usly; however, several studies have been conducted for other *CHRM2* gene polymorphisms with regard to asthma. In the study by Yamamoto et al. [17] on the group of 102 asthmatic patients, with 58 individuals without current asthma symptoms but symptomatic in childhood, and 70 healthy subjects, they found a "silent" A1050G change, with both variants coding serine. In the study of the Maltese population, two polymorphisms were described [18] in the coding gene region: A976C (both variants coding arginine) and T1197C (both variants coding threonine); however, none of them was associated with asthma in the group of 46 asthmatic patients and 46 control subjects. In their study, they also found the presence of common polymorphism in the 3' UTR gene region, analyzed in the present study; however, they did not perform association analysis for asthma. It was reported that this polymorphism did not lead to any alteration in the gene sequence recognized by transcription factors and, therefore, it is unlikely to affect gene expression. In the same study, in all analyzed individuals, the presence of arginine insertion was reported within a 100 bp distance of the aforementioned polymorphism, introducing consensus sequence for C-Rel/NF-κB transcription factors in the 3'UTR. Considering the proximity of those two variants, a linkage that might possibly influence gene expression cannot be excluded. The examples given here indicate no association of the *CHRM2* gene with asthma, which is consistent with our results; however, one should take into account that most of those studies (including this one) were conducted on a relatively small sample size (n ~100). Therefore, further analysis of the *CHRM2* gene on a larger population is required to verify the results obtained so far.

In the present study, we evaluated the relationship between *CHRM2* gene polymorphism and bronchodilator response to ipratropium bromide with no concurrent treatment with long-acting β_2-agonists (wash-out). We found that homozygotes for T allele demonstrated significantly worse bronchodilator response (lesser FEV1 increase) after ipratropium bromide application as compared to patients with the other genotypes ($p = 0.035$). To our knowledge, there are no other studies analyzing any *CHRM2* gene polymorphisms in relation to response to anticholinergic agents, so we cannot compare our results. However, due to the small sample size ($n = 32$) analyzed in this study, the results should be interpreted cautiously.

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