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The Effect of Polymorphisms of Beta2 Adrenoceptors on Response to Long-acting Beta2 Agonists in Iranian Asthmatic Patients

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ABSTRACT

The results of many studies suggested possible relationship between polymorphism at codons 16 and 27 and development of tolerance to beta-2 adrenoceptor agonist responses as well as disease severity in asthmatic patients. This study was designed to evaluate the effect of polymorphism of beta2 adrenoceptors on response to salmeterol and fluticasone (as inhaled Seretide).

Sixty-four patients with either mild or moderate-severe asthma were evaluated in this study. A four-week therapy with Seretide was conducted in moderate-severe asthmatics. The respiratory parameters and asthma score (based on GINA guidelines) were measured before and after run in period. Blood samples were genotyped at codons 16 and 27.

No significant difference was observed in genotypes neither at codon 16 nor at codon 27 between mild and moderate-severe asthma groups. However, Patients in Arg/Arg (n = 8) category showed significant improvement in asthma control parameters and lung function compared with Arg/Gly genotype (n =20).

These results suggest that genotyping may be useful in some asthmatic patients in order to better tailor asthma treatment plan.

Keywords: Asthma; Beta-adrenoceptor; Polymorphism

INTRODUCTION

The use of β2-agonists is an effective treatment for patients with persistent asthma.[1,2] β2 adrenoceptors (B2AR) are G protein-coupled receptors which have seven transmembrane-spanning α-helices.[3] The human

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B2ARs are polymorphic in their constitution. Some of the polymorphisms determine responsiveness to β2-agonists, whereas others may act as disease modifier [4]. To date, nine single nucleotide polymorphisms (SNPs) have been identified in human β2-adrenoceptor gene in which two polymorphisms, at codon 16, with substitution of Arg for Gly, and at codon 27, with substitution of Gln for Glu are more frequent.[5]

In vitro studies have shown that the receptor with Glycine amino acid at position 16 exhibits enhanced downregulation after exposure to β-agonists, but the data remain inconsistent.[6] Data analysis of clinical trials suggest that SNPs can influence the response to both short-acting and long-acting β2-agonists.[7-9] In contrast, some studies have reported no change in bronchodilator response to either short-acting [10] or long-acting β2-agonists [11] in asthmatic patients with varying B2AR genotypes.

On the other hand, B2AR polymorphisms have been shown to be associated with a variety of asthma-related phenotypes. This study was designed to evaluate the effect of genetic polymorphisms in the two mentioned positions of B2AR gene on individuals' response to a 4-weeks therapy with an inhaled preparation (Seretide) which consists of a long-acting B2AR agonist (salmeterol) along with a corticosteroid compound (fluticasone).

MATERIALS AND METHODS

Clinical Procedure

This study was performed based on ethnic guidelines for human studies issued by Shahid Beheshti University of Medical Sciences and was approved by local committee. Sixty-four patients who were admitted to Pulmonary Clinic at Masih Daneshvari Hospital entered the study and were classified based on Global Initiative for Asthma (GINA) guidelines[1, 12] as follows: thirty patients with moderate-to-severe asthma disease who entered a 4-week run-in period during which all participants received inhaled Seretide (50 µg salmeterol xinofoate and 250 µg fluticasone propionate) by evohaler twice a day and 34 subjects with the diagnosis of mild asthma. Patients in mild asthma group were not taking any regular medication (inhaled corticosteroids and long acting beta 2 agonists) for their asthma disease. Their treatment just included salbutamol as needed.

In the latter group, asthma was confirmed according to positive metacholine test results in 26 out of 34 patients (The test was not performed for the rest). Participants were excluded if they had any of the following conditions: diabetes mellitus, heart failure, chronic obstructive pulmonary disease (COPD), gastro-esophageal reflux, Churg-Strauss syndrome, bronchitis, occupational asthma, and lung infection within one month prior to the study procedure.

Also, patients with body mass index (BMI) over 30, smokers, and those who took angiotensin converting enzyme inhibitors (ACEIs), nonsteroidal anti-inflammatory drugs (NSAIDs) and β-blockers were not included in the study. Patients were advised to use salbutamol if acute asthma attacks occurred. Subjects who received Seretide were instructed to take the medication using a spacer[13] to make sure that all patients follow a standard procedure for drug administration. The respiratory parameters of FEV1, FEV/FVC, and asthma score were measured before and after run-in period, based on Global Initiative For Asthma.[12] Five factors were considered in the evaluation of asthma score: day time symptoms, limitation of activities, nocturnal symptoms/ awakening, salbutamol use, and how the patient rated his/her asthma control. Blood samples were collected and restored in -20˚C freezer for genotyping analysis.

Molecular Procedure

Genome Extraction and PCR Method

Genomic DNA was extracted from peripheral blood obtained using phenol chloroform method.[14] B2AR genotypes were determined by primer-induced restriction site assay.

The primers were selected according to previous study by Martinez et al [15] and were

5′-GCCTTCTTGCTGGCACCCCAT-3′ and 5′-CAGACGCTCGAACTTGGCCATG-3′

and then a PCR product which included the regions of β2AR-16 and the β2AR-27 polymorphisms were generated. PCR reactions were carried out in a volume of 50 µl containing 50 ng of genomic DNA, 10 mM Tris-HCl (PH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1.5 U of Taq polymerase, 0.2 mM of each deoxynucleotide triphosphate and 30 pm of each primer. Temperature cycling was 94˚C for 60 s. 60˚C for 60 s, 72˚C for 60s, for 44 cycles then a final extension for 7 min at 72˚C. The size of PCR product generated was 168 bp.

Polymorphism Detection

For detection of β2AR polymorphism, 15 µl of PCR product was digested with 1 U of NcoI (New England BioLabs), 2 µl PCR water, 2 µl of 50 mM NaCl, 10 mM Tris-HCl, 10mM MgCl2, 1 mM dithiothreitol (PH 7.9) at 37˚C for 12 h. NcoI cuts 22 bp from the 3'-end of both alleles and 18 bp from the 5'-end of the Gly-16 allele. The restriction digests were electrophoresed on 3% agarose gels and visualized with ethidium bromide staining and visualized by gel documentation. The Gln27Glu polymorphism was identified in a second restriction digest using another aliquot of the same PCR product. 12µl of the PCR product was digested with 2 U of BbvI (New England BioLabs), 5 µl PCR water, in 2 µl 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol (PH 7.9) at 37˚C for 12 h. BbvI digests the Gln-27 allele to produce 105- and 63-bp fragments which are separated from uncut Glu-27 alleles on 3% agarose gels.

Statistical Analysis

The primary hypothesis is that difference in genotype at either codon 16 or codon 27 produces different severity in asthma and different clinical response to Seretide during treatment of subjects with moderate-severe asthma. One-way ANOVA, Student's t-test or Mann-Whitney test was performed to test for difference among genotypes. Evaluation of the effect of genotype on asthma severity was performed using Chi-square or Fisher's exact probability test. *P*-values less than 0.05 were considered significant in all statistical analyses.

RESULTS

Baseline Distribution

Sixty four subjects completed the study protocol and blood samples were collected from them. Sixty two samples were successfully genotyped at position 16 and 27 (we did not able to genotype one sample at position 16 and one at 27). Due to small number of patients with Gly/Gly genotype (n=1), the related data was not considered in analysis. The prevalence of the alleles in all subjects combined were 0.32 for Gly16 (n = 62) and 0.71 for Gln27 (n = 61). Patients were divided into two groups; 30 subjects as moderate-severe asthma and 34 subjects as mild asthmatic. The genotypic distribution at position 16 had deviation from Hardy-Weinberg equilibrium (p=0.001), but allele frequency at codon 27 was consistent with the equilibrium.

Table 1. Demographic and baseline characteristics of subjects by Arg16Gly genotype

Characteritics	Genotype		*p*-value
	Arg/Arg	Arg/Gly	
Age, yr (mean±SD)	50.5±8.2	48.8±10.4	0.467[a]
Range	37-64	27-65	
Sex [n(%)]			
Male	2 (25%)	8 (40%)	0.593[b]
Female	6 (75%)	12 (60%)	
Lung function parameters			
FEV1 % predicted (mean±SD)	57.9±7.4	47.1±3.6	0.117
FEV1/FVC ratio	69.9	66.6	0.373
Asthma score parameters			
Day time symptoms (mean±SD)	4.37±0.3	3.85±0.3	0.648
Limitation of activities (mean±SD)	2.5±0.7	2±0.3	0.515
Nocturnal symptoms/ awakening (mean±SD)	4.25±0.5	3.25±0.4	0.100
Salbutamol use (mean±SD)	3.5±0.6 *	1.95±03	0.009
How patient rates his/her asthma control (mean±SD)	3.37±0.3 *	2.45±0.2	0.039
Total asthma score (mean±SD)	18±1.8	13.5±1.2	0.288

[a] Comparison was made using Fisher exact test.

[b] Comparison was made using independent *t*-test.

* $p<0.05$ significant difference compared to respective Arg/Gly group.

There was no significant difference in male/female ratio subjects between the two groups, however, patients in moderate-severe asthma group were significantly older than mild asthma group (49.3±9.6 and 30±8.2, respectively; $p<0.001$). Also, comparison of baseline demographic and pulmonary function characteristics between Arg16Gly genotypes in moderate-severe asthma group revealed no significant difference between groups, however, regarding clinical parameters, patients who fell in Arg/Arg category showed a significantly higher baseline need for reliever/rescue treatment and patient rating for asthma control (Table 1). The number of patients with Gly/Gly genotype was too small (n = 1) and it was not included in these analyses.

Asthma Severity and B2AR Genotypes

The B2AR allele frequencies for mild and moderate-severe asthmatic patients are mentioned in Table 2. The analysis indicated no significant difference in genotypes neither at codon 16 nor at codon 27 between mild and moderate-severe asthma groups.

Response to Therapy

The changes in respiratory outcomes and clinical symptoms from baseline were compared in the Arg16Gly and Gln27Glu subjects. During the treatment period, significant improvement in lung function and asthma score parameters were observed in subjects with Arg/Gly genotype.

Table 2. β2AR allele frequencies for mild and moderate-severe asthmatics

Type of asthma	Gly16					Gln27				
	N	Gly/Gly	Gly/Arg	Arg/Arg	Allele frequency	N	Glu/Glu	Glu/Gln	Gln/Gln	Allele frequency
Mild asthma	34	0 (0%)	20 (59%)	14 (41%)	0.30	33	2 (6%)	13 (39.4%)	8 (24.3%)	0.74
Moderate-severe asthma	28	0 (0%)	20 (71%)	8 (29%)	0.36	28	3 (10%)	13 (43%)	14 (47%)	0.68

Table 3. Change from baseline to the end of treatment period as well as difference between genotypes for each outcome in subjects treated with seretide in Arg/Arg and Arg/Gly subjects

Outcomes	Arg/Arg (n=8) Difference compared with baseline[a]	*P* value	Arg/Gly (n=20) Difference compared with baseline	*P* value	Arg/Arg vs Arg/Gly Difference between genotypes	*P* value
Lung function parameters[b]						
FEV1 % predicted	6 (11.90, 0.10)	0.047*	0.10 (19.08, 1.39)	0.026*	4.2 (-10.8, 19.3)	0.565
FEV1/FEC ratio	1.28 (6.50, -3.93)	0.570	3.44 (6.24, 0.63)	0.019*	2.2 (-3.0, 7.3)	0.393
Asthma score parameters [b,c]						
Day time symptoms	0.37 (1.26, -0.51)	0.351	1.05 (1.72, 0.38)	0.004**	0.7 (-0.5, 1.8)	0.240
Limitation of activities	1.00 (2.6, -0.61)	0.186	1.65 (2.42, 0.87)	<0.001***	0.7 (-0.8, 2.1)	0.380
Nocturnal symptoms	0.50 (1.76, -0.76)	0.381	1.40 (2.18, 0.62)	0.001**	0.9 (-5.0, 2.3)	0.197
Salbutamol use	1.13 (2.64, -0.38)	0.122	1.80 (2.57, 1.03)	<0.001***	0.7 (-0.8, 2.1)	0.348
Patient self-scoring	0.37 (1.00, -0.25)	0.197	1.75 (2.41, 1.09)	<0.001***	1.4 (0.3, 2.5)	0.015*
Total asthma score	3.38 (7.00, -0.25)	0.064	7.65 (9.69, 5.60)	<0.001***	4.3 (0.5, 8.0)	0.027*

[a] mean (95% confidence interval) of the difference

[b] comparison was performed using paired t-test

[c] asthma scores were graded by the subject from 0 for more severe symptoms to 5 for no symptoms

* $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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However, no significant improvement from baseline except in FEV1% was observed in lung function and asthma score parameters in Arg/Arg subjects at the end of treatment. Moreover, comparison between Arg/Arg and Arg/Gly genotypes revealed a significant difference in patient self-scoring (p=0.015) and total asthma score (p=0.027) changes before and after treatment with Seretide (Table 3).

As for codon 27, comparison of different genotypes revealed that patients with Gln/Glu showed improvement in all asthma score parameters. However, no improvement was observed in lung function parameters (FEV1% & FEV1/FVC). In patients with Gln/Gln lung function improvement was observed considering FEV1%.

Table 4. Change from baseline to the end of treatment period as well as difference between genotypes for each outcome in subjects treated with seretide in Gln/Gln, Gln/Glu and Glu/Glu subjects

	Gln/Gln (n=14)		Gln/Glu (n=13)		Glu/Glu (n=3)		Difference between genotypes	
Outcomes	**Difference compared with baseline[a]**	***P***	**Difference compared with baseline**	***P***	**Difference compared with baseline**	***P***	**Gln/Gln vs Gln/Glu Gln/Gln vs Glu/Glu Gln/Glu vs Glu/Glu**	***P***
Lung function parameters[b]								
FEV1 % predicted	6.5 (10.8, 2.3)	0.007 **	9.2 (23.1, -4.7)	0.171	1.9 (33.0, 5.7)	0.026	-2.6 (-18.4, 13.1) -12.8 (-36.8, 11.2) -10.2 (-34.2, 13.9)	0.907 0.390 0.547
FEV1/FEC ratio	2.6 (5.6, -0.4)	0.087	3.4 (8.0, -1.2)	0.128	3.0 (5.5, 0.5)	0.035 *	-0.8 (-6.6, 5.0) -0.4 (-9.4, -8.6) 0.4 (-8.7, 9.5)	0.933 0.993 0.993
Asthma score parameters [b,c]								
Day time symptoms	0.6 (1.2, 0.0)	0.088	1.3 (2.4, 0.2)	0.022 *	0	-[d]	-0.7 (-2.1, 0.6) 0.6 (-1.7, 2.8) 1.3 (-1.0, 3.6)	0.394 0.809 0.347
Limitation of activities	1.4 (2.5, 0.4)	0.010 *	1.3 (2.3, 0.3)	0.015 *	2.3 (7.5, -2.8)	0.192	0.1 (-1.6, 1.8) -0.9 (-3.7, 1.9) -1.0 (-3.8, 1.8)	0.982 0.699 0.636
Nocturnal symptoms	0.1 (0.3, -0.2)	0.583	2.1 (3.1, 1.1)	0.001 **	1.3 (7.1, -4.4)	0.423	-2.0 (-3.3, -0.7) -1.3 (-0.4, 0.9) 0.7 (-1.4, 2.9)	0.002 ++ 0.316
Salbutamol use	1.7 (2.8, 0.7)	0.004 **	1.5 (2.4, 0.5)	0.006 **	1.0 (5.3, -3.3)	0.423	0.3 (-1.4, 1.9) 0.7 (-2.0, 3.4) 0.5 (-2.2, 3.2)	0.666 0.921 0.788 0.906
Patient self-scoring	1.1 (1.9, 0.4)	0.007 **	1.8 (2.7, 0.8)	0.001 **	0.3 (3.2, -2.5)	0.667	-0.6 (-2.0, 0.7) 0.8 (-1.4, 3.1) 1.4 (-0.8, 3.7)	0.497 0.649 0.273
Total asthma score	4.9 (7.2, 2.7)	<0.001** *	7.9 (11.4, 4.5)	<0.001***	5.0 (12.5, -2.5)	0.102	-3.0 (-7.5, 1.5) -0.1 (-7.6, 7.4) 2.9 (-4.6, 10.5)	0.248 1.000 0.608

[a] Mean (95% confidence interval) of the difference.
[b] Comparison was performed using paired t-test.
[c] Asthma scores were graded by the subject from 0 for more severe symptoms to 5 for no symptoms.
[d] The p value cannot be computed because the standard error of the difference is 0.
[*] $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to baseline
[++] $P < 0.01$ significant difference between mentioned genotypes

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Moreover, the asthma score parameters except for day time and nocturnal symptoms (question 1 and 3, respectively) were also improved. Nocturnal symptoms (question 3) were significantly different (p=0.002) between these two genotypes after the treatment (Table 4).

DISCUSSION

Our data is the first results reflecting the pharmacogenetic characteristics of B2ARs in response to combination therapy of salmeterol and fluticasone in Iranian asthmatic patients. These results did not indicate a significant role of B2AR polymorphisms neither at Arg/Gly nor at Glu/Gln genotypes in the incidence of moderate/severe asthma. The Gly/Gly homozygote genotype was absent in the subjects with both mild and moderate/severe asthma who entered our study. This could suggest a relationship between Gly/Gly homozygote allele and asthma severity. Our results were not consistent with previous study which showed that Gly16/Gln27 haplotype was more prevalent in moderate/severe group compared to the group that had mild disease.[16] Moreover, Holloway and colleagues have shown that the Gly16 polymorphism may play a role in the pathogenesis of asthma severity, but they could not find any significant relationship between Gly16 polymorphism and development of asthma.[17] The differences between the results of present study and some mentioned previous studies possibly might be in part due to difference in inclusion criteria.

It should be noted that patients who fell in mild asthma group in our study were those with newly diagnosed asthma. As the disease progresseed, some individuals with mild asthma might fall in moderate-severe asthma group. Moreover, in some previous studies, mild asthmatic patients took inhaled corticosteroids (ICS) with medium doses[17,16] which was quite different from our inclusion criteria for mild asthmatics. Finally, many factors which could alter the net effect of B2AR polymorphism on asthma control were excluded in our study.

In this study, in a subset of asthmatic patients with Arg/Arg genotype, airway function and indices of asthma control did not improve with salmeterol/ICS use compared to patients with Arg/Gly genotype. One of the limitations in our study was the lack of Gly/Gly genotype which could affect our conclusion. However, according to prior studies, heterozygotes harboring the Arg/Gly genotype responded to a manner similar to that of Gly/Gly subjects to salmeterol therapy.[18,15] Several mechanisms have been suggested for such a difference in response to Long-acting beta agonists (LABAs) including difference in receptor downregulation between polymorphic variants of B2ARs,[19] genetic-specific difference in loss of bronchoprotection [20], difference in inflammatory cytokine expression[21] and finally effects on the signaling and function of other receptors that control airway contractility.[22] A significant decrease in the daily dose of salbutamol as well as in the frequency of nocturnal symptoms in asthmatic patients with Arg/Gly genotype and yet lack of such an effect in Arg/Arg genotype could suggest attenuation in the function of B2AR in patients with Arg/Arg genotype. Our results were consistent with those of previous studies which showed a genotype-related difference in response to SABAs.[8,7] However, our results were not consistent with the previous studies [23,24] conducted in a longer treatment period and on large number of asthmatic patients > 12 years of age which showed no significant pharmacogenetic effect of B2AR polymorphism on response to salmeterol in asthmatic patients. It is notable that in our study, only subjects who were between 18 and 65 years of age were assigned, which was based on some longitudinal epidemiological studies and clinical trials showing progression of asthma disease which varies in different age groups especially in children.[13] Also, only patients with same ethnic origin were entered in this study in order to eliminate the ethnic-specific pharmacogenetic differences, which could change the response of individuals to B2AR agonists [25,26]. Other confounding factors which could affect the results of the study such as obesity, the history of cardiovascular and gastrointestinal disease and the history of taking medication which might exacerbate asthma in patients (e.g. NSAIDs, non-selective beta-blockers) were excluded from our study. These factors could produce poor response to asthma control medications and decrease the asthma control level.[13]

Our data is consistent with the result of SOCS trial[18] in which patients with Arg/Arg genotype who took salmeterol without concurrent corticosteroid therapy showed significant difference in response to medication compared with Gly/Gly genotype. On the other hand, in SLIC trial [18], subjects with Arg/Arg genotype, appeared to initially benefit from addition of salmeterol to the medium dose of inhaled corticosteroid triamcinolone

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acetonide, but longer treatment caused deterioration in peak expiratory flow measurements in subjects with Arg/Arg genotype.

Our data was not consistent with the results of other study by Wechler et al.[27] in which patients who took 240 mcg beclomethasone (which is considered as medium dose of inhaled corticosteroid and equivalent to 500 mcg fluticasone used in our study) and 50 mcg salmeterol twice daily, showed no genotype related difference in response to salmeterol.

If we focus on treatment protocols, we could see that the type and dose of inhaled corticosteroid are important factors that could affect the response of individuals to LABAs.

There is evidence of effect of ICS in improvement of beta2-adrenoceptor mediated signaling, and increase in the receptor density.[28,29] Other studies reported reduction in functional desensitization of B2ARs by ICS.[30] Accordingly, such effects could be related to the chemical structure of ICS, so different ICS might produce different effects in this regard.

As for codon 27, a significant improvement was observed in %FEV1, limitation of activities, the number of salbutamol use per week, patient self-scoring and total asthma score in patients with Gln/Gln genotype compared with baseline values. In addition, patients with Glu/Gln genotype showed significant difference in all asthma score parameters. Patients with Glu/Glu genotype showed significant difference in FEV1 as well as FEV1/FVC ratio compared to baseline values which should be further studied due to small sample size (n=3) of this group. The results suggest that polymorphism at codon 27 has less important role than codon 16 in the response of asthmatic patients to combination of salmeterol and ICS therapy. However, improvement of some asthma score parameters such as day time and nocturnal asthma symptoms might be under influence of polymorphism at codon 27.

Taken together, the present study suggests no relationship between asthma severity and polymorphism at codon 16 and codon 27. However, asthmatic patients with different genotyping at codon 16 showed significantly different responses to LABAs. Such changes in response to LABAs were not clearly observed in patients with different genotypes at codon 27, which could be due to short period of trial and/or small number of patients with Gln/Glu genotype.

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