ORIGINAL ARTICLE

Polymorphisms of the β₂-Adrenergic Receptor Correlated to Nocturnal Asthma and the Response of Terbutaline Nebulizer

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Background: Inhaled β₂-adrenergic receptor (β₂-AR) agonists are the mainstay of treatment of acute asthma. Polymorphisms of the β₂-AR, especially codons 16, 27, and 164, may affect the functions of the receptor. This study was conducted to investigate whether different polymorphisms of the β₂-AR are related to the treatment responses of an inhaled β₂-AR agonist in children with nocturnal and nonnocturnal asthma in Taiwan.
Methods: The nocturnal asthma group consisted of 27 children (mean age of 10.3 ± 2.4 years), and the nonnocturnal asthma group consisted of 24 patients (mean age of 9.9 ± 3.0 years). Allele-specific polymerase chain reaction was performed to determine 16, 27, and 164 loci alleles of β₂-AR genetic polymorphisms, and peak expiratory flow (PEF) was measured before and 1 hour after inhalation of 0.2 mg/kg/dose of terbutaline to determine the treatment response in these patients.
Results: The polymorphisms of β₂-AR 27 but not 16 or 164 were significantly associated with the response to terbutaline nebulizer ($p < 0.05$). The polymorphism of β₂-AR 16 was associated with nocturnal asthma ($p = 0.027$). The Gly16 allele was more prevalent in the nocturnal asthma group (9/27; 33.3%) than in the nonnocturnal asthma group (3/24; 12.5%). Arg16 allele was less prevalent in the nocturnal asthma (3/27; 11.1%) than in the nonnocturnal asthma group (10/24; 41.7%). There was also a linkage disequilibrium found between β₂-AR 16 (Arg/Arg) and β₂-AR 27 (Gln/Gln).
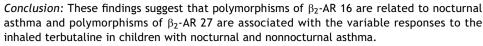
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Conclusion: These findings suggest that polymorphisms of β_2-AR 16 are related to nocturnal asthma and polymorphisms of β_2-AR 27 are associated with the variable responses to the inhaled terbutaline in children with nocturnal and nonnocturnal asthma.

1. Introduction

The caliber of the human airway is not a constant. It may increase during the day and decrease at night.[1] Evidence shows that this circadian fluctuation in the caliber of both upper and lower airways is amplified in disease states, such as asthma.[1] This is one of reasons why most in-hospital sudden deaths and episodes of ventilator arrest from asthma occur at night. Nocturnal asthma, a unique subset of patients with asthma, is of particular interest because patients with this disease show that their caliber of the airways decreases and causes peak dyspnea and wheezing between 2 and 6 AM.[2] Moreover, the disturbance of sleep because of nocturnal asthma may impair performance during the day. Therefore, it will be helpful if patients with nocturnal asthma can be early identified and receive proper treatment.

Although inhaled β_2-adrenergic agonists are the mainstay of treatment of acute asthma, it is still not clear why patients with asthma show various responses to the same inhaled β_2 agonists. After the β_2-adrenergic receptor (β_2-AR) gene was cloned in 1987,[3] the role of the β_2-AR gene in determining disease response and disease severity is now reasonably well accepted.[4] The gene encoding this G-protein-coupled β_2-AR is located on the chromosome 5q31-33 and is highly polymorphic. To date, nine distinct polymorphisms in the β_2-AR gene have been reported.[5] Each of these polymorphisms represents a single base pair substitution. Four of these polymorphisms result in amino acid substitutions at amino acids 16, 27, 34, and 164, whereas the other five are silent mutations located at amino acids 84, 175, 351, 366, and 413.[6] A question here is whether these polymorphisms might explain altered pharmacologic responses to β_2-AR agonist treatment. Indeed, studies have suggested that these polymorphisms may be associated with asthma of different severity.[7,8] Three of these polymorphisms have been found to alter the receptor function by site-directed mutagenesis and recombinant expression studies,[8–10] including substitutions of glycine for arginine at amino acid position 16 (Arg16 → Gly16), glutamic acid for glutamine at position 27 (Gln27 → Glu27), and isoleucine for threonine at position 164 (Thr164 → Ile164). Some studies suggest that the Gly16 allele showed enhanced downregulation of β_2-AR, whereas the Glu27 allele was relatively resistant to downregulation of β_2-AR during exposure to β_2-AR agonists.[8,9] The Ile164 variant markedly altered ligand binding and coupling properties.[9,10] All these *in vitro* findings are consistent with the concept that a defective β_2-AR may be a primary causal abnormality in asthma.

Using asthmatic children, we want to know the difference between β_2-AR polymorphisms at amino acid positions 16, 27, and 164 in Taiwanese children with nocturnal and nonnocturnal asthma. Moreover, to investigate the responses of patients with nocturnal and nonnocturnal asthma in the treatment with an inhaled adrenergic agonist, we used terbutaline, a selective β_2-AR stimulator, which increases the diameter of the airway via relaxation of bronchial smooth muscle within few minutes.[11] The polymorphisms and changes of the peak expiratory flow rate (PEFR) before and after inhaled terbutaline treatment were correlated with when the children required the use of bronchodilator in acute asthma.

2. Materials and Methods

2.1. Study participants

Children with a diagnosis of asthma for at least 1 year attending our emergency department were enrolled for the study. Asthma was defined using the criteria of the American Thoracic Society.[12] Exclusion criteria included use of oral or inhaled steroids, cromolyn, antibiotics, or any investigational drug within 2 weeks, moderate to severe asthma exacerbations or upper respiratory tract infection within 2 weeks, and presence of other lung or cardiac diseases as the cause of patient symptoms before this study. Home peak expiratory flow monitoring was performed. PEFRs were measured by using a peak flow meter (Mini-Wright; Armstrong Industries, Northbrook, IL, USA). Patients were separated into those with nocturnal asthma or those with nonnocturnal asthma. Nocturnal asthma was defined as a documented fall in PEFR of >20% on at least four of seven nights of testing at home[13] or on the history of early morning awakening, dyspnea, wheezing, and cough occurs between 2 and 6 AM on 3 consecutive days. The patients with nonnocturnal asthma were those with asthma attacks beyond the range of 2–6 AM. Informed consent was obtained from the parents in each case. Peak flow meter was performed before and 1 hour after inhalation of 0.2 mg/kg/dose of terbutaline (Brincanyl; Astra-Zeneca, London, UK) in a 3-mL isotonic sodium chloride solution administered with a handheld disposable updraft nebulizer (whisper Jet nebulizer; Intec, Marquest Medical products, Englewood, CO, USA).[14] $PEFR_0$ was the PEFR before the treatment with terbutaline nebulizer when the patients were sent to our pediatric emergency room for asthma attack. $PEFR_1$ was defined as the PEFR 1 hour after inhalation of terbutaline. $\Delta PEFR$ was the percentage change of $PEFR_0$ to $PEFR_1$.

2.2. Genotyping of β_2-AR polymorphism

Genomic DNA from peripheral whole blood was prepared by standard phenol/chloroform extraction procedures.[15]

Polymorphisms of the β_2-AR coding block were delineated using an allele-specific polymerase chain reaction (PCR) approach.[8] Allele-specific PCR was performed to assess polymorphisms at nucleic acids 46, 79, and 491, which result in changes in the encoded amino acids at positions 16, 27, and 164 of the receptor protein. The genotypes are abbreviated as Arg16, Gly16, Gln27, Glu27, Thr164, and Ile164. Genomic DNA was isolated from 2 mL of peripheral blood. PCRs were carried out in a volume of 50 μL using 100 ng of genomic DNA. To delineate the two polymorphisms at nucleic acid 46, the primer pairs used were 5′-CTTCTTGCTGGCACCCAATA-3′ (sense) and 5′-CCAATT TAGGAGGATGTAAACTTC-3′ (antisense) or the same antisense primer and 5′-CTTCTTGCTGGCACCCAATC-3′ (sense). The generated PCR product size using these primers was 913 base pairs (bp). The primer pair for delineating the two polymorphisms at nucleic acid 79 was 5′-GGACCACGACGT CACGCAGC-3′ (sense) and 5′-ACAATCCACACCATCAAGAAT-3′ (antisense) or the same antisense primer and 5′-GGAC CACGACGTCACGCAGG-3 (sense). Use of these primers resulted in a product with a molecular size of 442 bp. For the detection of polymorphisms at nucleic acid 491, the primer pair used was 5′-TGGATTGTGTCAGGCCTTAT-3′ (sense) and 5′-CACAGCAGTTTATTTTCTTT-3′ (antisense) or the same antisense primer and 5′-TGGATTGTGTCAGGCCT TAC-3′ (sense). The PCR product size from these primers was 662 bp. In general, a 0.1 μg (2 μL) of DNA template was added to 50 μL of reaction mixtures containing 0.5 μL of Takara Taq DNA polymerase (Takara Shuzo Co.; Kyoto, Japan), 1 μL of each primer, 1 μL of dinucleoside 5′-triphosphate, 5 μL of 10× PCR reaction buffer, and 39.5 μL of deionized water. The reaction consisted of an initial denaturation at 94°C for 5 minutes; followed by denaturation at 94°C for 2 minutes, 55°C for 1 minute, and 72°C for 1 minute for 35 cycles; and a final extension of 10 minutes at 72°C (DNA Thermal Cycler; Perkin-Elmer Co., Norwalk, CT, USA). The results of electrophoresis of the PCR products could effectively differentiate the polymorphisms in these three alleles. The allele-specific PCR technique was verified by direct dideoxy sequencing (PE Applied Biosystems, Foster City, CA, USA) of PCR products that were generated using sequencing primers that were different from those used in the PCR.

2.3. Statistical analysis

Demographic data, including age, sex, and initial PEFR, were recorded and analyzed. The patients' ages and pulmonary function test data were expressed as mean ± standard deviation. The association of the β_2-AR polymorphisms genotype between nocturnal asthma and nonnocturnal asthma patients was examined by the χ^2 and one-way analysis of variance tests (SPSS 16.0, SPSS Inc., Chicago, IL, USA). The p values <0.05 were considered statistically significant.

3. Results

3.1. Demographic data

There were a total of 51 participants in our study, 16 male and 35 female. Mean age of the participants was 10.1 ± 2.6 years. The nocturnal asthma group consisted of 27 patients (mean age of 10.3 ± 2.4 years; 8 male and 19 female), and the nonnocturnal asthma group consisted of 24 patients (mean age of 9.9 ± 3.0 years; 8 male and 16 female). No patients regularly used inhaled bronchodilator. Mean $PEFR_0$, $PEFR_1$, and $\Delta PEFR$ in the nocturnal asthma group were 204.8 ± 42.5%, 232.2 ± 46.2%, 14.0 ± 10.9%, respectively, whereas those in nonnocturnal asthma group were 185.6 ± 51.9%, 222.5 ± 58.6%, 9.6 ± 11.9%, respectively (Table 1). There were no significant differences in the demographic data and initial PEFR between the nocturnal and the nonnocturnal asthma groups.

3.2. Response to treatment with terbutaline nebulizer according to the polymorphisms of β_2-AR

The allele frequencies found in this study population were 0.49 for Gly16, 0.51 for Arg16, 0.41 for Gln27, 0.59 for Glu27, 0.5 for Thr164, and 0.5 for Ile164. When β_2-AR polymorphisms at amino acid positions 16, 27, and 164 were correlated with mean $\Delta PEFR$, the polymorphisms of β_2-AR 27 were significantly associated with mean $\Delta PEFR$ (homozygous Gln/Gln vs. heterozygous Gln/Glu, $p = 0.012$; homozygous Gln/Gln vs. homozygous Glu/Glu, $p = 0.001$). There was no correlation found between the polymorphisms of β_2-AR 16 or 164 and the $\Delta PEFR$ after terbutaline treatment (Table 2).

3.3. Correlation of genotypes with nocturnal and nonnocturnal asthma

The distribution of Arg-Gly16, Gln-Glu27, and Thr-Ile164 of β_2-AR polymorphisms with nocturnal asthma or nonnocturnal asthma patients is shown in Table 3. The polymorphisms at position 27 (Gln or Glu) and position 164 (Thr or Ile) were not different between both groups. A linkage disequilibrium was found in participants carrying Arg16, who also carried Gln27 (84.6%) (Table 4).

4. Discussion

Asthma is a polygenic disease, but so far no clear genotype-phenotype relationships have emerged. Although evidence

Table 1 Demographic data of nocturnal and nonnocturnal asthma groups

Classification of asthma	Sex (M/F)	Age (yr)	$PEFR_0$ (L/min)	$PEFR_1$ (L/min)	Δ PEFR (%)
Nonnocturnal asthma	8/16	10.3 ± 2.4	204.8 ± 42.5	232.2 ± 46.2	14.0 ± 10.9
Nocturnal asthma	8/19	9.9 ± 3.0	185.6 ± 51.9	222.5 ± 58.6	9.6 ± 11.9

Values given as mean ± standard deviation.
M/F = male/female; PEFR = peak expiratory flow rate; β_2-AR = β_2-adrenergic receptor.

Table 2 Response to treatment with terbutaline nebulizer according to polymorphisms of β₂-AR

Genotype		ΔPEFR (%)	p
β₂-AR 16	Homozygous Arg/Arg	12.2 ± 12.1	
	Heterozygous Arg/Gly	13.1 ± 9.4	NS
	Homozygous Gly/Gly	8.0 ± 14.9	
β₂-AR 27	Homozygous Gln/Gln	8.2 ± 9.4	
	Heterozygous Gln/Glu	10.7 ± 9.0	<0.05
	Homozygous Glu/Glu	23.4 ± 14.4	
β₂-AR 164	Homozygous Thr/Thr	3.8 ± 11.6	
	Heterozygous Thr/Ile	12.3 ± 11.3	NS
	Homozygous Ile/Ile	12.1 ± 14.2	

ΔPEFR is the percentage change of PEFR before and after 1 hour treatment with terbutaline nebulizer.
Values given as mean ± standard deviation.
NS = not significant; PEFR = peak expiratory flow rate.

supports that polymorphisms of the human β₂-AR are a critical determinant of functions of this receptor *in vitro*,[10] there are still inconsistencies between *in vitro* findings and asthma phenotypes. Therefore, it is of interest to investigate the associations between polymorphisms of β₂-AR and nocturnal asthma and clarify whether the variant β₂-AR receptors may affect asthmatic patients' response to inhaled β₂-AR agonists. Because the analysis of polymorphisms of β₂-AR from peripheral leukocyte can be surrogate for β₂-AR expressed on bronchial smooth muscle and other cell types relevant to asthma,[15] DNA of peripheral blood from 51 asthmatic children was analyzed by using an allele-specific PCR approach.[8] Our results show that the allele frequencies found in this study were 0.49 for Gly16, which was compatible with the data of Asian children with asthma (0.4 for Gly16).[16] The allele frequencies were 0.51 for Arg16, 0.41 for Gln27, 0.59 for Glu27, 0.5 for Thr164, and 0.5 for Ile164. The difference between our data and those of other studies[6,8,16,17] may be because of variability in allele frequencies of β₂-AR in different ethnic populations, which has yet to be elucidated.

Airway hyperresponsiveness to a variety of stimuli is one of the essential components of the manifestations of asthma. Interestingly, 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.[7] Children in Argentina with homozygous Gln27 developed significantly greater bronchodilator desensitization to β₂-AR agonist than those with homozygous Glu27.[18] In addition, Gln27 has been also reported to be associated with an elevated immunoglobulin E level[6] and may be more susceptible to inflammation and bronchoconstriction. Another study shows that the polymorphism of β₂-AR 27 has a linkage disequilibrium in a locus related to the control of immunoglobulin E levels nearby on chromosome 5q,[19] which contains a number of cytokine genes important in the generation of Type 2 T helper cell responses, including those for interleukin (IL)-4, IL-5, IL-9, and IL-13.[20] In contrast, Glu27 has been reported to be related to a lower degree of airway reactivity.[21] Site-directed mutagenesis experiments have shown that although agonist binding and coupling to adenylyl cyclase is intact in the Glu27 β₂-AR polymorphism, it was associated with impaired agonist downregulation.[6,10] In our study, the polymorphism of β₂-AR 27, although not related to individuals

Table 3 Association between the frequency of genotypes of the β₂-AR polymorphism and asthma groups

Genotypes		Frequency		p
		Nocturnal asthma, % (n = 27)	Nonnocturnal asthma, % (n = 24)	
β₂-AR 16	Homozygous Arg/Arg	11.1	41.7	
	Heterozygous Arg/Gly	55.6	45.8	0.027
	Homozygous Gly/Gly	33.3	12.5	
β₂-AR 27	Homozygous Gln/Gln	48.1	54.2	
	Heterozygous Gln/Glu	40.8	10.8	NS
	Homozygous Glu/Glu	11.1	25	
β₂-AR 164	Homozygous Thr/Thr	11.1	4.2	
	Heterozygous Thr/Ile	81.5	87.5	NS
	Homozygous Ile/Ile	7.4	8.3	

Values given as percentages.
β₂-AR = β₂-adrenergic receptor; NS = not significant.

Table 4   Association between genotypes of the polymorphisms of β_2-AR 16 and 27

Polymorphism 16	Polymorphism 27			
	Gln/Gln	Gln/Glu	Glu/Glu	Total
Arg/Arg	11 (84.6%)	0 (0%)	2 (15.4%)	13
Arg/Gly	8 (30.8%)	13 (50.0%)	5 (19.2%)	26
Gly/Gly	7 (58.3%)	3 (25.0%)	2 (16.7%)	12

Values given as number (%).
β_2-AR = β_2-adrenergic receptor.

with or without nocturnal asthma, showed a significant association with the mean ΔPEFR (Table 3). Individuals with homozygous Glu27 had a higher increase of ΔPEFR than those with homozygous Gln27. Therefore, patients with β_2-AR Glu27 may have a more augmented response to terbutaline inhalation than those with β_2-AR Gln27 in this study. We suggest that Glu27 may play a role in assisting β_2-AR to respond to an inhaled terbutaline in patients with asthma.

Recent clinical reports provide evidence that β_2-AR polymorphisms at position 16 have an important role. A cross-sectional survey revealed that possessing with homozygous Arg16 may predispose children and young adults to asthma exacerbation.[22] Another group found that PEFR was reduced in patients with homozygous Arg16.[23,24] A Chinese group reported that they detected 72% homozygous Gly16 in patients with nocturnal asthma.[25] In addition, a case report also supports the concept that homozygous Arg16 was connected to asthma in one patient with asthma in an uncontrolled situation.[26] However, Turki et al[8] reported that Gly16 allele appeared more frequently among pediatric patients with nocturnal asthma than among patients with nonnocturnal asthma, and Gly16 of β_2-AR was significantly associated with nocturnal falls in PEFR in individuals with asthma. Reihsaus et al[5] concluded that the Gly16 was associated with the more severe form of asthma, and patients with Gly polymorphism may correlate with a lack of benefit from β_2-AR agonist therapies, thereby necessitating the use of corticosteroids and/or immunotherapy. Cell study shows that Gly16 is associated with increased agonist-promoted downregulation of the β_2-AR compared with Arg16.[9,10] These results imply that when compared with patients without this genotype, asthmatic individuals with homozygous Gly16 may be more likely to show less improvement after treatment with short acting β_2-AR agonist, even leading to respiratory failure and death after inhalation of bronchodilators. This enhanced agonist-mediated β_2AR downregulation may also contribute to the pathogenesis of nocturnal asthma, especially in patients with Gly16 polymorphism.[6] Our data show that the Gly16 allele was more prevalent in the individuals with nocturnal asthma than in those with nonnocturnal asthma. Furthermore, a strong linkage disequilibrium between the two polymorphisms in patients, with the Arg16 allele having 84.6% of individuals carrying the Gln27 allele. In fact, as in previous reports, above 90% of all chromosomes carrying Arg16 also carried Gln27.[23,27] We think that the amino acid substitution at codons 16 and 27 of the β_2-AR sequence may result in conformational changes, which may possibly affect the function of β_2-AR and the incidence of nocturnal

asthma, and that more studies, such as knock-in variant codons of β_2-AR in vivo may answer these questions.

Amino acid 164 is the third most frequent β_2-AR polymorphism. This location may be important because it is within the fourth transmembrane domain and adjacent to serine 165, which has been proposed to interact with the β-carbon hydroxyl group of adrenergic ligand.[28] Site-directed mutagenesis studies show that the Ile164 protein displays significant dysfunction, with a 50% decrease in maximal stimulation of adenylyl cyclase compared with the wild-type receptor. In addition, the Ile164 form displayed decreased affinities for β-agonists. Therefore, it has been suggested that the Ile164 form may be associated with diminished responsiveness to β-agonists.[28] Our data show that there was no statistically significant association between 164 β_2-AR polymorphisms and nocturnal asthma and the change of PEFR. We think that 164 β_2-AR polymorphisms may play a minor role in the nocturnal asthma in our study.

In conclusion, the distribution of β_2-AR polymorphisms is variable among different ethnic groups. Based on our findings, these polymorphisms may be related to the phenotypic modulation of nocturnal asthma and in the determination of the treatment responses of β_2-AR agonists. We suggest that the specific polymorphisms of β_2-AR may distribute in the particular phenotype of asthma in Taiwanese children. The different genotypes of β_2-AR may affect the responses to extrinsic or intrinsic β_2-AR agonists. A large-scale prospective study assessing the effects of β_2-AR haplotypes on the response to chronic use of short- and long-acting β_2-AR agonists is required in the design of clinical trials involving a new generation β_2-AR agonists.

Acknowledgments

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