The N363S and I559N single nucleotide polymorphisms of the *h-GR/NR3C1* gene in patients with bronchial asthma

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Abstract. Bronchial asthma is a disease of multifactorial etiology. The natural variability of the DNA sequence within the *h-GR/NR3C1* gene affects both the conformation and the activity of glucocorticoid receptors. There are 2 major types of resistance to glucocorticoids (GCS)-resistant asthma failing to respond to treatment with high doses of inhaled and oral glucocorticoids. Type I GCS-resistant asthma is cytokine-induced or acquired. Type II GCS resistance involves generalized primary cortisol resistance, which affects all tissues and is likely to be associated with a mutation in the glucocorticoid receptor (GCR) gene or in genes that modulate GCR function. There are clear examples of glucocorticoid gene *h-GR/NR3C1* polymorphisms that can influence responses and sensitivity to glucocorticosteroids. Among the numerous polymorphisms observed within this gene, N363S and I559N single nucleotide polymorphisms (SNPs) may play an important role in the development of bronchial asthma and in the alteration of sensitivity to GCS in severe bronchial asthma. The aim of this research project was to study the correlation between the N363S and I559N polymorphisms of the *h-GR/NR3C1* gene and the occurrence of asthma in a population of Polish asthmatics. Peripheral blood was obtained from 210 healthy volunteers and 234 asthma patients. Structuralized anamnesis, spirometry and allergy skin prick tests were performed in all participants. Genotyping was carried out using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and PCR-HRM methods. In the healthy, non-atopic population, the GG variant of the N363S polymorphism was found with a 5.7% frequency. In asthma patients, GG SNP of N363S occurred with the frequency of 6.4%. In the groups of patients with uncontrolled moderate asthma and uncontrolled severe disease, the genotype distribution for the investigated polymorphisms were as follows: N363S, AA, AG, GG occurring with 0.8750/0.0834/0.0416 frequency and I559N, TT, TA, AA occurring with 1.000/0.000/0.000 frequency. The analysis demonstrated a significantly higher frequency of the A and G variants of the N363S polymorphisms in uncontrolled moderate asthma and uncontrolled severe disease than in the healthy population. No variant-related differences in the frequency of the studied I559N polymorphism were demonstrated in healthy controls and asthma patients. In conclusion, the N363S polymorphism of the *h-GR/NR3C1* gene is significantly associated with an increased sensitivity to glucocorticoids *in vivo* and susceptibility to the development of a moderate to severe form of uncontrolled bronchial asthma in the Polish population. This observation needs to be confirmed in a larger group of subjects.

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Abbreviations: ACT, asthma control test; AF_1, activation domain 1; AF_2, activation domain 2; AP-1, activator protein-1; ATS, American Thoracic Society; cAMP, cyclic adenosine monophosphate; CBP, CREB-binding protein; DBD, DNA binding domain; DNA, deoxyribonucleic acid; EAACI, European Academy of Allergy and Clinical Immunology; EDTA, ethylenediaminetetraacetic acid; ERS, European Respiratory Society; FEV_1, forced expiratory volume; FVC, forced vital capacity; GCR, glucocorticoid receptor; GCS, glucocorticoids; GINA, the Global Initiative for Asthma; GRE, glucocorticoid response elements; *h-GR/NR3C1*, human glucocorticoid receptor gene/nuclear receptor subfamily 3, group C, member 1 gene; HRM, high resolution melting; LBD, ligand-binding domain; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NLS, hinge region; NSAIDs, non-steroid anti-inflammatory drugs; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RNA, ribonucleic acid; SNP, single nucleotide polymorphism; SPT, skin prick tests

Key words: h-GR/NR3C1 gene, glucocorticoid receptor, glucocorticoid receptor gene polymorphism, SNP, RFLP, inflammation, resistance to steroids

Introduction

Bronchial asthma is a chronic inflammatory disease with an etiology determined by numerous factors, involving many cell types and substances secreted by them (1). The etiopathogenetic process leading to the development of bronchial

hyperreactivity, wheezing episodes, cough and dyspnea is triggered by interactions between hereditary and environmental factors (2,3). Among the individual factors influencing the course and expression of the disease, genetic determinants (genetic predisposition to atopy, genetic predisposition to bronchial hyperreactivity), degree of obesity and gender play an important role (2,3). Recent reports confirm the involvement of numerous genes, the roles of which may be different in various ethnic groups, in the pathogenesis of bronchial asthma (3-5). The clinical presentation is a result of complex gene-gene and gene-environment interactions (6). It has been estimated that approximately 100 genes are involved in the etiopathogenesis of asthma. Nevertheless, the genetic component of hereditary variance is estimated at 35-95% (7,8). It is noteworthy that increasing incidence of allergic diseases and bronchial asthma has been observed during the last dozen or so years (7). The reason for the increased incidence of severe asthma has not been fully elucidated from the epigenetic point of view. This phenomenon cannot be explained only by significant changes of the genetic factors.

There are currently over 300 million subjects with asthma all over the world. In that number, patients with severe asthma account for approximately 5%, and those with very severe asthma - for approximately 1% (3 million) (9). Resistance to glucocorticoids (GCS) is observed in approximately 50% of patients with severe and difficult-to-treat bronchial asthma (9). Nevertheless, it should be emphasized that GCS resistance among asthma patients is generally rare and is estimated to occur in 1 out of 1,000 subjects (9). Among the numerous causes of glucocorticoid receptor (GCR) GCS, the following ones are mentioned: GCR genetic mutations (familial GCS resistance syndrome), GCR ligand-binding defects, GCR nuclear translocation disturbances and disturbances of GCR binding to glucocorticoid response elements (GRE) (10).

Resistance to GCS is the extreme form of all possible reactions to these drugs (6). There are two forms of GCS resistance: type 1, induced by cytokines (acquired, reversible reduction of GCS binding potential of T lymphocytes) and type 2, mutations and polymorphisms of glucocorticoid receptor gene (mutations and polymorphisms of the *h-GR/NR3C1* gene, or genes modulating the functions of GCR, GCR ligand-binding defects, decrease in the number of GCR) (11-18). As evidenced from recent research, non-receptor transcriptional factors such as: [activator protein-1 (AP-1) and nuclear factor (NK)-κB protein] play an important role in the development of GCS resistance (6,9,10,19). Impaired synthesis of inhibitor proteins for transcriptional factors leads to an increase of their concentration in cells and GCR blockade (9).

The *h-GR/NR3C1* gene [NR3C1; nuclear receptor subfamily 3, group C, member 1 gene (human glucocorticoid receptor gene); HGNC ID: HGNC:7978] is located on 5q31-q32 (12-14,17). The product of the *h-GR/NR3C1* gene expression is mRNA, on the basis of which GCS receptor isoforms (GCRα, GCRβ, GCRδ, GCRγ), with only the α variant active, are formed as a result of alternative splicing (6,20-22). The GCS receptor consists of 777 amino acids and contains five domains (AF_1, activation domain 1; DBD, DNA binding domain; NLS, hinge region; LBD, ligand-binding domain; and AF_2, activation domain 2) (6,23,24). The structure and biological activity of the GCS receptor domains is determined by the structure and composition of the appropriate exons of the *h-GR/NR3C1* gene, respectively: AF_1 domain, exon 2; DBD domain, exons 3 and 4; NLS and LBD domain, exons 5 to 9 (25). Nucleotide changes in the DNA sequence of the analyzed gene may lead to gene transcription activation disturbances, changes in RNA splicing, changes in the secondary or tertiary structure of GCS receptor domains, translation initiation disturbances and disturbances of GCS receptor mRNA stability.

Table I presents amino acid changes in the GCS receptor structure resulting from the occurrence of single nucleotide polymorphisms (SNPs).

Polymorphism N363S (A>G) is localized in exon 2 of the NR3C1 gene at position 1220. The occurrence of SNP causes asparagine to serine change in codon 363 (26). In human peripheral mononuclear cells *in vitro* the presence of the N363S mutation form has been correlated with significantly higher transactivation capability of the genes responsible for the GCS-GCR complex (27). This mutation has also been associated with increased sensitivity to GCS *in vivo* (27,28). The N363S SNP modifies the AF_1 immunogenic domain of the glucocorticosteroid receptor influencing the activation of receptor function and possibility of interactions with the transcriptional factors, AP-1 and NF-κB. It should be emphasized that AP-1 and NF-κB do not constitute part of signaling cascades, for which coactivator proteins p300/CBP are macromolecular docking 'platforms'. Homologous p300, cAMP-responsive element binding protein (CREB)-binding protein (CBP) and the family of p160 nuclear receptor coactivators participate in the transformation of the GCS ligand-receptor complex into a transcription initiation complex (25,29,30). Cytokine-activated AP-1 demonstrates high affinity to DNA sequences of the regulatory parts of the genes, the same parts that also bind the GCS-GCR complex. AP-1 and the GCS-GCR complex mutually abolish each other's regulatory effects on gene expression.

Polymorphism I559N (T>A) is localized in exon 5 of the *h-GR/NR3C1* gene at position 1808. The occurrence of SNP causes an isoleucin to asparagine change in codon 559 in the LBD domain of GCR (25). The occurrence of I559N SNP does not determine ligand binding, but exerts a dominant negative effect on the wild-type receptor by forming a heterocomplex (25,32). Specifically, it causes a delay of wild-type receptor translocation to the cell nucleus (25,32).

The aim of the present study was to assess the respective frequencies of N363S and I559N polymorphisms of the *h-GR/NR3C1* gene in the groups of healthy subjects and bronchial asthma patients. The correlations between N363S and I559N *h-GR/NR3C1* gene polymorphisms and the severity of bronchial asthma, the control of the disease, the Asthma Control Test (ACT), and sensitivity/resistance of the patients to glucocorticosteroid therapy were examined.

Materials and methods

The study was approved by the local ethics committee (Consent of Research Review Board at the Medical University of Lodz, Poland, no. RNN/133/09/KE). At the commencement of the study, participants were invited to attend voluntarily. Before enrollment, written informed consent was obtained from every patient.

Table I. Amino acid change in glucocorticoid receptor structure caused by SNPs, MIM ID +138040.

No.	Phenotype	Gene (amino acid change)	dbSNP
1	Glucocorticoid resistance, familial	*h-GR/NR3C1* (ASP641VAL)	rs104893908
2	Glucocorticoid resistance, familial	*h-GR/NR3C1* (IVS6DS, 4-BP DEL)	-
3	Glucocorticoid resistance, cellular	*h-GR/NR3C1* (LEU753PHE)	rs121909726
4	Glucocorticoid receptor polymorphism	*h-GR/NR3C1* (ASN363SER)	rs56149945
5	Glucocorticoid resistance, generalized	*h-GR/NR3C1* (ILE559ASN)	rs104893909
6	Glucocorticoid resistance, familial	*h-GR/NR3C1* (ILE747MET)	rs104893910
7	Pseudohermaphroditism, female, with hypokalemia, due to glucocorticoid resistance	*h-GR/NR3C1* (VAL571ALA)	rs104893911
8	Glucocorticoid resistance, relative	*h-GR/NR3C1* (198G-A, 200G-A)	rs6189/rs6190
9	Glucocorticoid resistance, generalized	*h-GR/NR3C1* (LEU773PRO)	rs104893912
10	Glucocorticoid resistance, generalized	*h-GR/NR3C1* (ARG477HIS)	rs104893913
11	Glucocorticoid resistance, generalized	*h-GR/NR3C1* (GLY679SER)	rs104893914
12	Glucocorticoid resistance, generalized	*h-GR/NR3C1* (PHE737LEU)	rs121909727
13	Glucocorticoid sensitivity, metabolic profile	*Bcl*I gene *h-GR/NR3C1* promoter (Intron B)	rs41423247

Data are based on materials obtained from the Online Mendelian Inheritance in Man®, 1966-2011 Johns Hopkins University with partial modification by the authors.

The study was conducted in a group of 234 patients with bronchial asthma. Asthma diagnosis was established according to the Global Initiative for Asthma (GINA) recommendations, based on clinical asthma symptoms and pulmonary function test. The level of asthma severity and control was determined on the basis of GINA report guidelines. All the participants underwent subjective examinations (structuralized anamnesis including, besides the element of subjective examination, also the analysis of factors such as: gender, obesity, tobacco smoking, duration of bronchial asthma, allergy to house dust mites, animal fur, mold spores, cockroach allergens, hypersensitivity to non-steroid anti-inflammatory drugs (NSAIDs), etc., in order to determine their role in the development of resistance to glucocorticoids, as well as to establish whether they were primary, or secondary to genetic factors in character) and objective examinations.

The results of pulmonary function tests and allergological tests were obtained from the individual medical records of the patients. If there were no results of spirometry or allergological tests available, such examinations were additionally performed during the recruitment visit. Subjects suffering from clinically significant exacerbations, using drugs which might induce resistance to glucocorticoids (such as rifampicin, phenobarbital, phenytoin, ephedrine), subjects with signs of viral infections, either generalized, or affecting the respiratory tract, as well as subjects failing to comply with the doctor's recommendations, were excluded from the patient group. The control arm included a group of 210 healthy adults, who met the following criteria: no history or symptoms of either bronchial asthma, or other pulmonary diseases, no history or symptoms of allergy, no history or symptoms of atopic dermatitis, no history or signs of hypersensitivity to aspirin, negative results of skin tests for 12 common allergens, no first-degree relatives with bronchial asthma or atopic disorders.

According to the standards developed by the Polish Society for Pulmonary Diseases, the best result of three spirometry maneuvers was selected for the analysis of obstructive disorders and disease severity. The correlation analysis took into consideration the forced expiratory volume (FEV_1) values expressed in liters, FEV_1% (A/N%, percentage ratio of the measured to expected value) and FEV_1% FVC index (FEV_1 to FVC ratio, forced vital capacity) expressed as absolute numbers. Spirometry tests were conducted in the Outpatient Department according to European Respiratory Society (ERS)/American Thoracic Society (ATS) standards, and allergological tests according to the guidelines of European Academy of Allergy and Clinical Immunology (EAACI).

The level of asthma control was assessed with the Asthma Control Test (ACT), which is clear and easy for patients. ACT consists of five questions and was developed by Nathan *et al* in cooperation with general practitioners and specialists in diagnostics and therapy of asthma. The bronchial asthma control level was calculated on the basis of the following patients' results obtained in ACT: 0-19 points, no asthma control; 20-24 points, partially controlled asthma; and 25 points, well-controlled asthma.

The study was carried out on 444 participants. The control group consisted of 139 women (66.2%) and 71 men (33.8%). The youngest participant was 18 and the oldest 85 years-old. The mean age in the control group was 45.62 years, the median 47.00, and the SD ± 16.52. The case group consisted of 146 women (62.4%) and 88 men (37.6%). The youngest participant was 19 and the oldest 82 years-old. The mean age in the case group was 48.81 years, the median 51.00 and the SD ± 15.95. Non-severe asthma was diagnosed in 151 patients (64.53%) whereas 83 (35.47%) had a severe form of the disease. A comprehensive comparison of the analyzed groups is presented in Tables II and III. The whole group of patients participating in the study was stratified and subjected to statistical analysis.

Table II. Descriptive statistics of the analyzed parameters in the controls and cases of the general asthma population.

Parameter	Control group	Group of bronchial asthma patients
N	210	234
Gender		
Female/male	139/71	146/88
Female/male, %	66.2/33.8	62.4/37.6
Age		
Mean ± SD, years	45.62±16.52	48.81±15.95
Min, years	18	19
Max, years	85	82
Kurtosis	-0.96	-0.94
Skewness	0.14	-0.13
Median	47.00	51.00
FEV_1 (L)		
Mean ± SD	2.97±0.79	2.21±0.85
Median	2.87	2.17
Mean ± SD (%)	96.26±12.60	72.85±19.72
Median (%)	96.00	74.00

Table III. Descriptive statistics of the analyzed parameters in the cases of the general asthma population.

Parameter	Bronchial asthma patients n (%)
Asthma	234 (100.00)
Non-severe	151 (64.53)
Severe	83 (35.47)
Chronic mild	35 (14.95)
Chronic moderate	116 (49.57)
Chronic severe steroid-dependent	73 (31.19)
Chronic severe steroid-resistant	10 (4.29)

Table IV. Description of Hardy-Weinberg equilibrium for control and case groups.

	Tests for deviation from Hardy-Weinberg equilibrium	
SNP	Controls	Cases
N363S		
nAA	165 (156.87)	191 (179.59)
nAG	33 (49.26)	28 (50.81)
nGG	12 (3.87)	15 (3.59)
fa1	0.86±0.019	0.88±0.018
F	0.33014	0.44895
p (Pearson)	$1.716x10^{-6}$	$6.529x10^{-12}$
p (Llr)	0.000030	$9.155x10^{-9}$
p (Exact)	0.000030	$1.101x10^{-8}$
I559N		
nTT	210 (0.00)	234 (0.00)
nTA	0 (0.00)	0 (0.00)
nAA	0 (0.00)	0 (0.00)
fa1	1.00±0.000	1.00±0.000
F	0.00000	0.00000
p (Pearson)	$0.000x10^{+00}$	$0.000x10^{+00}$
p (Llr)	$0.000x10^{+00}$	$0.000x10^{+00}$
p (Exact)	$0.000x10^{+00}$	$0.000x10^{+00}$

Tests for deviation from Hardy-Weinberg equilibrium were performed using free software provided by Institut für Humangenetik, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (Germany). The tests for association were adapted from Sasieni (37). fa1, frequency of allele 1 ± standard deviation; F, inbreeding coefficient; p (Pearson), Pearson's goodness-of-fit χ^2 (degree of freedom, 1); p (Llr), log likelihood ratio χ^2 (degree of freedom, 1); p (Exact), Exact test; n, number.

Venous blood samples were collected from the participants onto EDTA K3 and DNA was obtained from the peripheral blood leukocyte fraction. The genetic material was isolated using the QIAamp DNA Blood Mini kit (Qiagen Inc.) according to the guidelines provided by the manufacturer. The investigated polymorphisms were analyzed using the PCR-RFLP method.

Exponential amplification of DNA segments for the N363S polymorphism was carried out using a forward primer (5'-CCA GTA ATG TAA CAC TGC CCC-3') and a reverse primer (5'-TTC GAC CAG GGG AAG TTC AGA-3') according to a standard PCR protocol (33). Starter binding to complementary DNA matrix sites was conducted at 56˚C. Amplified DNA sequences of 357 bp length were obtained. The material was incubated with FastDigest® *Tsp*509I (*Tas*I) restriction enzyme (Fermentas International, Inc.) at 65˚C for 1 h (33). DNA fragments of 135, 73, 70, 60 and 19 bp, were obtained as a set of representative, typical (wild-type) alleles, whereas segments of 135, 92 (73+19 bp), 70 and 60 bp, constituted the set of polymorphic alleles (33). RFLP products were separated by electrophoresis on an 8% polyacrylamide gel, stained with ethidium bromide and observed in UV light. Representative, typical homozygotes, as well as heterozygotes were sequenced and used as internal control.

Detection of I559N was conducted using the PCR-high-resolution melting (HRM) technique. Exponential amplification of DNA segments for the I559N polymorphism was carried out using a forward primer (5'-GAG GTT ATT GAA CCT GAA GTC-3') and a reverse primer (5'-GGT ATT GCC TTT GCC CAT TTC-3') according to a standard PCR protocol. Starter binding to complementary DNA matrix sites was conducted at 52˚C.

The amplified PCR product was diluted 1:50 to obtain the matrix. The first stage of HRM analysis involved amplification of the investigated DNA fragment containing the analyzed I559N SNP on the 1:50 matrix using a forward primer (5'-GAT ATG ATA GCT CTG TTC CAG-3') and a reverse primer

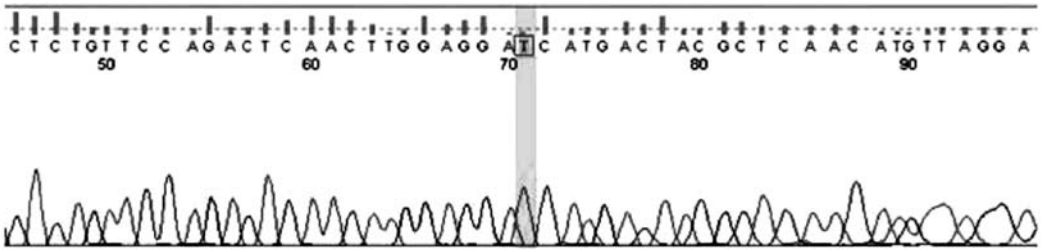

Figure 1. I559N SNP sequencing result, sample no. 315.

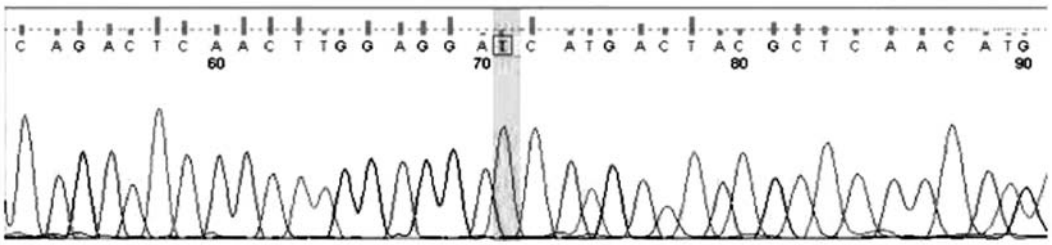

Figure 2. I559N SNP sequencing result, sample no. 340.

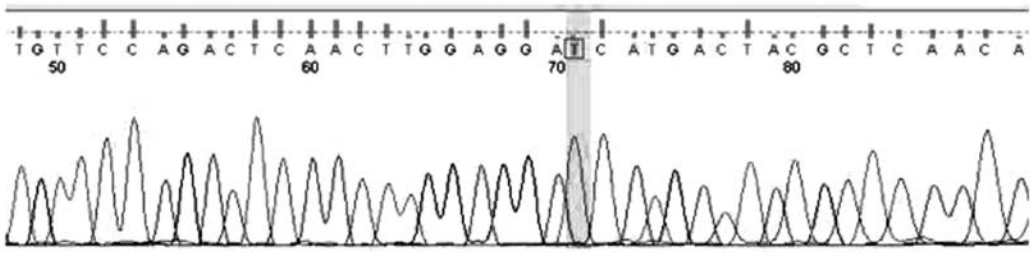

Figure 3. I559N SNP sequencing result, sample no. 411.

(5'-GCC GCC CTC CTA ACA TGT TGA-3'), followed by denaturation and slow renaturation to form a heteroduplex. At the last stage, the mixture was subjected to precise denaturation in the presence of intercalating stain, and identification of DNA fragments (I559N SNP) was based on the analysis of melting curves. The LightScanner® High Sensitivity Master Mix (Idaho Technology, Inc.) was used in the reaction. It is a specialized master mix containing LCGreen Plus® dye and internal temperature calibrators. The obtained product was subjected to internal control using a molecular probe phosphorylated at the 3'-terminal portion (unlabeled, 3' blocked oligonucleotide; 5'-CAA CTT GGA GGA TCA TGA CTA CGC T-Pho-3'). HRM analysis with the probe utilized the LightScanner Master Mix (Idaho Technology, Inc.). The selected SNP samples were verified once again by sequencing to confirm the presence of the appropriate PCR-HRM reaction product, as well as to confirm the I559N polymorphism variant. The examples of sequencing for I559N *h-GR/NR3C1* gene polymorphism are presented in Figs. 1-3.

The obtained results were subjected to descriptive statistical analysis with calculation of arithmetic means and standard deviations. The significance of differences between the mean values was determined by means of χ^2 test, with $P<0.05$ adopted as the significance level.

Results

In a healthy, non-atopic population, within the N363S gene, a polymorphism of N363S: AA, AG, GG was found to occur with 0.786/0.157/0.057 frequency; polymorphisms of I559N, TT, TC, CC were occurring with a 1.000/0.000/0.000, frequency. In the cases (general asthma population analyzed in the study) two polymorphisms were identified in exon 2 at position 1220 in the *h-GR/NR3C1* gene: N363S (AA, AG, GG occurring with 0.816/0.120/0.064 frequency) and in exon 5 at 1808 position in the *h-GR/NR3C1* gene: I559N (TT, TA, AA occurring with 1.000/0.000/0.000 frequency).

The distributions of N363S and I559N polymorphisms of the *h-GR/NR3C1* gene were consistent with Hardy-Weinberg equilibrium. Tables IV-VI illustrate distributions of the investigated genotypes: N363S and I559N of the *h-GR/NR3C1* gene (34-39). Fig. 4 illustrates de Finetti distributions of the investigated N363S polymorphism of the *h-GR/NR3C1* gene (34-39).

A lower frequency of the N363S polymorphism AA homozygote was observed in the healthy population (AA=78.57% in comparison with the group of bronchial asthma patients AA=81.62%). The GG homozygote was found to occur more frequently in cases than in controls (GG cases vs. GG controls 6.41 vs. 5.71%).

Table V. Distribution of N363S polymorphism allele frequency in bronchial asthma cases stratified according to disease severity.

	Chronic asthma			
	Mild	Moderate	Severe steroid-dependent	Severe steroid-resistant
AA, n (%)	25 (71.43)	96 (82.76)	62 (84.93)	8 (80.00)
AG, n (%)	6 (17.14)	14 (12.07)	7 (9.59)	1 (10.00)
GG, n (%)	4 (11.43)	6 (5.17)	4 (5.48)	1 (10.00)

Table VI. Distribution of N363S polymorphism allele frequency in bronchial asthma cases stratified according to disease severity and control of symptoms (Asthma Control Test).

	Asthma											
	Chronic mild			Chronic moderate			Chronic severe steroid-dependent			Chronic severe steroid-resistant		
	NC	PC	C	NC	PC	C	NC	PC	C	NC	PC	C
AA, n	14	10	1	51	37	8	47	15	0	7	1	0
AA, %	40.0	28.6	2.8	43.9	31.9	6.9	64.4	20.5	0	70.0	10.0	0
AG, n	3	3	0	6	8	0	4	1	2	0	1	0
AG, %	8.6	8.6	0	5.2	6.9	0	5.5	1.4	2.7	0	10.0	0
GG, n	2	2	0	1	4	1	4	0	0	0	1	0
GG, %	5.7	5.7	0	0.9	3.4	0.9	5.5	0	0	0	10.0	0

NC, non-controlled asthma according to Asthma Control Test (ACT); PC, partially controlled asthma according to ACT; C, well-controlled asthma according to ACT.

The population of patients with uncontrolled bronchial asthma and uncontrolled severe asthma demonstrated higher frequency of N363S SNP AA homozygote in comparison with the control group (87.50 vs. 78.57%). In the group of healthy participants of the study, a higher frequency of GG homozygote for the N363S polymorphism was observed in comparison with the group of patients with moderate to severe asthma with uncontrolled profile of clinical symptoms (5.71 vs. 4.16%). A statistically significant difference in the prevalence of alleles of the analyzed N363S polymorphism was observed (G vs. A: OR, 0.57; CI, 0.33-0.99; χ^2, 4.07; P=0.04; and A vs. G: OR, 1.72; CI, 1.01-2.95; χ^2, 4.07; P=0.04). Table VII and Fig. 5 illustrate the distribution of alleles for the N363S polymorphism of the *h-GR/NR3C1* gene in the group of patients with uncontrolled moderate asthma and uncontrolled severe asthma vs. controls. As estimated on the basis of *GR/NR3C1* N363S polymorphism frequency analysis using the χ^2 test, the genetic component - allele A of N363S SNP is associated with the risk of development of uncontrolled asthma, moderate to severe. The presence of allele G correlated with lower risk of development of the disease of uncontrolled moderate and severe asthma phenotype (AG vs. AA: OR, 0.47; CI, 0.22-1.00; χ^2, 3.90; P=0.04; AG+GG vs. AA: OR, 0.52; CI, 0.27-0.98; χ^2, 4.09; P=0.04).

The analysis of the investigated I559N polymorphism of the *h-GR/NR3C1* gene demonstrated the lack of any heterozygotes or mutants (genotype TA or AA). Only wild-type homozygotes (genotype TT) were observed. No correlations between I559N polymorphic variants of the *h-GR/NR3C1* gene in the case group and controls were found (P>0.05). No correlations of asthma course severity phenotype and the extent of control of the disease for the I559N polymorphism were demonstrated in the analyzed populations.

Discussion

Glucocorticosteroids constitute the basic group of anti-inflammatory drugs used in bronchial asthma. According to the guidelines developed by GINA, they are used in all patients with chronic asthma at the doses appropriate for the severity of symptoms. The response to GCS therapy depends on many factors. The key element, considering the control of asthma symptoms, is the patients' biochemical response to drugs determined by genetic variances of the hereditary component. Binding of GCS-GCR homodimer with the regulatory DNA sequences of the genes encoding proteins synthesized within the framework of cellular response to these drugs is dependent on AP-1 activity and AP-1 affinity to GCR, leading to enhancement of their interactions. The GCS-GCR complex can also bind to NF-κB. This leads to impairment of the antiinflammatory effect of GCS. It should be emphasized that the transcriptional factors bind to the AF_1 domain of GCR,

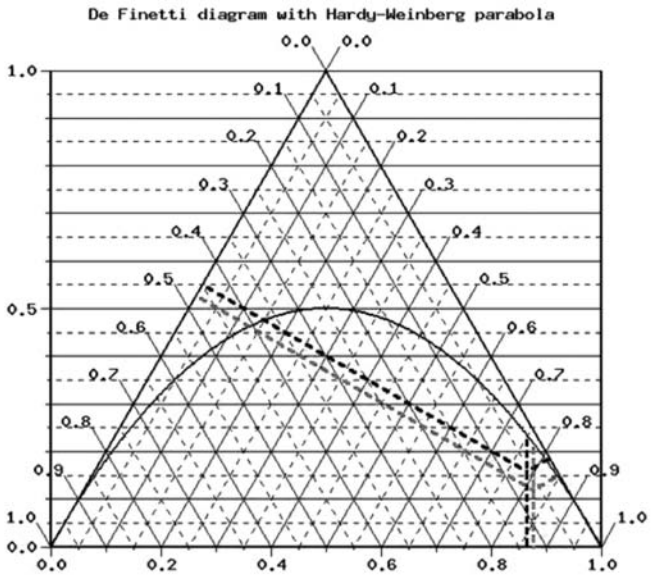

Figure 4. Description of diagram: black line, controls; gray line, cases. The de Finetti diagram with a Hardy-Weinberg parabola was drawn using free software provided by Institut für Humangenetik, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (Germany).

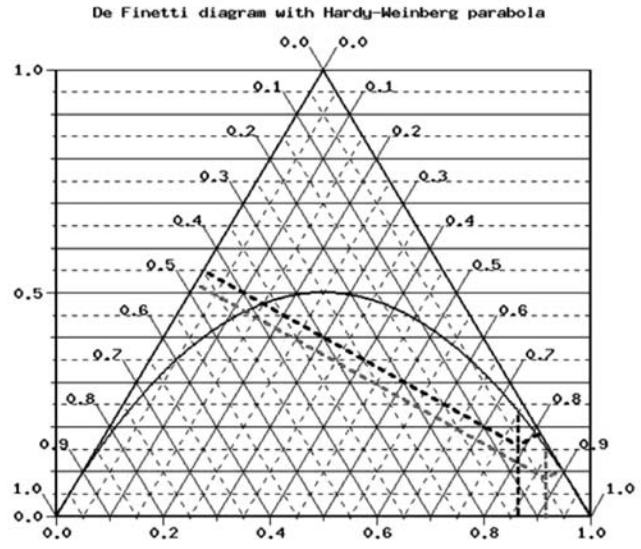

Figure 5. Description of diagram: black line, controls; gray line, cases (moderate and severe asthma, ACT score ≤19). The de Finetti diagram with a Hardy-Weinberg parabola was drawn using free software provided by Institut für Humangenetik, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (Germany).

Table VII. Tests for association (CI, 95% confidence interval) for the control and case groups (moderate and severe asthma, ACT score ≤19).

Tests for deviation from Hardy-Weinberg equilibrium		
	Controls	Cases
nAA	165 (156.87)	105 (100.83)
nAG	33 (49.26)	10 (18.33)
nGG	12 (3.87)	5 (0.83)
fa1	0.86±0.019	0.92±0.022
F	0.33014	0.45455
p (Pearson)	1.716×10^{-6}	6.382×10^{-7}
p (Llr)	0.000030	0.000156
p (Exact)	0.000030	0.000206

Tests for association (CI, 95% confidence interval)			
	Allele freq. difference	Heterozygous	Allele positivity
	G vs. A	AG vs. AA	AG+GG vs. AA
Risk allele G			
OR	0.57	0.47	0.52
CI	0.33-0.99	0.22-1.00	0.27-0.98
χ^2	4.07	3.90	4.09
p	0.04	0.04	0.04
	A vs. G	AG vs. GG	AA+AG vs. GG
Risk allele A			
OR	1.72	0.72	1.39
CI	1.01-2.95	0.20-2.56	0.47-4.05
χ^2	4.07	0.25	0.37
p	0.04	0.61	0.54

Tests for association (CI, 95% confidence interval) were performed using free software provided by Institut für Humangenetik, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (Germany). Partial modification by the authors. The tests for association were adapted from Sasieni (37). fa1, frequency of allele 1 ± standard deviation; F, inbreeding coefficient; p (Pearson), Pearson's goodness-of-fit χ^2 (degree of freedom, 1); p (Llr), log likelihood ratio χ^2 (degree of freedom, 1); p (Exact), exact test; n, number of patients.

whereas steroid receptor coactivator-1 protein (SRC-1), a part of the signaling cascades, binds to the AF_2 domain. The normal activity of coactivator proteins determines loosening of DNA strands and binding of RNA polymerase II. The effect of the GCS/GCR interaction with GRE may be a selective repression of specific inflammatory genes, induced by pleiotropic effects on specific promotor fragments of NF-κB genes and AP-1 activation complex. The *in vivo* experiments conducted by Huizenga *et al* (28) demonstrated a correlation of N363S polymorphism with increased sensitivity to glucocorticosteroids. The presence of the N363S variant did not alter the capacity of repression of target genes via negative the glucocorticoid-responsive elements or via the aforementioned AP-1 and NF-κB.

Our research has demonstrated and confirmed, that patients with uncontrolled inflammation phenotype and moderate or severe asthma are characterized by more frequent occurrence of the variant AA of the N363S polymorphism, associated with reduced cellular sensitivity to GCS in comparison with the control group. The population of patients with uncontrolled moderate to severe asthma demonstrated lower frequency of AG and GG variants of the N363S polymorphism, which

was associated with poorer response to anti-inflammatory treatment with GCS. The measurable consequence of loss of N363S polymorphic variants of the *h-GR/NR3C1* gene was loss of control of bronchial asthma symptoms, confirmed by the results of the ACT tests. The presence of codon 363 variants AG or GG correlates with better anti-inflammatory effects of GCS therapy and reduced risk of development of uncontrolled asthma (severe and moderate).

The analysis of I559N polymorphism did not demonstrate the occurrence of allelic variants in the studied population of healthy subjects and bronchial asthma patients. The results were verified and confirmed by sequencing. Thus, it is conceivable that I559N polymorphism is very rare and it should be rather regarded as a mutation. Obviously, the confirmation of this fact requires increased numerosity of the studied cohorts and multicenter analyses.

It is noteworthy that the group of patients with moderate and severe asthma was selected from among all bronchial asthma patients. Significant correlations were obtained only for the selected phenotype subgroup and not for the whole analyzed asthmatic population. Therefore, it should be concluded that the loss of N363S polymorphic alleles in the case group probably determines the moderate to severe asthma phenotype, and only its uncontrolled forms (ACT score ≤19). Thus, the RFLP marker for N363S SNP seems to have a clinical significance. It may help to create the profile of patients requiring more intensive anti-inflammatory treatment from the very beginning.

Considering the problem of GCS resistance in bronchial asthma patients, a comprehensive analysis whether it is a primary phenomenon (e.g. polymorphic variants of the *h-GR/NR3C1* gene), or a consequence secondary to effects of environmental factors, exacerbation of an allergic inflammatory process, a systemic inflammation of another etiology, use of high doses of β_2-agonists, a viral infection, or long-term glucocorticosteroid therapy should be attempted in each case. Resistance to steroids is sometimes also associated with receptor density reduction mechanism, which is observed in the case of administration of very high GCS doses for severe/difficult-to treat asthma.

In conclusion, the effect of the GCS-GCR complex interaction with GRE may be selective activation or repression of specific inflammation genes. An activating or inhibitory effect of GCS on protein synthesis is determined by interactions with GRE-positive, GRE-negative sequences, as well as with transcriptional factors (NF-κB or AP-1).

The latest reports point at the role of non-receptor transcriptional factors in the development of resistance to GCS therapy. AP-1 and NF-κB are the modulators of activity of numerous genes responsible for the development of inflammation; by binding to GCR, they block GCS-GCR binding with DNA sequences of the regulatory gene domains.

Our results indicate that N363S polymorphism is associated with an additional alteration in the *h-GR/NR3C1* gene, which determines some phenotypic chronic asthma traits. Polymorphism N363S correlates with increased sensitivity to GCS. The observed reduction of its occurrence in the case group correlates with the severity of inflammation and the phenotype of asthma characterized by the loss of control of the disease by means of treatment, as well as with the moderate to severe forms of asthma.

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