Study the Possible Role of β_2 Adrenergic Receptor Gene in the Pathogenesis of Bronchial Hyperresponsiveness in Asthmatic Patients and its Relation to Disease Severity and Treatment Response

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Abstract: Background and objectives: Several identified β_2-adrenergic receptor (β_2AR) gene polymorphisms, including the amino acid substitution from arginine (Arg) to glycine (Gly) at codon 16 and from glutamine (Gln) to glutamic acid (Glu) at codon 27, are linked with functional changes in the β_2AR in the respiratory system. The objective of this study was to investigate the association between single nucleotide polymorphisms (SNPs) in β_2AR gene in asthmatic patients with bronchial hyperresponsiveness (BHR), asthma severity, and response to inhaled long acting β_2 agonists. **Methods:** This case-control association study involved 60 patients with asthma and 60 healthy subjects. Thirty asthmatics patients of them received inhaled long acting $\beta_2$agonists. The β_2AR gene polymorphisms at codon 16 and 27 were assessed on the genomic DNA obtained from the whole blood. Genotyping was carried out by a PCR based restriction fragment length polymorphism technique. **Results:** 1- The combined genotype Gly-Gly/Gln-Glu was positively associated with BHR ($P < 0.05$). 2- No association between β_2AR polymorphism at codon 16 and asthma severity was observed. However; homozygous Gln27 genotype was significantly associated with severe asthma either individually or when combined with homozygous Gly16. In contrast; Glu 27 was found to reduce asthma severity as a significant negative association was observed between Gln-Glu, Gly-Gly/Gln-Glu and clinical severity score, and between Gly-Gly/Gln-Glu and the airway reactivity score ($P < 0.05$). 3- Glu27Glu genotype had significant positive association with the improvement in BHR (assessed by percentage of change in $PD_{20\text{-}FEV1}$) after long acting β_2 agonist administration for 14 days ($P <0.05$). **Conclusions:** β_2AR gene can confer genetic susceptibility to BHR and the combinations of β_2AR genotypes are more informative than individual SNPs in their predictive power. Polymorphisms in β_2AR gene are important in the modulation of asthma severity and response to β_2 agonist. The predetermination of single β_2AR genotypes might have a utility for predicting long-acting $\beta_2$agonist response.

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1. Introduction

Asthma is a complex disorder characterized by both phenotypic and genotypic heterogeneity. Many genes are involved in the pathogenesis of asthma, and none of these genes has an effect on susceptibility that is strong enough to emerge as a major contributor in asthma [(1)].The strategy for identifying candidate genes offers opportunities to further specify persons at increased risk for susceptibility to bronchial hyperreactivity, severity of asthma symptoms, and response to therapy[(2)].

Bronchial hyperresponsiveness (BHR) is a cardinal feature of asthma, and one of the strategies in the dissection of genetic risks of asthma is to investigate genes that contribute to this intermediate phenotype to give further insight into the causes and mechanisms of the disease. The severity of BHR generally correlates with the severity of asthma [(3)]. No single causative defect has been reported to underlie the phenomenon of BHR.

There are many reasons to evaluate β_2AR gene as a candidate gene in asthma including clinical and laboratory functional data which showed that β_2AR dysfunction is a hallmark of asthma. Thus β_2 adrenergic receptor gene is an attractive candidate gene for asthma, particularly within families in which a genetic cause of asthma is suspected. It has been classified as a gene involved in lung function, airway remodeling, and disease severity [(4)].

The β-adrenoceptor is a member of the seven-transmembrane family of receptors related to bacteriorhodopsin, which was used for the early structural work [(5)]. β_2ARs are present in increased numbers on asthmatic airway smooth muscle.

β_2AR activation promotes bronchodilatation through β_2AR agonists that can act on the receptor to rapidly reverse bronchoconstriction by mediating

relaxation of the smooth muscle and resulting in bronchodilation[6].

Small changes in the amino acid sequence including single amino acid substitutions in the β_2AR gene, single nucleotide polymorphisms (SNPs), could result in significant alterations in receptor function. The main clinical interest in these polymorphisms lies in the possibility that they may determine the extent to which the receptor is down regulated in the airways and as such may modify bronchodilator responses through changes in the expression and coupling of $\beta_2$receptors in airway cells[4]. Two of these polymorphisms have now been studied in some details. The initial studies focused[7] on amino acid 16, which can be either arginine (Arg) or glycine (Gly), depending on whether base 46 is A or G. The second polymorphism is at codon27, which exists as either glutamine (Gln) or as glutamate (Glu), depending on whether base 76 is C or G.

It is well known that asthma severity is related to environmental exposures, such as chemicals or allergens. However, recent research suggests that genetic factors may play a role in determining asthma severity, with conflicting results as regards the association between β_2 polymorphism and the severity of asthma

Among the anti asthma therapies available today, β_2AR agonists are the most commonly prescribed therapeutic agents for the management of asthma[8]. It is the most effective bronchodilators used for the relief of symptoms in patients with asthma [9]. β_2 Agonists are able to relax airway smooth muscle. Long acting β_2 agonists (LABA), such as salmeterol and formoterol, improve asthma control, reduce asthma exacerbations and provide long term protection against bronchoconstrictor stimuli [10]. Long acting β_2 agonists are introduced at an earlier point in asthma management and can be given in combination with an inhaled corticosteroid.

β_2 adrenoceptor agonists are used by virtually all asthmatic patients as rescue bronchodilator [11]. Long acting β_2 agonists exhibit protective effects against a variety of direct and indirect bronchoconstrictor stimuli. However, regularly scheduled treatment with β_2 agonists is associated with tachyphylaxis to the functional antagonism against bronchoconstrictor stimuli.

In the early 1990s, regularly scheduled LABA use was found to worsen asthma control,[12] but a similar study with the beta-agonist in mild asthma found no harmful effects[13].

Bronchial asthma is a heterogeneous disorder, but even in patients with an apparently identical clinical phenotype, response to drug treatment may be remarkably variable. It is common for some patients to respond in a salutary fashion to a given treatment while others fail to manifest such a response. The basis for this variable treatment response is not known with certainty, but there is good reason to believe that a significant component of the variability is genetic in nature [14].

β-agonists were known to exert their effects through the β_2-adrenergic receptor gene, thus this gene was investigated in the present study to examine whether phenotypic outcomes including bronchial hyperresponsiveness, the severity of the disease, and the response to inhaled long acting β_2 agonists were related to polymorphisms of β_2AR gene.

2. Materials and Methods:

Subjects: The study sample consisted of sixty patients (52 females and 8 males), with age ranged between 18–54 years old. They were previously diagnosed with asthma as defined by American Thoracic Society [15]. All patients attended to the Clinical Physiology Unit in the Medical Research Institute for asthma assessment and management in the period from December 2009 to April 2010. At the time of the study, all subjects were asymptomatic and in a clinically stable condition. None had had respiratory infections in the month proceeding to the study. All patients were free from parasitic infections, and they were non smokers. Inhaled long acting bronchodilators, oral bronchodilators, and corticosteroid medications were withheld during the 14 days prior to the study. Thirty asthmatic patients of the sixty received a long acting beta agonist (12 µg formoterol fumarate) twice daily for fifteen days, after which clinical severity and airway reactivity were reevaluated. The pulmonary function and methacholine challenge tests were also repeated.

Control: This group comprised of sixty healthy volunteers (47 females and 13 males), with age ranged between 18 – 55 years old. All of them were non smoker, apparently healthy with no history of asthma or any other allergic diseases. None of them had displayed symptoms of respiratory infection in the 2 months preceding the study. They had no family history of asthma and/or atopic diseases. All were free from parasitic infection. Pulmonary function tests revealed no abnormalities. Controls were not receiving any form of medication between the start and conclusion of the study.

Assessment for asthma severity

Airway reactivity was assessed using a questionnaire about the effects of 22 nonspecific inhaled irritants. Each subject was asked, “Is your asthma worsened by, or does the following produce wheezing, chest tightness, and/or coughing. All irritants on the list are substances participants were likely to encounter in their day to day environment. The numbers of positive responses were recorded as

the patients' airway reactivity score. A score of zero indicated that all responses were negative; a score of 22 indicated that all responses were positive [16].

Clinical severity score:

For each patient, disease severity was quantitatively determined by summing the value of nine parameters describing clinical features of asthma regularly present during the month preceding the study [16]. Each parameter was scored on a 5 point Linsker scale in which the highest and lowest values represented maximum and minimum severity.

Patients with scores (up to 21, up to 33, and more than 33) were considered as indicative of mild, moderate and severe asthma; respectively.

Pulmonary function tests:

Pulmonary flow rates were measured using a computerized dry spirometer (JAEGER GmbH, Hoechberg, Germany) with automatic dosimeter. Spirometry measurements included forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC%, forced expiratory flow at 25% ($FEF_{25\%}$), forced expiratory flow at 50% ($FEF_{50\%}$), forced expiratory flow at 75% ($FEF_{75\%}$) of FVC, and maximum mid expiratory flow rate (MMEF).

All measurements were performed adhering to the European Respiratory Society & American Thoracic Society protocol for spirometry standardization [17].

For each subject; at least three artifact-free forced expiratory curves with an acceptable start of test were recorded. For each test; the spirometer software automatically selected the best-forced expiration and calculated and displayed measured values, represented as percentages of the predicted values based on age, sex, weight and height [17].

Methacholine inhalation challenge

Bronchial hyperresponsiveness was assessed using methacholine inhalation challenge test. It was performed, for controls, asthmatic patients and after LABA treatment, using the five-breath dosimeter protocol according to guidelines recommendation of American Thoracic Society for methacholine inhalation challenge test [18]. Methacholine dose response curves were plotted. The results were represented as the percent decrease in FEV_1 from baseline associated with different concentrations of methacholine ($PD_{20\text{-}FEV1}$). Cases in which the reduction in FEV_1 was less than 20% below baseline with the highest dose of methacholine were recorded as negative responses.

Genotyping

Genomic DNA was extracted from peripheral blood using the DNA modified salting out procedure [19]. β_2AR polymorphisms (Arg16Gly), and (Gln27Glu) were determined by direct sequencing of PCR products obtained with the following gene-specific primers.

Forward primer: 5'- GCC TTC TTG CTG GGC ACC CAT-3';

Reverse primer: 5'-CAT ACG CTC GAA CTT GGC CAT C-3'.

DNA amplification was performed in a Thermohybraid PCR Express Thermalcycler, with a total reaction volume of 25 µl containing:

Milli Q water: 12.5 µl
DNA: 300 ng
Forward primer (Bioron): 20 Pico moles
Reverse primer (Bioron): 20 Pico moles
PCR master mix: 12.5 µl

- Amplification was performed using initial denaturation at 95°C for 3 minutes followed by 35 cycles of 94° C for 30 seconds, 66°C for 30 seconds, and 72°C for 30 seconds with a final extension of 70°C for 6 minutes.
- Following amplification, 9 µl of the PCR product were mixed with 2 µl of 6 x loading buffer (0.09 % bromophenol blue, 0.09 xylene cyanol, 60 Mm EDTA in 60% glycerol) and loaded on agarose gel 2% (containing ethidium bromide 20 ng/ µl).
- 5 µl of 50 bp ready to use DNA ladder (MBI Fermentas) was loaded in a separate lane. Products were visualized on a UV Transilluminator.

Restriction digestion was performed using the NcoI (MBI Fermentas) for Arg16Gly and the BbvI restriction enzyme (MBI Fermentas) for Gln27Glu[20].

Statistical analysis

Statistical analysis was performed using SPSS version 17 Chicago, IL, USA for Windows. Mean values for different variables of controls and asthmatic patients were compared using the t test for independent samples. Mean values for asthmatic patients pre and post LABA treatment was compared using a paired two tailed t-test. Alleles frequencies were estimated and the genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium [21]. Codon16/Codon 27 revealed 9 different combinations. Association between specific genotypes and patients/controls status were tested using 2×2 contingency tables comparing individual genotype against a pool of all other genotypes. Significance was measured using the chi square test. Reported results include odds ratios and 95% confidence intervals. In statistical tests alpha was set to 0.05. Results with $P \leq 0.05$ were considered to be statistically significant.

3. Results:

The anthropometric data of patients and controls are presented in table (1). There were no significant difference between patients and control groups as regards the age, weight, and height.

Table (1): Anthropometric data of patients and controls: (mean ± SD).

	Age	Weight	Height
Patients (n=60) (mean ± SD)	35 ± 9	81 ± 16	163 ± 7
Controls (n=60) (mean ± SD)	34 ± 10	79 ± 16	164 ± 8
P	> 0.05	> 0.05	> 0.05

Severity of the disease:

Airway reactivity score: The airway reactivity scores in the 60 asthmatic patients ranged between 3–22 with a mean (14±5). However; for asthmatic patients groups who had received inhaled long acting β_2 agonist treatment, the airway reactivity score post treatment was significantly decreased as compared to their corresponding values pre treatment ($P < 0.05$).

Clinical severity score: The clinical severity scores in the asthmatic patients ranged between 9–39 with a mean (24 ±7). According to the clinical severity score, the asthmatic patients were classified into mild (24, 40%), moderate (29, 48%), and severe asthma (7, 12%).

For asthmatic patients groups who had received inhaled long acting β_2 agonist treatment, the clinical severity score was significantly decreased as compared to their corresponding values pre treatment ($P< 0.05$).

Post LABA treatment, the proportion of patients classified as having mild asthma increased from 7 (23%) to 23 (77%); moderate asthma fell from 18 (60%) to 7 (23%); and severe asthma fell from 5 (17%) to zero.

Pulmonary function tests

Pulmonary function tests in patients and control groups are presented in table (2) as percent predicted values. There were significant differences between asthmatic patients and control groups in all pulmonary flow rates measured.

For asthmatic patients subgroups who had received inhaled long acting β_2 agonist treatment the pulmonary flow rates measured were significantly increased post LABA treatment.

Table (2): Pulmonary function tests:

	Patients & Controls			Patients subgroup*		
	Patients (n=60)	Control (n=60)	*P* value	Pre Treatment (n=30)	Post Treatment (n=30)	*P* value
FVC	88 ± 12[a]	96 ± 14	$P < 0.05$	87± 10	92 ± 9[a]	$P < 0.05$
FEV_1	86 ± 10[a]	100 ± 13	$P < 0.05$	85± 8	90 ± 8[a]	$P < 0.05$
FEV_1/FVC%	85 ± 10[a]	92 ± 6	$P < 0.05$	84 ± 8	89 ± 8[a]	$P < 0.05$
$FEF_{25\%}$	74 ± 16[a]	91 ± 17	$P < 0.05$	71 ± 15	81 ± 14[a]	$P < 0.05$
$FEF_{50\%}$	71 ± 19[a]	98 ± 17	$P < 0.05$	67 ± 17	78 ± 21[a]	$P < 0.05$
$FEF_{75\%}$	72 ± 32[a]	108 ± 38	$P < 0.05$	66 ± 25	79± 32[a]	$P < 0.05$
MMEF	71 ± 22[a]	100 ± 23	$P < 0.05$	67 ± 17	79 ± 22[a]	$P < 0.05$

[a] statistically significant difference $P <0.05$

* Including only patients who received inhaled long acting $\beta_2$agonist (n=30)

FVC: Forced vital capacity, FEV_1: Forced expiratory volume in one second, $FEF_{25\%}$: Forced expiratory flow at 25%, $FEF_{50\%}$:Forced expiratory flow at 50%, $FEF_{75\%}$:Forced expiratory flow at 75% of FVC, MMEF: Maximum mid expiratory flow rate.

Methacholine inhalation challenge:

All controls responded negatively to the methacholine inhalation challenge test. For the asthmatic patients the $PD_{20\text{-}FEV1}$ ranged between 0.0003 – 0.3599 mg with a mean value of 0.0319±0.06. For asthmatic patients subgroups who had received inhaled long acting β_2 agonist treatment the $PD_{20\text{-}FEV1}$ was significantly improved after LABA. Two patients out of thirty responded negatively to the methacholine inhalation challenge.

3. Molecular studies

The distribution of alleles, and genotypes frequencies of β_2AR gene at codon16 and codon27 among patients and control groups were not deviated from Hardy-Weinberg equilibrium, and showed no significant difference ($P > 0.05$). (Table 3).

Table (3):β_2AR polymorphisms distributions among patients and control:

	Allele/ Genotypes	Patients (n=60)	Controls (n=60)	χ^2	P	OR	95% CI
Alleles	Gly	70 (58%)	66 (55%)	0.27	0.602	1.145	0.687 – 1.909
	Arg	50 (42%)	54 (45%)			0.873	0.524 – 1.455
	Glu	56 (47%)	62 (52%)	0.6	0.439	0.819	0.493 – 1.359
	Gln	64 (53%)	58 (48%)			1.222	0.736 –2.028
Codon 16	Gly-Gly	19(32%)	12 (20%)	2.131	0.144	1.85	0.81 – 4.27
	Arg-Gly	32(53%)	42(70%)	3.525	0.06	0.49	0.231 – 1.037
	Arg-Arg	9 (15%)	6 (10%)	0.686	0.41	1.59	0.53 – 4.78
Codon 27	Glu-Glu	9 (15%)	8 (13%)	0.069	0.793	1.147	0.41 – 3.206
	Gln-Glu	38 (63%)	46 (77%)	2.54	0.111	0.526	0.237 – 1.165
	Gln-Gln	13 (22%)	6 (10%)	3.06	0.08	2.489	0.877 – 7.07
Codon 16/ Codon 27	Arg-Gly/Gln-Glu	24 (40%)	32 (54%)	2.143	0.143	0.583	0.283 – 1.203
	Arg-Gly/Gln-Gln	5 (8.5%)	5 (8%)	0.00	1.0	1.0	0.274 – 3.650
	Arg-Gly/Glu-Glu	3 (5%)	5 (8%)	0.536	0.464	0.579	0.132 – 2.54
	Gly-Gly/Gln-Glu	9 (15%)	8 (13%)	0.069	0.793	1.147	0.41 – 3.206
	Gly-Gly/Gln-Gln	6 (10%)	1 (2%)	3.793	0.051	6.556	0.764 – 56.22
	Gly-Gly/Glu-Glu	4 (7%)	3 (5%)	0.152	0.697	1.357	0.29 – 6.341
	Arg-Arg/Gln-Glu	5(8.5%)	6 (10%)	0.1	0.752	0.818	0.236 – 2.841
	Arg-Arg/Gln-Gln	2 (3%)	0 (0%)	–	–	–	–
	Arg-Arg/Glu-Glu	2 (3%)	0 (0%)	–	–	–	–

The distribution of genotypes frequencies of β_2AR gene at codon16, codon 27, and codon16/codon27 in mild, moderate and severe patients presented in tables (4-6), Figures(1-3).

Table (4): Distribution of genotypes frequencies of β_2AR gene at codon16, codon27, and codon16/codon 27 among mild patients and controls:

	Genotype	Mild (n=24)	Control (n=60)	χ^2	*P*	OR	95% CI
Codon 16	Gly-Gly	8 (33%)	12 (20%)	1.68	0.195	2	0.694–5.764
	Arg-Gly	12 (50%)	42(70%)	2.99	0.08	0.429	0.162–1.133
	Arg-Arg	4 (17%)	6 (10%)	0.726	0.394	1.8	0.46–7.05
Codon 27	Glu-Glu	2 (8%)	8 (13%)	0.41	0.523	0.591	0.166–3.01
	Gln-Glu	17 (71%)	46 (77%)	0.311	0.577	0.739	0.255–2.143
	Gln-Gln	5 (21%)	6 (10%)	1.768	0.184	2.368	0.647–8.663
Codon 16/ Codon 27	Arg-Gly/Gln-Glu	8 (33%)	32 (53%)	2.75	0.097	0.438	0.163–1.176
	Arg-Gly/Gln-Gln	2 (8.5%)	5 (8%)	0.0	1	1	0.18–5.54
	Arg-Gly/Glu-Glu	2 (8.5%)	5 (8%)	0.0	1	1	0.18–5.54
	Gly-Gly/Gln-Glu	7 (29%)	8 (13%)	2.93	0.087	2.676	0.845–8.48
	Gly-Gly/Gln-Gln	1 (4%)	1 (2%)	0.461	0.497	2.565	0.154–42.75
	Gly-Gly/Glu-Glu	0	3 (5%)	–	–	–	–
	Arg-Arg/Gln-Glu	2 (8.5%)	6 (10%)	0.06	0.814	0.818	0.153–4.37
	Arg-Arg/Gln-Gln	2 (8.5%)	0 (0%)	–	–	–	–
	Arg-Arg/Glu-Glu	0 (0%)	0 (0%)	–	–	–	–

Table (5): Distribution of genotypes frequencies of β_2AR gene at codon16, codon27, and codon16/codon 27 among moderate patients and controls:

	Genotype	Moderate (n=29)	Controls (n=60)	χ^2	*P*	OR	95% CI
Codon 16	Gly-Gly	8 (28%)	12 (20%)	0.65	0.422	1.524	0.543–4.27
	Arg-Gly	16 (55%)	42(70%)	1.89	0.169	0.527	0.211–1.319
	Arg-Arg	5 (17%)	6 (10%)	0.95	0.331	1.875	0.521– 6.75
Codon 27	Glu-Glu	6 (21%)	8 (13%)	0.798	0.372	1.696	0.528–5.45
	Gln-Glu	18 (62%)	46 (77%)	2.062	0.151	0.498	0.191–1.3
	Gln-Gln	5 (17%)	6 (10%)	0.95	0.331	1.875	0.521–6.75
Codon 16/ Codon 27	Arg-Gly/Gln-Glu	13 (45%)	32 (53%)	0.57	0.452	0.711	0.292–1.73
	Arg-Gly/Gln-Gln	2 (7%)	5 (8%)	0.06	0.813	0.815	0.148 –4.475
	Arg-Gly/Glu-Glu	1 (4%)	5 (8%)	0.74	0.389	0.393	0.044–3.53
	Gly-Gly/Gln-Glu	2 (7%)	8 (13%)	0.81	0.367	0.481	0.096–2.43
	Gly-Gly/Gln-Gln	3 (10%)	1 (2%)	3.43	0.064	6.808	0.676–68.564
	Gly-Gly/Glu-Glu	3 (10%	3 (5%)	0.89	0.346	2.192	0.414–11.601
	Arg-Arg/Gln-Glu	3 (10%)	6 (10%)	0.003	0.96	1.038	0.241–4.48
	Arg-Arg/Gln-Gln	0 (0%)	0 (0%)	–	–	–	–
	Arg-Arg/Glu-Glu	2 (7%)	0 (0%)	–	–	–	–

The distribution of genotypes frequencies of β_2AR gene at codon16, codon27, and codon16/codon27 among severe patients and control groups showed that Gln-Gln, and Gly-Gly/Gln-Gln genotypes frequencies distribution were significantly increased in severe patients [(χ^2=5.82, *P*< 0.05), and (χ^2=10.61, *P*< 0.05); respectively] when compared to control group.

Table (6): Distribution of genotypes frequencies of β_2AR gene at codon16, codon27, and codon16/codon27 among severe patients and controls:

	Genotype	Severe (n=7)	Controls (n=60)	χ^2	*P*	OR	95% CI
Codon 16	Gly-Gly	3 (43%)	12 (20%)	1.89	0.17	3	0.591 – 15.24
	Arg-Gly	4(57%)	42(70%)	0.48	0.488	0.571	0.116 – 2.818
	Arg-Arg	0 (0%)	6 (10%)	–	–	–	–
Codon 27	Glu-Glu	1 (14%)	8 (13%)	0.005	0.94	1.083	0.115 – 10.22
	Gln-Glu	3 (43%)	46 (77%)	3.65	0.056	0.228	0.046 – 1.144
	Gln-Gln	3 (43%)	6 (10%)	5.82	0.016	6.75	1.2 – 37.63
Codon 16/ Codon 27	Arg-Gly/Gln-Glu	3 (43%)	32 (53%)	0.28	0.6	0.656	0.135 – 3.188
	Arg-Gly/Gln-Gln	1 (14%)	5 (8%)	0.27	0.602	1.833	0.183 – 18.41
	Arg-Gly/Glu-Glu	0 (0%)	5 (8%)	–	–	–	–
	Gly-Gly/Gln-Glu	0 (0%)	8 (13%)	–	–	–	–
	Gly-Gly/Gln-Gln	2 (29%)	1 (2%)	10.61	0.001	23.6	1.81 – 307.79
	Gly-Gly/Glu-Glu	1 (14%)	3 (5%)	0.96	0.326	3.167	0.283 – 35.42
	Arg-Arg/Gln-Glu	0 (0%)	6 (10%)	–	–	–	–
	Arg-Arg/Gln-Gln	0 (0%)	0 (0%)	–	–	–	–
	Arg-Arg/Glu-Glu	0 (0%)	0 (0%)	–	–	–	–

Figure (1): The distribution of genotypes frequencies of β_2AR gene at codon16 among asthmatic patients and control groups.

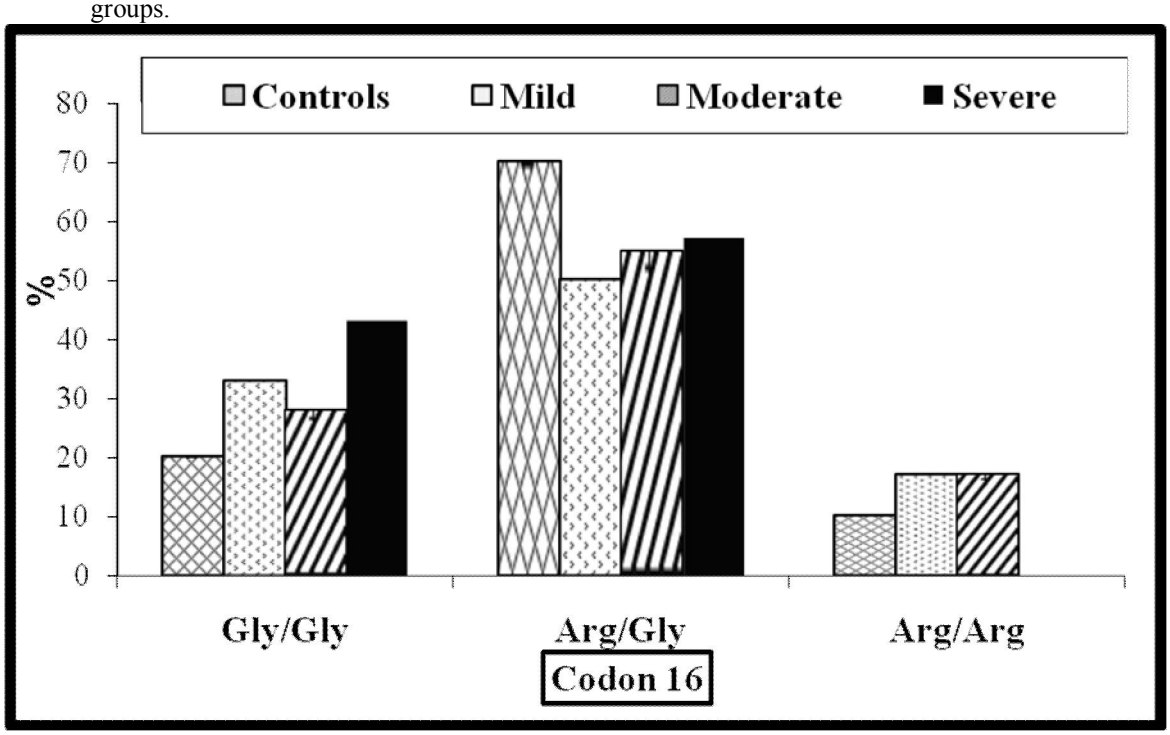
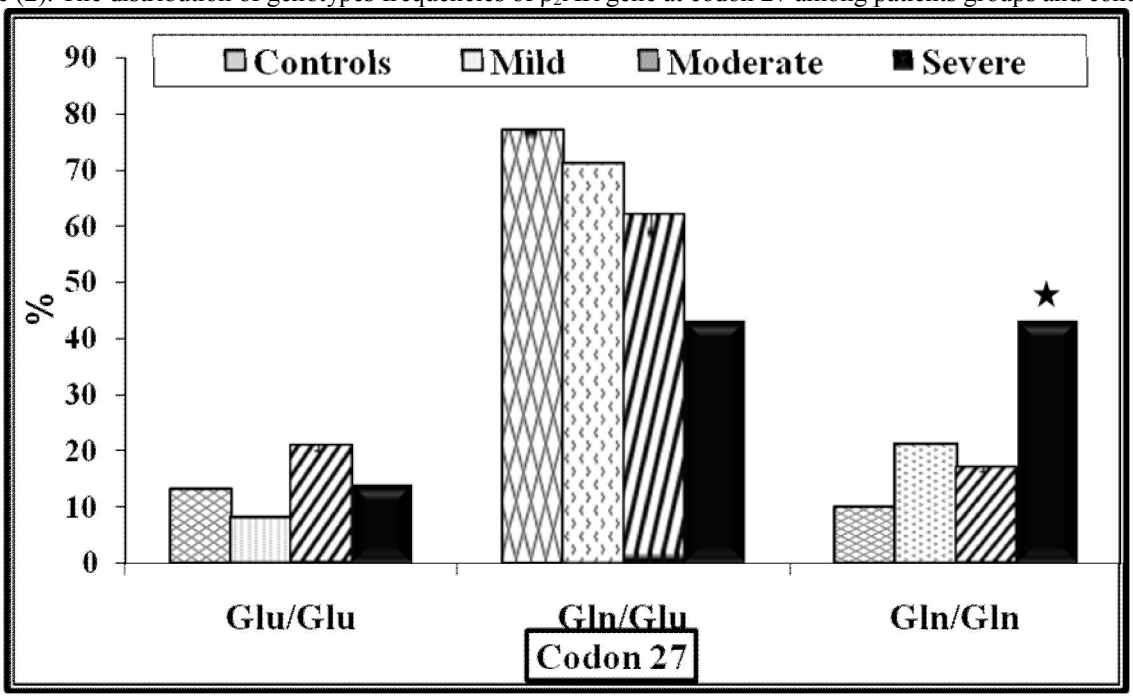
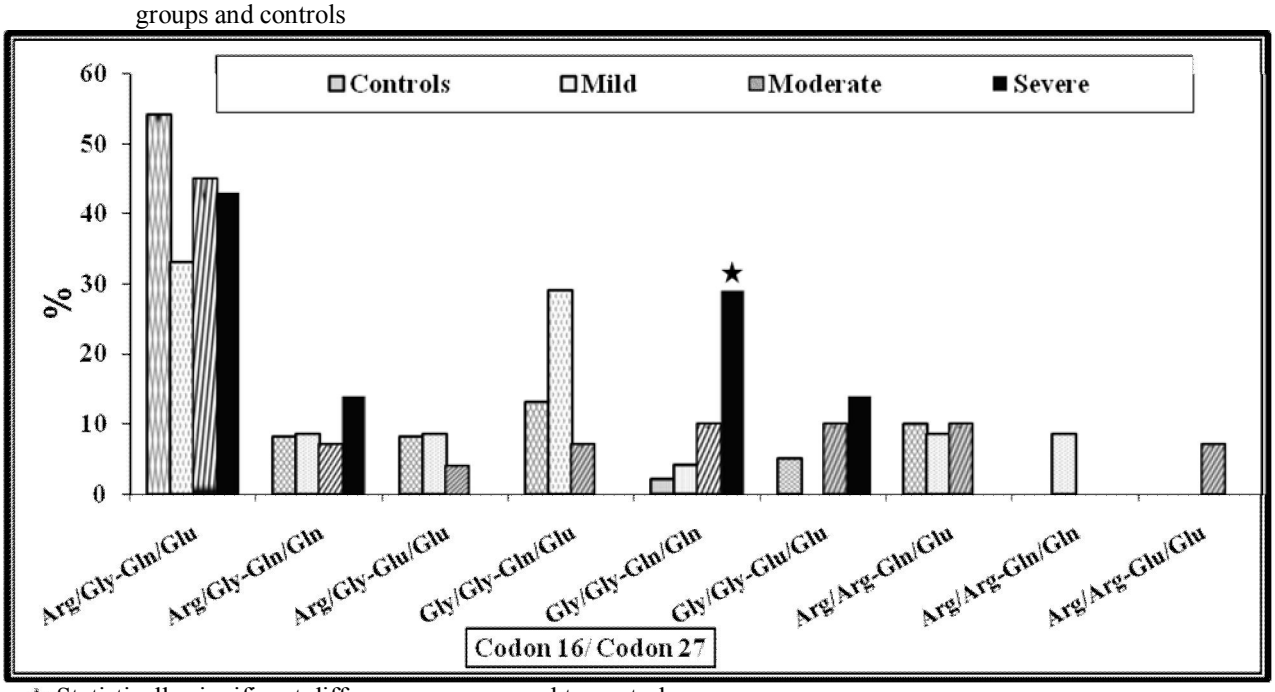


Figure (2): The distribution of genotypes frequencies of β_2AR gene at codon 27 among patients groups and control.



★ Statistically significant difference as compared to control group.

Figure (3): The distribution of genotypes frequencies of β_2AR gene at codon16/codon27 among asthmatic patients groups and controls



★ Statistically significant difference as compared to control group.

Association of β_2AR gene with bronchial hyperresponsiveness.

Gly-Gly/Gln-Glu genotype was significantly correlated to the provocational dose of methacholine that causes 20% reduction in FEV_1 ($PD_{20\text{-}FEV1}$). (χ^2 = 4.9, *P*=0.028).

Association of β_2AR gene with disease severity.

A significant positive association was found between Gly-Gly/Gln-Gln genotype and clinical severity score (χ^2= 5.5, *P* =0.019).

A significant negative association was found between Gln-Glu, and Gly-Gly/Gln-Glu genotypes and clinical severity score (χ^2 =3.9, *P* =0.048), and (χ^2= 9.2, *P* =0.002); respectively.

A significant negative association was found between Gly-Gly/Gln-Glu genotype and airway reactivity score (χ^2= 4.5, *P* =0.035)

Association of β_2AR gene with the response to treatment.

A significant positive association was found between Glu-Glu genotype and the percent of change in the provocational dose of methacholine that causes 20% reduction in FEV_1 ($PD_{20\text{-}FEV1}$) after inhaled long acting β_2 agonist treatment (χ^2= 3.9, *P* =0.047).

4. Discussion:

The present study showed that the distribution of allelic frequencies of β_2AR gene at codon 16, and 27 among asthmatic patients and controls were in Hardy-Weinberg equilibrium, with no significant difference among patients and control groups. This was in consistent with previous studies [21,23-29].

Many previous studies [30-35] have investigated possible associations between asthma and polymorphisms in the coding region of β_2AR gene and have yielded conflicting results. The present study showed no significant association between β_2AR polymorphisms at both codons 16 & 27 and the susceptibility to asthma. This study confirms two previous studies done on Chinese population from southwest China [25] and Hong Kong [27]. It also agreed with many association studies from different ethnic and racial groups including Iceland population [28], German population [29], Caucasian subjects [30,31], North Indian subjects [32], Japanese families [33], Israelis subjects[34], and Hutterite [35] populations.

Studying the relationship between BHR and different β_2AR genotypes in the current study and in previous studies [21,29,36-38] revealed no association when studied individually at both codons. However, the combined genotype Gly-Gly/Gln-Glu in the present study was positively associated with bronchial hyperresponsiveness (assessed by $PD_{20\text{-}FEV1}$).

Other authors have shown differences in BHR to methacholine in subjects depending on β_2AR polymorphisms. In studying combined genotypes, D'Amato et al. [39] showed that Gly16/Gln27 haplotype was associated with a higher prevalence of BHR. However; Zhang *et al.* [40] found that it is Arg16Gly haplotypes associated with increased BHR.

Litonjua *et al.* [21] examined the effect of haplotype on BHR in case control study of more than 500 American Caucasian subjects and showed that Gly16/Gln27 was negatively associated to BHR to methacholine. Wilson *et al.* [41], and Joos *et al.* [38] studies did not find any association between β_2AR haplotypes and BHR.

Ramsay *et al.* [42] and Hall *et al.* [43] studies done on single nucleotide polymorphisms at β_2AR gene showed that homozygous Glu27 genotype has been linked to decreased BHR. While; Fowler *et al.* [44] found that homozygous Gly16 genotype has been associated with increased BHR. The conflicting evidence regarding the association of β_2AR polymorphisms and bronchial hyperresponsiveness between studies may in part be due to subjects' selection, sample size, or difference in the ethnic groups. In the present study sixty asthmatic patients were selected according to the American Thoracic Society guidelines [16], they were asymptomatic, and in clinically stable conditions. D'Amto *et al.* [39] studied 285 Italian men as a part of an extended program of preventive medicine devoted to investigate the individual and environmental risk factors for allergy and asthma in the military the study population were selected according to a physical examination which did not include allergy or pulmonary function tests. Zhang *et al.* [40] performed a prospective study on lung function and airway hyperresponsiveness including 180 children unselected for asthma and representative for the general population in Australia. Ramsay *et al.* [42] studied 332 subjects from 76 Australian families to investigate the associations between β_2AR polymorphisms and asthma related parameters in a large, phenotypically well characterized population which was unselected for asthma and the subjects were characterized using physiological assessments, immunological data and information obtained from questionnaire[42].

In addition; Fowler *et al.* [44] performed a retrospective analysis for 487 asthmatic patients diagnosed as having asthma from their primary or secondary care physician, based on symptoms, and being prescribed at least one anti asthma drug.

Moreover; difference in the assessment of bronchial hyperresponsiveness between studies was based on different cutoffs for the maximal or cumulative methacholine dose in challenge tests. In the present study methacholine inhalation challenge test was performed using four concentration of methacholine with the maximum dose of 16mg/ml. However; Fowler *et al.* [44] used PD_{20} value of 500 mg equates to a PC_{20} value of 5 mg/ml as a cut-off for determining the degree of bronchial hyperresponsiveness to methacholine.

It was reported[36] that difficulties in defining the asthmatic phenotype might be a reason for differences between studies as the interpretations of phenotypes between studies likely differ for many reasons: (1) a limited understanding of the interplay between allergy, asthma, and airway hyperresponsiveness; (2) although airway hyperresponsiveness is considered a hallmark of asthma, this may be altered by therapy; (3) not all patients with airway hyperresponsiveness exhibit clinical asthma; and (4) studies of different ethnic groups may yield different genetic linkage results.

The results of the present study together with the previously mentioned studies[29,38,39,42-46] may suggest that the β_2AR gene can confer genetic susceptibility to BHR, and that combined Gly-Gly/Gln-Glu genotype may have a significant influence on asthma phenotypes, and may play an important role in modifying clinical characteristics. Also it could suggest that the combinations of β_2AR genotypes are more informative than individual SNPs in their predictive power.

The severity of asthma and its response to treatment can be determined by the interaction of asthma features including the variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation[47]. However, it is possible that β_2AR variants influence certain intermediate phenotypes of asthma and that environmental exposures may interact with the β_2AR gene in conferring severity of asthma[27].

The present study agreed with previous studies[7,27,34,48,49] and found no association between β_2AR polymorphism at codon 16 and asthma severity. In contrast; other studies [50-52] have reported association between Gly 16 and the severity of asthma.

On the other hand; and in consistent with previous findings[27,43,48,49], the present study found that homozygous Gln27 genotype was significantly associated with severe asthma. Moreover; the frequencies of combined genotype Gly/Gly-Gln/Gln were always higher in the severe group than in the moderately and mild groups suggesting that it was Gln27 polymorphism that is associated with asthma severity. This was confirmed as well; by the significant positive association observed in the current study between clinical severity score and Gly-Gly/Gln-Gln genotype.

In addition; the present study showed that the presence of Glu27 polymorphism in asthmatic patients renders them to have a lower risk of having severe asthma. This was confirmed by the significant negative correlation found between clinical severity score and both Gln-Glu, and Gly-Gly/Gln-Glu

genotypes, also between the airway reactivity score and Gly-Gly/Gln-Glu genotype.

Interestingly; the effect of β_2AR polymorphisms on the functional properties of the corresponding β_2AR variants has been studied in β_2AR transfected Chinese hamster fibroblasts[53] and in human bronchial smooth muscle cell lines [6]. It was reported [53] that individuals with Gln27 may have augmented airway hyperresponsiveness to endogenous catecholamines, resulting in increased airway sensitivity to proinflammatory stimuli and leading to some extent of long-term airway inflammation and bronchoconstriction.

It was also shown that amino-terminal polymorphisms 16 and 27 affect the agonist-promoted downregulation in that Gly16 confers increased downregulation when compared with Arg16, as does Gln27 when compared with Glu27[7,53]. Thus, the association of the Gly-Gly/Gln-Gln β_2AR genotype with clinical severity score in the current study could be explained by the fact that this combination has the greatest potential for receptor downregulation, and increasing the severity on the disease.

On the other hand; previous studies [43, 45] have shown that asthmatic patients who were homozygous Glu27, which is predicted to protect against receptor desensitization, have less reactive airways than those with the Gln27 homozygotes of the β_2AR. Previous study [46] also showed that when Gln is present at position 27, the risk of asthma is the same regardless of what allele is present at position 16. However, with Glu at position 27, the risk of asthma is lower, and this decreased risk is modified by the allele at position 16, being lower with Arg16 than with Gly16.

Thus, the current study together with previous studies[27,34,43,45,46,48,54,55] could suggest that polymorphisms in β_2AR gene are important in the modulation of asthma severity, and that Gln27 genotype is associated with severe form of asthma, while Glu 27 has a protective effect against the severity of the disease. In addition the effect of Glu 27 variant prevails over the opposite effect of Gln27 when both variants are concomitantly presented either alone or when combined with polymorphism at codon 16.

Inhaled β_2 agonists are the most potent bronchodilators currently available for asthma treatment. Genetic factors controlling β_2AR function may be very important determinant for the response to bronchodilator therapy and thus the severity and duration of asthmatic symptoms [56,57]. Previous studies [58-61] have investigated the effect of specific mutations of the β_2AR gene on response to β_2 agonists. Several studies [30,38,61-68] failed to find any association between β_2AR polymorphisms and response to β_2 agonists.

The present study showed that inhaled long acting β_2 agonists (LABA) significantly improved the pulmonary function, and reduce the severity of the disease in all asthmatic patients. A significant positive correlation was found between Glu27Glu genotype and the improvement in bronchial hyperresponsiveness (assessed by percentage of change in $PD_{20\text{-}FEV1}$) after long acting β_2 agonist. On the other hand; no association was found between β_2AR polymorphisms at codon 16 and the response to inhaled long acting β_2 agonists.

There is discordance in the findings between investigative groups who found an effect of β_2AR polymorphisms on the response to β_2 agonists' treatment. Previous studies showed that polymorphisms at codon 16 of β_2AR gene, either Gly 16[69,70] or Arg 16 [59,71-76] were reported to achieve higher bronchodilating response after inhaled β_2 agonists. Polymorphisms at codon 27 of β_2AR gene, Glu was reported [56, 58] to have better response to β_2 agonists. On the other hand; other authors [73,77,78] found that β_2AR haplotypes at codon 16 and 27 together have an influence on bronchodilation and bronchoprotection. The contrasting results between studies can be attributed to various factors, including sample size, differences in study design, asthma severity, intrinsic activity of the $\beta_2$agonist evaluated, concomitant use of an inhaled corticosteroid, and to pharmaco-ethnic difference. In addition; the inconsistencies across studies on the β_2AR polymorphisms may have resulted from not taking into account environmental factors that could modulate receptor activity.

It was indicated that Glu27 allele enhances resistance to downregulation after prolonged stimulation with β_2 agonists [79-80]. Thus; Glu27 allele appears to be protective against asthma, and reducing the risk of asthma [81]. It was found in a recent study [58] that Glu27 may play a role in assisting β_2AR to respond to an inhaled terbutaline in patients with asthma. Also previous study [82] found that Glu homozygous individuals respond more rapidly to treatment with β_2 agonists. In addition; Green *et al.* [53] reported that the combination Arg16/Glu27 showed complete absence of agonist driven downregulation of β_2AR. Also, Cho *et al.* [73] found that Arg16/Glu27 was positively associated with bronchodilating response.

On the other hand; the homozygosity for both Gly 16 [83], and Gln27 [84] polymorphisms were associated to a desensitization of the receptor with a decline in the bronchodilator response to β_2 agonists. Literature suggests that the down-regulation effect is influenced by the use of long acting β_2 agonists and

that it is more visible after salmeterol [85] than formoterol [67].

Previous study [66] reported that the therapy used on a long term basis led to decrease in bronchial hyperresponsiveness and improved bronchial patency. However; chronic repetitive administration of long acting β_2 agonists has been associated with tolerance [86-89], an increase in AHR to allergen [90], poor asthma control [14] and death[91].

The result of the current study together with the previously mentioned studies[52,57,72,78-81] suggested that β_2AR polymorphisms at codon 16 has no effect on the response to β_2 agonists. On the other hand; the presence of Glu27 seems to be protective factor against tachyphylaxis, and Glu 27 has significant effects on improving bronchial hyperresponsiveness after inhaled long-acting $\beta_2$agonist treatment. Thus, the predetermination of single β_2AR genotypes might have a utility for predicting long-acting $\beta_2$agonist response.

Conclusion:

It can be concluded that polymorphisms at codon 27 of $\beta_2$adrenegric receptor gene were associated with bronchial hyperresponsiveness, disease severity and respond to β_2 agonists. Glu 27 variant prevails over the opposite effect of Gln27 when both variants are concomitantly presented either alone or when combined with polymorphism at codon 16. Thus, studying $\beta_2$adrenergic receptor polymorphisms may have profound implications for the understating of the genetic factors determining asthma severity and response to asthma therapy. The predetermination of single $\beta_2$adrenergic receptor genotypes might have a utility for predicting long acting $\beta_2$agonist response.

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