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ADRB2 and *ACE* Gene Polymorphisms in COPD Susceptibility

Chimedlkhamsuren Ganbold[1], Jambaldorj Jamyansuren[1], Odonchimeg Puntsag[3], Ochbadrakh Batjargal[1], Ichinnorov Dashtseren[2], Sarantuya Jav[1]

[1]Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Department of Pulmonology, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [3]Department of Pulmonology, Third State Central Hospital, Ulaanbaatar, Mongolia

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Corresponding Author
Jambaldorj Jamyansuren, MD, PhD
Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, Mongolian National University of Medical Sciences, Zorig Street, Ulaanbaatar 14210, Mongolia.
Tel: +976-8989-4417
E-mail: jambaldorj@mnums.edu.mn

Objectives: Recent studies have established that rs1042713 and rs1042714 polymorphisms in the *ADRB2* and rs4646994 in the *ACE* gene may have an influence on chronic obstructive pulmonary disease (COPD) development and its severity. The aim of this study was to investigate the association of *ACE* and *ADRB2* gene polymorphisms with COPD. **Methods:** 100 patients with COPD and 100 healthy volunteers were randomly involved in this case-control study. The gene polymorphisms were identified by polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis. **Results:** No significant differences were observed for allele and genotype frequencies of the rs4646994 polymorphism in the *ACE* gene between case and control groups ($p > 0.05$). Genotype frequencies of Gly/Gly_{16} (OR = 2.31, 95% CI = 1.23-4.32, p = 0.027) and Gln/Gln_{27} (OR = 2.04, 95% CI = 1.14-3.63, p = 0.044) of the *ADRB2* gene were more prevalent in COPD patients than the controls. Also, the proportion of the $Gly_{16}+Gln_{27}$ haplotype was statistically different between both groups (OR = 2.62, 95% CI: 1.65-4.15, $p < 0.001$). **Conclusion:** There is no relation between the rs4646994 polymorphism in the *ACE* gene and COPD, but the homozygote Gly/Gly_{16} and Gln/Gln_{27} variations of the *ADRB2* gene may increase the carrier's susceptibility to the development of COPD.

Keywords: Chronic Obstructive Pulmonary Disease, Single Nucleotide Polymorphism, Angiotensin Converting Enzyme, Beta-2 Adrenergic Receptors

Introduction

Chronic obstructive pulmonary disease (COPD) is a multifactorial disorder that is influenced by environmental and genetic risk factors. The major environmental risk factor is cigarette smoking but not all chronic smokers will develop the disease [1]. Thus, we hypothesized that genetic variation contributes to COPD development. An α1-antitrypsin deficiency is a definitively proven genetic determinant of COPD susceptibility. Variants in other candidate genes have been studied which may influence COPD susceptibility, including *SERPINA1*, *ADRB2*, *ACE*, *GSTs* and *TNF*α genes [2].

In this study, we examined the association of rs4646994 in the *ACE* gene and rs1042713 and rs1042714 polymorphisms in the *ADRB2* gene with COPD. Angiotensin I-converting enzyme (*ACE*) plays an essential role in the renin-angiotensin system (RAS), leading to the production of angiotensin II (Ang-II). Most studies have focused on an insertion/deletion (Ins/Del rs4646994) polymorphism in intron 16. It consists of insertion (Ins) and deletion (Del) of 287 bp fragment that determines three genotypes (Ins/Ins, Ins/Del, and Del/Del). In 1990, Rigat et al. reported that *ACE* activity levels in Del/Del carriers were approximately two-fold higher compared with Ins/Ins individuals, with significantly higher Ang-II level [3]. Overexpression of Ang-II will impact the pro-inflammatory response which up-regulates the expression of the endothelial adhesion molecules ICAM-1, VCAM-1 and T cell cytokine secretion [4]. There is evidence that lower *ACE* level by Ins allele may be advantageous in delaying *disease* progression [5].

The beta$_2$ (β_2) adrenergic receptor (β_2-AR) is expressed in airway smooth muscle cells and it is closely related to physiologic responses of the airways. This receptor is the target of β_2-agonists, which have been used as clinically important drugs in the treatment of asthma and COPD. However, not all patients with COPD have the same response to β_2-agonists. Several studies showed that genetic variations of the *ADRB2* gene associated with poor responsiveness to β_2-agonist drugs leads to susceptibility to the development of COPD [6].

The two common non-synonymous polymorphisms in the *ADRB2* gene, $Arg_{16}Gly$ (+46A>G, rs1042713) and $Gln_{27}Glu$ (+79C>G, rs1042714), were investigated in the present study. *In vitro* studies demonstrated that rs1042713 and rs1042714 single nucleotide polymorphisms (SNPs) have influence on the agonist-stimulated downregulation of β_2-AR [7, 8]. Hegab et al. published that the rs1042714 SNP was associated with COPD in Egyptians [9]. Also, Ho et al. highlighted that rs1042713 is related to COPD susceptibility and the +79C allele may be associated with severity of the disease in Chinese [10]. The aim of this study was to identify genetic variations on these genes which are contributors to COPD occurrence and development.

Materials and Methods

1. Study subjects

From January 2014 to February 2015, 100 patients, who had been referred to the Third Central Hospital of Ulaanbaatar, Mongolia for COPD, were involved in this study. All patients had a history of chronic or recurrent productive cough for ≥2 years and decreased maximum expiratory flow that had been slowly progressive and irreversible. The inclusion criteria for the COPD group were in accordance with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. Spirometric inclusion criteria for COPD patients were as follows: individuals with an FEV_1 <70% of predicted, an FEV_1/FVC ratio of <70%, and an increase in FEV_1 of <12% 15 min after the inhalation of 400 µg Ventolin (albuterol sulfate). A total of 100 unrelated, age-matched healthy volunteers, who had no known medical illness or family disorders and were taking no medications, were the control subjects. Exclusion criteria were that patients had been previously or currently diagnosed with any other disease of the respiratory system, such as asthma, lung cancer, sarcoidosis, tuberculosis and lung fibrosis. All participants gave written informed consent to participate in the study.

2. Sample preparation

Genomic DNA was extracted and purified from whole blood using a DNeasy Blood and Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol. Samples were kept at 4 °C for short-term storage or at -20 °C for long-term storage.

3. Genotyping

The rs4646994 polymorphism was identified from genomic DNA by polymerase chain reaction (PCR) using a pair of specific primers, which were performed as previously described [11]. PCR reactions were carried out using the AccuPower® PCR PreMix Kit (BioNeer Corporation, Korea) according to the manufacturer`s protocol. Thermal cycling was carried out using the following steps: 1 cycle at 94 °C for 3 minutes followed by 35 cycles at 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, with a final elongation which included 1 cycle at 72 °C for 10 minutes (Biometra Corporation, Germany). PCR products were analyzed by electrophoresis with a 1% agarose gel (catalogue number V3125, Promega Corporation, USA) and visualized with ethidium-bromide staining. The Ins was detected as a 490 bp band and the Del as a 190 bp band.

The *ADRB2* gene polymorphisms were determined by PCR based restriction fragment length polymorphism (RFLP) analysis. Forward and reverse primers were 5'-GCC TTC TTG CTG GCA

CCC CAT-3′ and 5′-CAG ACG CTC GAA CTT GGC CAT G-3′, respectively. The reaction consisted of an initial denaturation at 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, with a final extension for 5 minutes at 72 °C. The size of the generated product was 168 bp. The amplified product was digested at an introduced restriction site to determine the single nucleotide changes at +46 with *Nco*I and at +79 with *Fnu4*HI (New England BioLabs, USA). The reverse primer contains a complete restriction site and thus *Nco*I digests the PCR product from both alleles which served as a control for assessing whether digestion was complete. The same PCR product was digested with *Fnu*HI to identify the single nucleotide changes at the +79. The digested products were resolved by electrophoresis on 3% agarose gel, stained with ethidium bromide, and then visualized.

4. Statistical analysis

Analyses were performed using STATA 13.0 (StataCorp, USA) and Microsoft Excel (Microsoft Corporation, USA) software. Comparisons of age, body mass index (BMI), FEV_1, FVC, and FEV_1/FVC ratio were analyzed by the Student's t-test, ANOVA or Mann-Whitney U test. A Pearson`s chi-squared test (x^2) for 2x2 or 3x2 contingency tables and the Fisher's exact test were used to analyze the distribution of allele and genotype frequency. A p-value of 0.05 was considered statistically significant. Odds ratios (OR) with a 95% confidence interval (CI) were estimated by logistic regression to quantitatively assess the degree of association observed. Haplotype frequencies and linkage disequilibrium parameters were estimated by using SNPAlyze program (Dynacom, Japan). The Online Encyclopedia for Genetic Epidemiology calculator was used to test the Hardy-Weinberg equilibrium. A logistic regression analysis adjusted for age, sex, BMI and pack years (number of cigarettes smoked per day divided by 20 and multiplied by the duration of smoking years) was performed to assess the relation between SNPs and COPD. We used a method-four model strategy developed by Horita and Kaneko for genetic model selection [12].

Results

A total of 100 patients and 100 controls were included in the analysis. The baseline characteristics of all participants are summarized in Table 1. There were no significant differences between groups in terms of distribution of age, gender, BMI and pack years, suggesting that the frequency matching was adequate. SNP analysis of the *ADRB2* gene rs1042713 and rs1042714 polymorphisms showed that the frequencies of Arg_{16} and Gly_{16} alleles in our study population were 0.48 (n = 192) and 0.52 (n = 208) while those of Gln_{27} and Glu_{27} alleles were 0.65 (n = 260) and 0.35 (n = 140), respectively. The distribution of rs1042713, rs1042714 and rs4646994 genotypes among patients and controls was found in accordance with those expected by the Hardy-Weinberg equilibrium (p >0.05).

Table 1. Baseline characteristics of the study groups

Variables	Patients (n = 100) (Mean ±SD)	Controls (n = 100) (Mean ±SD)	p-value[a]
Age (years)	62.2 ±10.2	60.3 ±8.7	0.15
Male/female sex	57/43[b]	54/46[b]	0.29
BMI (kg/m^2)	24.9 ±2.4	25.6 ±3.0	0.11
Pack years	31.0 ±3.0	29.2 ±2.9	0.09
FEV_1, predicted (%)	44.5 ±10.9	82.4 ±2.3	<0.001
FVC, predicted (%)	49.9 ±9.7	94.9 ±4.9	<0.001
FEV_1/FVC ratio	0.53 ±0.11	0.86 ±0.04	<0.001

[a]Calculated by Student's t-test and Mann-Whitney U test [b]number

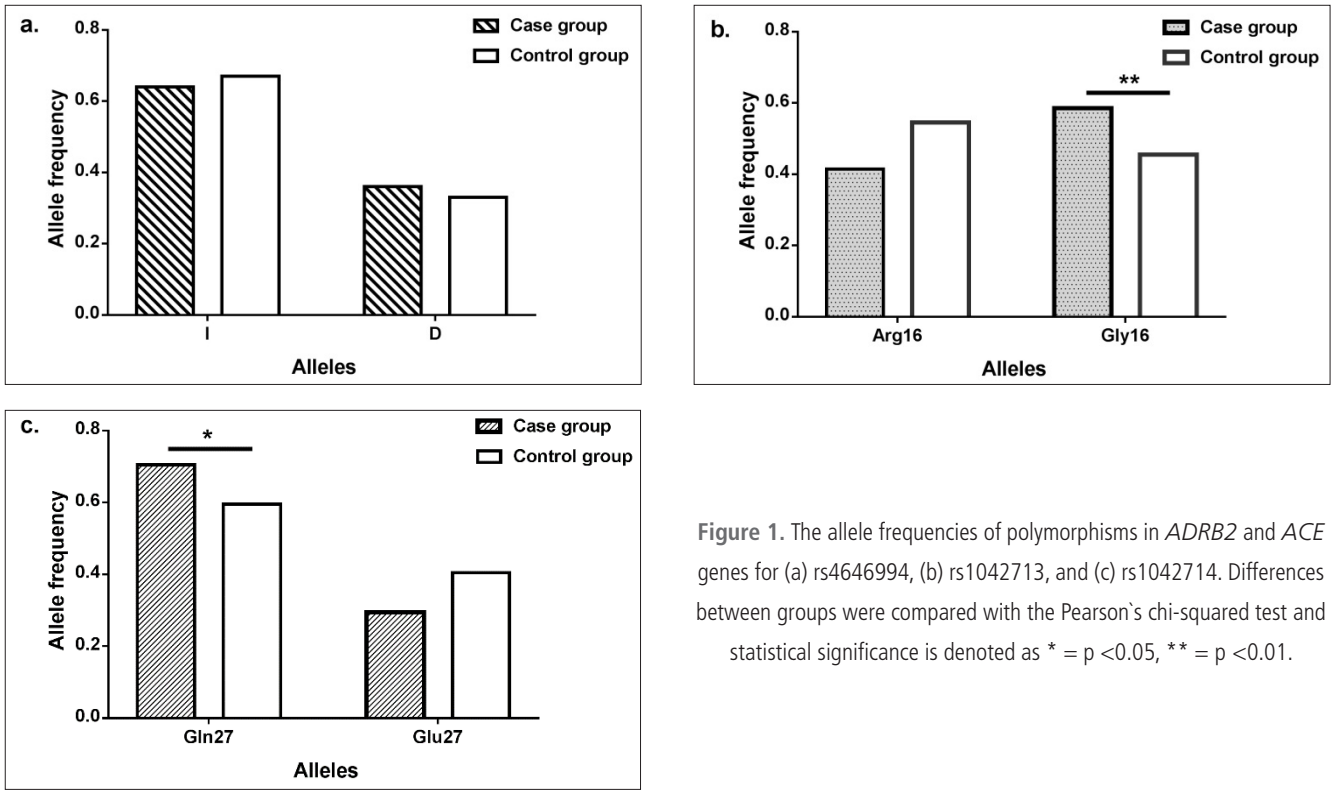

Figure 1. The allele frequencies of polymorphisms in *ADRB2* and *ACE* genes for (a) rs4646994, (b) rs1042713, and (c) rs1042714. Differences between groups were compared with the Pearson`s chi-squared test and statistical significance is denoted as * = p <0.05, ** = p <0.01.

Table 2. Genotype frequencies of rs4646994, rs1042713 and rs1042714 polymorphisms

Genotypes	Patients (n = 100) (number (frequency))	Controls (n = 100) (number (frequency))	OR (95% CI)[a]	Adjusted OR (95% CI)[a,b]
rs4646994 polymorphism of *ACE* gene				
Ins/Ins	42 (0.42)	42 (0.42)	1.00 (0.57-1.75)	1.01 (0.58-1.79)
Ins/Del	44 (0.44)	50 (0.50)	0.78 (0.45-1.37)	0.81 (0.47-1.4)
Del/Del	14 (0.14)	8 (0.08)	1.87 (0.74-4.68)	1.81 (0.72-4.59)
rs1042713 polymorphism (+46A>G of *ADRB2* gene)				
Arg_{16}/Arg_{16}	21 (0.21)	30 (0.30)	0.62 (0.32-1.18)	0.59 (0.31-1.14)
Arg_{16}/Gly_{16}	41 (0.41)	49 (0.49)	0.72 (0.41-1.26)	0.76 (0.43-1.35)
Gly_{16}/Gly_{16}	38 (0.38)	32 (0.32)	2.31 (1.23-4.32)*	2.29 (1.2-4.37)*
rs1042714 polymorphism (+79C>G of *ADRB2* gene)				
Gln_{27}/Gln_{27}	49 (0.49)	32 (0.32)	2.04 (1.14-3.63)*	2.07 (1.15-3.73)*
Gln_{27}/Glu_{27}	43 (0.43)	55 (0.55)	0.62 (0.35-1.07)	0.6 (0.34-1.05)
Glu_{27}/Glu_{27}	8 (0.0z8)	13 (0.13)	0.58 (0.23-1.47)	0.61 (0.24-1.58)

[a]p-values calculated by two-tailed Chi-squared test and statistical significance is denoted as * = p <0.05 [b]Calculated by logistic regression and adjusted for age, gender, BMI and pack years

Table 3. Pairwise haplotype combinations of rs4646994 in the *ACE* gene and rs1042713 and rs1042714 in the *ADRB2* gene

Variables	Patients		Controls		OR (95% CI)[a]
	Number	Frequency	Number	Frequency	
Arg_{16}+Gln_{27}	68	0.34	83	0.42	0.72 (0.48-1.06)
Gly_{16}+ Gln_{27}	73	0.37	36	0.18	2.62 (1.65-4.15)***
Gly_{16}+ Glu_{27}	44	0.22	55	0.28	0.74 (0.47-1.17)
Arg_{16}+Glu_{27}	15	0.07	26	0.12	0.54 (0.28-1.05)*
Gly_{16}+Ins	72	0.36	65	0.33	1.16 (0.77-1.76)
Arg_{16}+Ins	56	0.28	69	0.34	0.73 (0.48-1.13)
Gly_{16}+Del	45	0.22	26	0.13	1.94 (1.14-3.29)*
Arg_{16}+Del	27	0.14	40	0.2	0.62 (0.36-1.06)
Gln_{27}+Ins	99	0.5	80	0.4	1.47 (0.98-2.18)
Glu_{27}+Ins	29	0.14	54	0.27	0.45 (0.28-0.76)**
Gln_{27}+Del	42	0.21	39	0.19	1.09 (0.67-1.78)
Glu_{27}+Del	30	0.15	27	0.14	1.13 (0.64-1.98)

[a]p-values calculated by two-tailed Chi-squared test and statistical significance is denoted as * = p <0.05, ** = p <0.01, *** = p <0.001

ADRB2 gene Gly_{16} allele frequency was more frequent (OR = 1.69, 95% CI = 1.14-2.5, p <0.01) in COPD patients than in controls. Also, Gln_{27} allele frequency was significantly different between COPD and control groups (OR = 1.63, 95% CI = 1.07-2.46, p = 0.021). Figure 1 shows allele frequencies of rs1042713 and rs1042714 SNPs in the *ADRB2* gene and rs4646994 polymorphism in the *ACE* gene. No significant differences were observed for the rs4646994 allele and genotype frequencies between the case and control groups.

For the rs1042713 SNP, Gly/Gly_{16} was significantly the most prevalent genotype in the COPD patients (OR = 2.31, 95% CI = 1.23-4.32, p = 0.027). In addition, the homozygote Gln/Gln_{27} genotype was more frequent in COPD patients (OR = 2.04, 95% CI = 1.14-3.63, p = 0.044) than in the control group. Genotype frequencies in COPD and control groups are shown in Table 2.

The frequency of the Gly_{16}+Gln_{27} haplotype was significantly different between the COPD and control groups (OR = 2.62, 95% CI = 1.65-4.15, p <0.001) as shown in Table 3. In addition, the Ins+Glu_{27} (OR = 0.45, 95% CI = 0.28-0.76, p = 0.016) haplotype was more frequent in the control group than COPD patients for the rs1042714 and rs4646994 combination. This result suggests that the Ins+Glu_{27} haplotype may have a protective effect in COPD. We assessed the pairwise linkage disequilibrium for rs1042713, rs1042714, and rs4646994 using the parameter of r^2. Similar linkage disequilibrium patterns and low intensity were observed for both groups, with the pairwise r^2 for the three SNPs being 0.063, 0.017 and 0.001, respectively.

Discussion

Susceptibility to COPD likely results from multiple genetic and environmental effects. Previous investigations have established that the genetic variation of certain genes may have an influence on COPD development. *ACE* and *ADRB2* genes have been widely studied as candidate genes for COPD development. In the present study, we examined the allele frequency, genotype and haplotype variations of *ACE* and *ADRB2*, which were analyzed in 100 COPD patients and 100 healthy subjects in the Mongolian population. No association was observed for allele and genotype frequencies of the rs4646994 polymorphism with COPD. Similar results have reported by Zhang et al. who suggested that the *ACE* gene is not associated with exercise ventilatory responses

in COPD patients [13].

As for the rs4646994 polymorphism, Lee et al. reported no significant association between *ACE* activity and risk of COPD [14]. This is in contrast to previous studies, which established that the rs4646994 polymorphism was associated with COPD [15-16]. Busquets et al. reported that the Del/Del genotype of the *ACE* gene was associated with an increased risk of smokers to develop COPD [15]. Also, Shaw et al. investigated genetic variation in genes encoding vasoactive mediators and COPD [16]. They showed that Ins/Ins or Ins/Del genotypes were associated with a lower FEV_1% predicted than the Del/Del genotype and concluded that *ACE* genotype variations were associated with COPD disease severity [16].

ADRB2 allele frequencies and genotype variations were significantly different between the two groups. For instance, it was confirmed that the Gly/Gly_{16} genotype had a higher prevalence (OR = 2.31, 95% CI = 1.23-4.32, p = 0.027) in the COPD group compared to the controls. In addition, our result showed a higher frequency of the Gln/Gln_{27} genotype among patients (OR = 2.04, 95% CI = 1.14-3.63, p = 0.044). These findings are similar to previous studies, which investigated different ethnic populations (Chinese, Japanese, Egyptian, and Caucasian) [9, 10, 17, 18]. Vacca et al. reported that the Gly/Gly_{16} genotype was more frequent in Caucasian COPD patients compared with the healthy smokers [17]. Additionally, Papatheodorou et al. suggested that distribution of the homozygote Gly_{16} was higher in the severe COPD group [18]. *In vitro* studies demonstrated that Gly_{16} is associated with increased agonist-promoted downregulation of the β_2-adrenoreceptor number compared with Arg_{16} [7, 8]. Other investigations showed that the frequency of Arg_{16} was higher in the COPD group compared with the controls [19-23]. Probably, this discrepancy was due to different ethnic groups.

In this study, the Gly_{16}+Gln_{27} haplotype was more frequent (OR = 2.62, 95%CI = 1.65-4.15, p <0.001) in the COPD group. The distribution of the Ins+Glu_{27} (OR = 0.45, 95% CI = 0.28-0.76, p = 0.016) haplotype was higher in the control group. Joos et al. highlighted that Gln/Glu_{27} heterozygote may be protective against a decline in lung function [6]. Also, Ahsan et al. reported that lower levels of *ACE* by Ins allele may be advantageous for disease by increasing vasodilation [5]. These findings show that the Ins+Glu_{27} haplotype may have some positive effect on COPD development. From these results, we conclude that rs1042713 and rs1042714 SNPs in *ADRB2* gene may be contributing to COPD development, increasing the carrier's susceptibility to the development of COPD. A potential limitation of this study is that we had a small sample size. Further studies of genetic risk factors of COPD could lead to the development of novel prognostic and screening methods for COPD.

Conflict of Interest

The authors state no conflict of interest.

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