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No association of glucocorticoid receptor polymorphisms with asthma and response to glucocorticoids

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ABSTRACT

Purpose: Glucocorticoids are the most effective anti-inflammatory drugs in asthma therapy. They act via receptors localized in target cells that after activation by glucocorticoids may affect expression of inflammatory genes thus reducing inflammation in asthma. However, 10-20% of patients, particularly with severe, difficult-to-treat asthma may not respond well to glucocorticoids and remain symptomatic even after being treated with high doses of inhaled or systemic glucocorticoids. Therefore, we investigated if polymorphisms known to affect expression or function of the glucocorticoid receptor may be responsible for lower efficacy of steroid therapy and the need to use high doses of inhaled drug.

Material and Methods: We analyzed 113 pediatric patients in age from 6 to 18 with diagnosed asthma, including 54 children with severe, difficult-to-treat asthma. The diagnosis was based on clinical manifestation, a lung function test, increased IgE level and positive skin prick tests. We also analyzed 123 healthy control subjects. The polymorphisms were genotyped with the use of PCR-RFLP method. Linkage disequilibrium analysis was performed using Haploview.

Results: We did not observe any significant differences between asthmatic and healthy children for any of the polymorphisms analyzed. Weak linkage between two of the four polymorphisms studied: rs41423247 and rs6195 (D'=1.0; LOD=2.91, r^2=0.044) was found in linkage disequilibrium analysis. We did not find any association of GR polymorphisms with the dose of inhaled glucocorticoids needed to achieve asthma control in the group of patients.

Conclusion: The results may suggest that studied polymorphisms of the GR gene are not associated with asthma susceptibility and do not influence response to inhaled glucocorticoids in our sample.

Key words: asthma, glucocorticoid receptor, gene, polymorphism, association, response to glucocorticoids

INTRODUCTION

In asthma treatment, glucocorticoids are the most commonly used anti-inflammatory drugs which effectively reduce airway inflammation. However, there is a subgroup of 10-20% of less responsive patients, who do not improve, even after being treated with high doses of steroids, and are classified as having severe or difficult-to-treat asthma [1].

Glucocorticoids binding efficacy depends on the GR receptor, present in two isoforms: α and β that are produced in alternative splicing. Isoform α is expressed in almost all cells and tissues and within the respiratory system, including endothelium, smooth muscles, airway epithelial cells and inflammatory cells [2,3]. A decrease in sensitivity of target tissue correlates with reduced receptor expression [4], followed by loss in receptor sensitivity and lack of response to corticosteroid treatment, which has been confirmed by in vitro [5,6] and in vivo [7,8] studies. Isoform GR-β acts as a negative regulator of transcription and inhibitor of signal transduction and its enhanced expression is predominant in the airways of asthmatic patients insensitive to glucocorticoid treatment [9]. In many inflammatory diseases (including steroid-dependent asthma), a higher expression level in peripheral blood cells and bronchoalveolar lavage fluid was reported [9-11]. However, this was not confirmed for steroid insensitive interstitial lung diseases where it was GR-α expression that seemed to be responsible for differences in steroid response [12].

GR gene encodes glucocorticoid receptor located in the long arm of chromosome 5 (5q31-q32). Its location in the chromosomal region linked to asthma [13] suggests its involvement in the asthma pathogenesis. GR gene consists of 9 exons and its activity is regulated by three separate promoters containing several GC boxes binding transcription factor Sp-1 [14,15]. Using chemical mutational analysis, no mutations in the GR cDNA sequence of corticosteroid-resistant patients were detected by Lane et al. [16], so it seems unlikely that receptor structure is responsible for corticosteroid resistance in asthma. However, several polymorphisms in the nucleotide sequence of the *GR* gene have been identified and found to affect response to glucocorticoids. The most frequently cited include rs6190 (Arg23Lys), rs6195 (N363S), rs41423247 (BclI C/G) and rs10052957 (-3807 C/T orTth111I). The first of them is located in the exon 2 and leads to 23Arg/Lys substitution. In subjects with Lys allele, decreased sensitivity to glucocorticoids has been observed after dexamethasone application [17]. The rs6195 polymorphism is also located in exon 2 and results in asparagine to serine change in codon 363. This polymorphism was associated with increased glucocorticoid sensitivity [18] determined by specific haplotype [19]. However, this polymorphism is rather rare in Caucasians (4.5%) [20].

Another frequently analyzed polymorphism is rs41423247 leading to C>G substitution located in intron 2, 646 nucleotides downstream exon 2 that destroys recognition site for the BclI restriction enzyme. It has been observed that homozygous GG and heterozygous subjects are more sensitive to glucocorticoids, leading to suppressive influence on adrenal activity [21].

Frequently analyzed polymorphism from the promoter region is rs10052957, responsible for C>T substitution, 3807 nucleotides upstream transcription start, which destroys recognition site for the Tth111I restriction endonuclease described by Detera-Wadleigh et al. [22].

The aim of this study was to analyze the possible association of four of the GR polymorphisms (rs6190, rs6195, rs41423247 and rs10052957) with the presence of asthma as well as with an increased demand for high doses of inhaled glucocorticoid in patients with severe asthma.

MATERIALS AND METHODS

Patients

The study was performed on a Polish sample of 113 asthmatic patients of Caucasian origin aged between 6 to 18 years old (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). Patients were recruited from inpatients from the Wielkopolska region, treated for asthma in the Department of Pediatric Pulmonology, Allergy and Clinical Immunology at Poznan University of Medical Sciences. An asthma diagnosis was made according to GINA recommendation, based on clinical asthma symptoms and a lung function test (bronchodilator responsiveness). The bronchodilator response was assessed 20 minutes after administration of 200 mcg of Salbutamol MDI via a holding chamber (Volumatic). A $\geq$ 15 % increase in FEV1 was diagnostic. Clinical diagnosis of atopy was defined as described previously [23].

Separately, we analyzed a subgroup of children with severe asthma (n=54), that demand high doses of inhaled glucocorticoids (ICS). Severe asthma was defined as follows: symptoms requiring daily therapy with high-dose inhaled glucocorticoids (> 800 mcg budesonide or > 500 mcg 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 bursts per year. Compliance with treatment was verified during routine visits (at least 1 per 3 months) and was based on demand for inhalers and parental monitoring of drug administration. Response to glucocorticoids in this subgroup was evaluated on the basis of the dose of ICS according to the GINA guideline necessary to maintain control of asthma symptoms. A parameter of worse response was defined as a necessity of taking high doses of ICS ie. > 800 mcg of budesonide and > 500 mcg of fluticasone propionate. In all patients included in the study, asthma control was obtained after inhaled therapy with glucocorticoids, so there were no presumptions of chronic systemic therapy.

Control group

The 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 and 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 have given written informed consent. The local ethics committee accepted the project. The study was performed in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Genotyping

The DNA was extracted from 10 ml of EDTA anticoagulated whole blood using the salting out method [24]. The *GR* polymorphisms were analyzed using the PCR-RFLP method. The conditions of PCR and sequences of the primers for the following polymorphisms were used as described previously: rs6190 [25], rs41423247 [26], rs6195 [27]. For the rs10052957 polymorphism, the conditions and primers used were designed by our group. A volume of 5 µl of each PCR product (482 bp for rs6190; 153 bp for rs10052957; 206 bp for rs41423247 and 249 bp for rs6195) was then digested overnight in a total volume of 10 µl at 37˚C with appropriate restriction endonuclease (MnlI for rs6190 polymorphism; PsyI for rs10052957 polymorphism; BclI for rs41423247 polymorphism and TasI for rs6195 polymorphism). The uncut

Table 1. **Genotype distributions and allele frequencies of the four analyzed polymorphisms of the *GR* gene for asthmatic patients versus control group (figures in parentheses indicate percentages).**

Gene	Polymorphism		Asthma (n=113)	Control (n=123)	P value
rs6190	genotypes	GG	105 (94.59)	111 (90.24)	0.212
		AG	6 (5.41)	12 (9.76)	
	alelles	G	216 (97.30)	234 (95.12)	0.240
		A	6 (2.70)	12 (4.88)	
rs41423247	genotypes	GG	12 (10.62)	18 (14.63)	0.620
		CG	58 (51.33)	58 (47.15)	
		CC	43 (38.05)	47 (38.21)	
	alelles	G	82 (36.28)	94 (38.21)	0.703
		C	144 (63.72)	152 (61.79)	
rs10052957	genotypes	TT	16 (14.16)	11 (8.94)	0.272
		CT	57 (50.44)	58 (47.15)	
		CC	40 (35.40)	54 (43.90)	
	alelles	T	89 (39.38)	80 (32.52)	0.125
		C	137 (60.62)	166 (67.48)	
rs6195	genotypes	AG	17 (15.04)	15 (12.30)	0.539
		AA	96 (84.96)	107 (87.70)	
	alelles	G	17 (7.52)	15 (6.15)	0.586
		A	209 (92.48)	229 (93.85)	

Table 2. **Haplotype frequencies for two *GR* polymorphisms (rs41423247 and rs6195, respectively) in group of patients versus control subjects.**

Haplotype	Block frequency	Frequency ratio cases:control	X^2	p
CA	0.559	0.562 : 0.565	0.017	0.8970
GA	0.373	0.363 : 0.382	0.187	0.6652
CG	0.068	0.075 : 0.062	0.331	0.5653

PCR products for rs10052957 and rs41423247 were digested twice to confirm the results. A control of RFLP analysis was also performed (25% of randomly chosen samples from both groups).

Statistical analysis

The 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. For polymorphisms containing < 5 observations per cell, we performed Freeman-Halton exact test with use of StatsDirect statistical software v.2.6.2 (trial). Power analysis was also done. Calculations were performed using the Statistica version 7.1. Odds ratios were calculated using a demo of GraphPad InStat 3. Concordance with Hardy-Weinberg law was performed using „Utility Programs For Analysis Of Genetic Linkage" application (Copyright © 1988 J. Ott). We also performed linkage disequilibrium analysis using free online software Haploview version 3.32: http://www.broad.mit.edu/mpg/haplview/index.php [28].

RESULTS

Genotype distributions for all studied polymorphisms in the *GR* gene were in concordance with the Hardy-Weinberg law in both cases and control subjects for all studied polymorphisms (p=0.769 and p=0.569 for rs6190; p=0.548 and p=0.409 for rs10052957; p=0.241 and p=0.987 for rs41423247; p=0.387 and p=0.469 for rs6195, respectively). We did not find any significant differences in genotype distribution and allele frequencies for any of the polymorphisms studied (*Tab. 1*).

In linkage disequilibrium analysis, two of the polymorphisms analyzed (rs41423247 and rs6195) had D' value of 1, however, r^2 coefficient, affected by the rarity of rs6195 polymorphism (MAF=0.06 in the control group and 0.075 in the patient group) was very low (r^2=0.044), so the true linkage between them remains controversial. On the basis of high D' value, it was possible to define haplotypes, however, there were no statistical differences in frequencies of particular haplotypes between the asthmatic and the control subjects (*Tab. 2*).

In the analysis of relationship between *GR* polymorphisms and response to inhaled glucocorticoids (defined as demand for ICS), we have not found any significant differences neither for genotypes nor for alleles when comparing patients with severe asthma who required therapy with high doses of ICS to maintain asthma control to the patients with moderate asthma responding well to lower doses. The results are shown in *Tab. 3*.

Power analysis for the analyzed polymorphisms in our group was 7% for rs41423247 and rs6195; 24 % for rs6190 and 35% for rs10052957.

Table 3. **Genotype distributions and allele frequencies of the four analyzed polymorphisms of the *GR* gene for patients with severe asthma that demanded high doses of glucocorticoids to maintain asthma control versus patients with moderate asthma treated with low doses of glucocorticoids (figures in parentheses indicate percentages).**

Polymorphism			Severe asthma(n=54)	Moderate asthma (n=53)	P value
rs6190	genotypes	GG	52 (96.30)	50 (94.34)	0.678
		AG	2 (3.70)	3 (5.66)	
	alleles	G	106 (98.15)	103 (97.17)	0.681
		A	2 (1.85)	3 (2.83)	
rs41423247	genotypes	GG	6 (11.11)	6 (10.91)	0.995
		CG	27 (50.00)	28 (50.91)	
		CC	21 (38.89)	21 (38.18)	
	alleles	G	39 (36.11)	40 (36.36)	1.000
		C	69 (63.89)	70 (63.64)	
rs10052957	genotypes	TT	5 (9.26)	10 (18.18)	0.398
		CT	30 (55.56)	27 (49.09)	
		CC	19 (35.19)	18 (32.73)	
	alleles	T	40 (37.04)	47 (42.72)	0.409
		C	68 (62.96)	63 (57.28)	
rs6195	genotypes	AG	11 (20.37)	4.(7.27)	0.055
		AA	43 (79.63)	51 (92.73)	
	alleles	G	11 (10.18)	4 (3.63)	0.065
		A	97 (89.82)	106 (96.37)	

DISCUSSION

The first finding of this study is a lack of association of four polymorphisms of the *GR* gene with asthma in the analyzed population. We also have not found any relationship between the studied polymorphisms of *GR* gene and increased demand for glucocorticoids in the patients with severe asthma. In linkage disequilibrium analysis, we observed D' value suggestive for linkage for two of the analyzed polymorphisms (rs41423247 and rs6195); however, based on low r^2 value it seems unlikely to detect true linkage between them.

Regardless its chromosomal location in a region linked to asthma and atopy (5q) [13], there were no association studies analyzing the relationship between asthma and polymorphisms in the *GR* gene. In this study, we have not observed an association of any of the four analyzed polymorphisms with the presence of disease. It is difficult to verify our results as there have been no association studies of *GR* gene polymorphisms with asthma thus far. In the case of two of the analyzed polymorphisms, rs6190 and rs6195, lack of positive association may result from the fact that they are rare in our relatively small sample (MAF=0.05 and 0.06, respectively). The low frequencies of alleles for these polymorphisms were also found in other studies [20]. Therefore, for at least these two polymorphisms, false negative results should be taken into account.

In the linkage disequilibrium analysis of *GR* polymorphisms, we observed D' value suggestive for linkage (D'=1.0) between rs41423247 and rs6195 polymorphisms. However, considering low r^2 value for that haplotype block (r^2=0.044), which results from low allele frequency of rs6195 polymorphism, it is difficult to identify the real strength of linkage between the two polymorphisms. Therefore, this haplotype was not informative in our group. There are no data reporting on rs41423247 and rs6195 haplotypes in asthma, so we cannot verify our results.

In vitro studies have demonstrated that T cells from patients with glucocorticoid-resistant asthma showed decreased nuclear binding affinity and reduced receptor numbers [29]. It may indicate that *GR* gene polymorphisms affecting its affinity to steroids may play an important role in the treatment response to glucocorticoids. In the analysis of a relationship between the *GR* polymorphisms and poor response to glucocorticoids, of the four polymorphisms was associated in our sample. Results concerning rs6190 were not consistent with the findings by van Rossum et al. [17], as they demonstrated the relationship with decreased glucocorticoid sensitivity. A possible mechanism of reduced sensitivity of the lysine variant to glucocorticoids has been recently explained by Russcher et al. [30], which was confirmed in clinical studies demonstrating that patients with Lys23 have decreased response to dexamethasone [17]. For rs41423247 polymorphism, we also did not observe any association with a decreased sensitivity to glucocorticoids. There were no other studies analyzing patients with severe asthma, so it is not possible to compare the results obtained in our study. In genetic analysis, so far, no relationship between rs10052957 polymorphism and changes in glucocorticoid sensitivity confirmed by dexamethasone suppression test was reported [31], which is consistent with our results. Rs6195 polymorphism is related to increased transactivating capacity, both in vitro and in vivo [30], which is consistent with a correlation between G allele and increased glucocorticoid sensitivity [18,21]. Substitution of asparagine by serine creates

a potential phosphorylation site, which may be crucial for DNA binding [32,33]; however, the exact influence remains to be elucidated. Lack of a relationship between this polymorphism and glucocorticoid sensitivity obtained in this study confirmed the previous findings by Koper et al. [25] and Tissing et al. [34].

We demonstrate here for the first time, the lack of association of *GR* polymorphism with asthma and response to glucocorticoid therapy. However, due to a relatively small sample size, false negatives cannot be excluded; therefore our results need verification in a larger population of severe asthmatic patients that demand high doses of inhaled steroids to maintain asthma control. As similar reports in other steroid-resistant inflammatory diseases are lacking, we should be careful in the interpretation of results presented here treating them as the preliminary findings that await confirmation.

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