The Relationship Between Arginase Genes Polymorphisms and Preschool Wheezing Phenotypes

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Abstract

Background: The association between arginase I (ARG1) and arginase II (ARG2) genes and asthma has been reported in previous studies, but associations between polymorphisms in ARG genes and preschool wheezing (PSW) phenotypes are unknown. Objective: To examine the association between genetic variation in ARG1 and ARG2 genes and PSW phenotypes and to compare results with asthmatic patients. Methods: We enrolled 102 patients and 86 healthy controls. The patient group included three subgroups: episodic wheezing (EW) (n = 42, median age 41 months), multiple-trigger wheezing (MW) (n = 41, median age 39 months), and asthma (n = 19, median age 72 months). We genotyped six single nucleotide polymorphisms (SNPs) in ARG1 and six SNPs in ARG2. Eighteen haplotypes for ARG1 and 31 haplotypes for ARG2 were constituted, and the distributions of SNPs and haplotypes in patients and controls were analyzed. Results: The frequency of homozygote cytosine-cytosine genotype of the ARG1 rs2781667T>C SNP in the EW group was significantly lower than controls (p = 0.006) [OR (95% CI): 0.26 (0.10-0.66)], the MW group (p = 0.002) [OR (95% CI): 0.19 (0.06-0.52)], and asthmatics (p = 0.025) [OR (95% CI): 0.22 (0.06-0.75)]. ARG1 haplotype 4 was more frequent in the MW group, asthmatics, and controls than in the EW group (p < 0.0001) [OR (95% CI): 7.77 (2.54-23.7)], (p = 0.036) [OR (95% CI): 4.31 (1.15-16.15)], and (p = 0.030) [OR (95% CI): 3.44 (1.20-10.0)]. Conclusion: Variations in ARG1 gene may be useful in discriminating PSW phenotypes.

MAIN TEXT

INTRODUCTION

Preschool wheeze (PSW) is a highly prevalent clinical problem¹ that presents within a wide spectrum of severity and is associated with considerable healthcare costs². Asthma has its origins in early childhood; however, different patterns of childhood wheezing vary in their association with subsequent asthma, atopy, and bronchial hyper-responsiveness. Wheezing phenotypes used in epidemiological studies are frequently defined retrospectively and are therefore not usable in clinical practice³. In 2008, the European Respiratory Society’s preschool study group divided wheezing phenotypes into episodic (viral) wheeze (EW) and multiple-trigger wheeze (MW)⁴. According to this classification, wheezing attacks triggered only by viral respiratory tract infections with no complaints between attacks are classified as EW. Wheezing attacks triggered by allergens, exercise, cold weather conditions, or smoke, in addition to viral respiratory tract infections, with complaints between attacks are classified as MW⁴. However, wheeze patterns in young children vary over time and with treatment, rendering the distinction between EW and MW unclear in many patients⁵. Therefore,
we need predictive markers that are useful for differentiating between wheezing phenotypes and predicting asthma development in children with PSW as a guide for clinicians in early treatment decision-making.

There has been increasing interest among researchers to find a relationship between different genes and wheezing phenotypes and persistence and the development of childhood asthma. Arginase (\textit{ARG}) genes have been investigated in this context. Arginase, an essential enzyme in the hepatic urea cycle, is involved in L-arginine homeostasis by catalyzing the reaction in which L-arginine is converted to L-ornithine and urea. Arginase is also expressed in nonhepatic tissues, including in the airways. In humans, two arginase isoenzymes have been identified, arginase 1 and arginase 2, and both are constitutively expressed in the airways. Arginase and nitric oxide synthases (NOS) compete for their common substrate, L-arginine. Increased arginase expression and low L-arginine levels have been found to be associated with asthma and airway remodeling. After defining the important contributions of arginase in asthma, genetic studies have attempted to identify an association between genetic variation in \textit{ARG} genes and susceptibility for asthma, atopic sensitization, and bronchodilator response (BDR) in different populations. These studies have reported many associations between different single nucleotide polymorphisms (SNPs) in \textit{ARG1} or \textit{ARG2} genes and atopic sensitization, risk of asthma, asthma severity, bronchodilator/steroid response in asthma, and the arginase-NOS pathway, although some of these results have not been replicated. To date, associations between polymorphisms in \textit{ARG} genes and PSW phenotypes are unknown. Therefore, this study aimed to investigate whether there is any relationship between polymorphisms in \textit{ARG} genes and PSW phenotypes among Turkish children and compare these results with those of asthmatic children over five years old.

**METHODS**

**Study population**

This cross-sectional study was conducted at the Pediatric Allergy Department of Trakya University between May 2017 and May 2019 and included 102 patients and 86 healthy controls. The patient group included three subgroups: EW (n = 42; median age 41 months), MW (n = 41; median age 39 months), and asthma (n = 19; median age 72 months). Asthma diagnoses were based on the Global Initiative for Asthma (GINA) guidelines. Wheezing phenotypes in young children were discriminated, as proposed by the European Task Force. The control group included 86 (median age 20 months) non-allergic, unrelated, healthy outpatients with no history of recurrent wheezing and no individual or family history of allergic illness (e.g., asthma, atopic dermatitis, allergic rhinitis, allergic rhinoconjunctivitis, and food allergy). The inclusion criteria were the presence of ≥4 EW or MW episodes in a year for children up to five years of age or having asthma for children over five years of age. Patients with any other chronic disease or wheezing-associated diseases, such as cystic fibrosis, ciliary dyskinesia, anatomic abnormalities, and immunodeficiency disorder, and preterm-born children were excluded from the study.

Study forms were completed by pediatric allergists during patient visits at our pediatric allergy outpatient clinic. These forms included demographic data (i.e., age and gender) tobacco exposure, wheezing or asthma exacerbations per year, history of atopic dermatitis, presence of allergic rhinitis symptoms (i.e., nasal congestion, nasal itching, rhinorrhea, and sneezing), parental asthma, or other allergic diseases.

**Laboratory tests**

Serum total IgE levels in the patient group were measured by chemiluminescent immunometric assay (AU5800; Beckman Coulter Inc., CA, USA). Complete blood count analysis was performed by an automatic analyzer (UniCell DxH 800 Coulter Cellular Analysis System; Beckman Coulter Inc.) within one hour.
of blood sampling. The original manufacturer’s kits were utilized for the laboratory tests.

**Skin tests**

Aeroallergen and food allergen sensitivity were determined using a skin prick test. The following aeroallergens, common in Turkey, and food allergens were used: Dermatophagoides pteronyssinus and farinae, Felis domesticus, Canis familiaris, Blattella germanica, Dactylis glomerata, Festuca pratensis, Poa pratensis, Lolium perenne, Phleum pratense), Corylus avellana, Betula verrucosa, Alnus glutinosa, Artemisia vulgaris, Chenopodium album, Plantago lanceolata, Parietaria judaica, Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium, milk, egg white, egg yolk, wheat, hazelnuts, peanuts, walnuts, almond, and cacao (ALK-Abelló, Madrid, Spain). Histamine was used as a positive control and saline as a negative control. Skin reactions were evaluated 15 minutes after the skin test. A positive reaction was defined as wheal diameter ≥ 3 mm.

**Genetic analyses**

DNA isolations and real-time PCR studies were carried out in the Genetic Diseases Diagnosis Center’s Molecular Genetics Laboratory of Trakya University Medical Faculty.

**SNPs selection**

We searched PubMed and open-access databases for the keywords “arginase, asthma genetics, atopy, asthma severity, SNP, bronchodilator response.” SNPs in ARG1 and ARG2 were found associated with an increased risk of atopic sensitization, risk of asthma, asthma severity, and bronchodilator/steroid response in asthma in previous studies. These were selected for investigation in our study.

**Genomic DNA isolation**

Peripheral blood samples from both the patient and control groups were collected using tubes containing EDTA. Genomic DNA isolation from peripheral blood samples was performed using a PureLink Genomic DNA Mini Kit (Invitrogen, Catalog number: K182001; ThermoFisher Scientific) according to the optimized protocol specified in the manufacturer’s manual. Quality control and the purity of the isolated genomic DNA samples were determined using a DNA spectrophotometer (NanoDrop 2000C; ThermoFisher Scientific Inc., MA, USA). High-quality samples that had A260/280 values of 1.8–2.0 were included in the study, while low-quality samples were re-isolated from the blood samples.

**Genotyping with real-time PCR**

The real-time polymerase chain reaction (RT-PCR) method was used for allelic discrimination for the genotyping. The discrimination of the genotypes in the SNPs was performed using a PCR machine (Applied Biosystems StepOnePlus™ Real-Time PCR system; ThermoFisher Scientific) and TaqMan Assays (ThermoFisher Scientific). The RT-PCR reaction was performed according to the manufacturer’s recommended protocols for six SNPs in the ARG1 gene and six SNPs in the ARG2 gene, respectively (rs2781668, rs3756780, rs2781659, rs2781665, rs2781667, and rs2246012 and rs3759757, rs742869, rs3742879, rs17249437, rs3742880, and rs6573788) (see Figure 1).

Each PCR reaction was prepared in a 20 μl total volume of 10 μl 2xPrecision MasterMix, 1 μL TaqMan Assay, 4 μL RNase/DNase free water, and 5 μL genomic DNA. The PCR conditions were 95 °C for eight minutes for enzyme activation, 95 °C for 10 s for denaturation, and 60 °C for 60 s for the first extension in 10 cycles followed by 95 °C for 10 s for a second denaturation and 68 °C for 60 s for the second extension in 35 cycles. The ROX™ channel and VIC™ channel of the RT-PCR machine were coded for the wild-type probe or mutant-type probe with sequences determined by the TaqMan Assay® manufacturer. According
to the signals from the canals, the SNPs were considered either homozygous mutant, homozygous wild, or heterozygous. Fluorogenic data for each of the 12 polymorphisms were collected through the ROX and VIC channels at the end of each cycle of the second extension and determined individually.

**Haplotype analysis**

After determining the homozygote and heterozygote genotypes for the selected SNPs in ARG1 and ARG2, 18 haplotypes for ARG1 and 31 haplotypes for ARG2 were constituted. The GVSc names and H19 localizations of the selected SNPs and haplotypes in the ARG1 and ARG2 genes are shown in Figure 2.

**Ethical considerations**

This study was approved by Trakya University’s ethical committee (approval number 2016/264). Written informed consent was obtained from either parents or children more than seven years old.

**Statistical analyses**

Mean, standard deviation, median, minimum, maximum, and number (percentage) values were used for the descriptive statistics. Variable distribution was checked using the one-sample Kolmogorov–Smirnov test. Kruskal–Wallis and Mann–Whitney U tests with Bonferroni correction were used for a comparison of the quantitative data. Comparisons of genotype and haplotype frequencies and other categorical data were performed using Pearson’s chi-squared test or Fisher’s exact test when the sample size was small. The Hardy–Weinberg equilibrium test was used to calculate genotype frequencies. P-values < 0.05 were considered statistically significant. IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp) was used for the statistical analyses.

**RESULTS**

**Demographic characteristics and laboratory results**

The mean age of the total patient group was 46.3 ± 25.9 months (median 46), and the mean age of the control group was 23.9 ± 13.4 months (median 20) (p < 0.001). In the patient group, 37 (36.3%) of the cases were female, and 35 (40.7%) of the patients in the control group were female; (p = 0.534). A comparison of the demographic characteristics and laboratory results of the patient subgroups is shown in Table 1.

**Genetic workup with real-time PCR**

1. Genotype analysis of the studied SNPs

1a. Comparison of total patient group and controls

The genotypes were grouped as homozygote, heterozygote, or wild. After determining the genotypic distribution, we performed a genetic comparison according to the autosomal recessive inheritance pattern of the ARG1 and ARG genes between the patients, controls, and patient subgroups. There was no difference between the total patient group and the healthy controls in terms of the homozygote genotype of the SNPs in the ARG1 and ARG2 genes (Table 2).

1b. Comparison of patient subgroups

The homozygote cytosine-cytosine (CC) genotype of the ARG1 rs27816667T>C SNP in the EW group was significantly lower than for the controls (p = 0.006) [OR (95% CI): 0.26(0.10–0.66)], the MW group (p = 0.002) [OR (95% CI): 0.19(0.06–0.52)], and the asthmatics (p = 0.025) [OR (95% CI): 0.22(0.06–0.75)]. There
was no difference in the distribution of the other studied SNPs in the *ARG1* and *ARG2* genes between the patient subgroups and the controls (Table 2).

1c. Association between the SNP analysis and allergic rhinitis, aeroallergen sensitivity, number of aeroallergens detected as positive in the skin prick test, and tobacco exposure

The homozygote CC genotype of the *ARG1* rs2781667T>C SNP was more frequent in patients with AR than in those without AR (49.1% and 21.3%, respectively, \( p = 0.007 \)) [OR (95% CI): 3.56(1.48–8.56)].

There was no difference in the presence of aeroallergen sensitivity, the number of aeroallergens detected as positive in the skin prick test, tobacco exposure, and homozygote genotype distribution of any SNPs among all the patient groups.

2. Haplotype analysis

2a. Comparison of total patient group and controls

There was a statistically significant difference in terms of *ARG1* haplotype 5 (CT/T/G/T/T/CT) and *ARG1* haplotype 9 (C/T/G/T/T/T) between the healthy controls and the patient group. The frequency of *ARG1* haplotype 5 in the patient group was 9.8% and 1.2% in the control group (\( p = 0.028 \)) [OR (95% CI): 9.23(1.15–73.70)]. The frequency of *ARG1* haplotype 9 in the patient group was 2% and 11.6% in the control group (\( p = 0.016 \)) [OR (95% CI): 0.15(0.03–0.71)]. There was no difference between the patient groups and the controls in terms of other haplotypes in the *ARG1* and *ARG2* genes (see Tables 3A and 3B).

2b. Comparison of patient subgroups

The frequency of the *ARG1* haplotype 2 (CT/T/AG/AT/CT/CT) was significantly higher in the EW group than in the healthy control and MW groups (\( p = 0.041 \)) [OR (95% CI): 2.76(1.13–6.76)] and (\( p = 0.014 \)) [OR (95% CI): 5.88(1.49–25.0)], respectively. The frequency of haplotype 5 was significantly higher in the EW group than in the controls (\( p = 0.005 \)) [OR (95% CI): 14.16(1.64–121.93)]. The *ARG1* haplotype 4 (C/T/A/A/C/T) was more frequent in the MW, asthmatics, and control groups than in the EW group (\( p < 0.0001 \)) [OR (95% CI): 7.77(2.54–23.7)], (\( p = 0.036 \)) [OR (95% CI): 4.31(1.15–16.15)], and (\( p = 0.030 \)) [OR (95% CI): 3.44(1.20–10.0)], respectively (see Tables 3A, 3B).

2c. Association between SNP analysis and allergic rhinitis, aeroallergen sensitivity, number of aeroallergens detected as positive in the skin prick test, and tobacco exposure

*ARG1* haplotype 4 was more frequent in patients with allergic rhinitis than those without allergic rhinitis, 41.8% and 21.3%, respectively (\( p = 0.046 \)) [OR (95% CI): 2.65(1.10–6.41)].

There was no difference in terms of haplotype distribution and presence of aeroallergen sensitivity, the number of aeroallergens detected as positive in the skin prick test, and tobacco exposure among all the patient groups.

DISCUSSION

To our knowledge, this is the first study to look at PSW phenotypes and *ARG* gene polymorphisms. We hypothesized that there would be relationships between a PSW phenotype and polymorphisms of the *ARG1* and *ARG2* genes. Our study revealed that the frequency of the homozygote C-allele rs2781667 in *ARG1* was significantly higher in the MW and asthma groups than in the EW group. Li et al. reported that four SNPs in *ARG1* (rs2781659, rs2781665, rs2781667, rs2246012) and rs 3742880 in *ARG2* were associated with the number of positive skin tests, and carrying two copies of the minor allele for rs3759757 and rs6573788 SNPs in *ARG2* were found to be associated with the presence and severity of asthma. Litonjua et al. reported that rs2781659 in *ARG1* was significantly associated with BDR in asthmatic children. In another study, each variant allele of rs3742879 in *ARG2* was found to be associated with increased asthma risk.
controls. Taken together, we conclude that variations in ARG1 haplotype 5 were significantly higher in the EW group, while haplotype 9 was more frequent in the healthy ARG1 In conclusion, our study revealed that 1 haplotype 4 in lung and nasal mucosa. ARG no information about the functionality of the homozygote CC genotype of the ARG1 haplotype 4 in lung and nasal mucosa. ARG1

Acknowledgement

We also found that the homozygote CC genotype of the ARG1rs2781667T>C SNP was more frequent in patients with allergic rhinitis than in those without allergic rhinitis (p = 0.004). While it is known that the arginase-NOS pathway is associated with allergic rhinitis 23,24, we were unable to find any study related to the association of allergic rhinitis and ARG1 genes until now. Furthermore, we have no data for whether ARG1rs2781667 is a functional polymorphism and associated with changes in arginase expression in the nasal mucosa.

Our haplotype analyses revealed that the frequencies of the ARG1 haplotype 2 and haplotype 5 were significantly higher in the EW group than in the control group. Two recent studies suggest an interaction between arginase expression and viral airway infections25,26. Santiago-Olivares et al.25 investigated the impact of Respiratory Syncytial Virus (RSV) infection on nitric oxide production in an experimental model and found that ARG1 overexpression contributes to the maintenance of the RSV genome in the host in persistent infection. Wieczfinska et al.26 found that the expression of TGF-β and arginase was increased due to rhinovirus infection. To date, we have no data for viral-induced wheezing and genetic variations in ARG1 genes, but new studies could advance this. The ARG1 haplotype 4 was more frequent in the MW group than in the EW group. Interestingly, ARG1 haplotype 4 carried the variant C-allele for rs2781667 in ARG1, so it may be useful for differentiating between EW and MW. Salam et al.15 reported that the association between arginase variants and childhood asthma may be related with haplotypes rather than SNPs. Duan et al.19 studied haplotypes were identified according to the SNPs (rs2781659, rs2781663, rs2781665, and rs60389358) in the ARG1 and they have reported that two haplotypes may alter BDR and differentially regulate gene expression. The haplotypes that we estimated according to the studied SNPs were different from those used in these studies, so it is impossible to make a comparison between haplotype-based results.

The diagnoses of asthma cases and the classification of patients according to a PSW phenotype were performed by pediatric allergists at a tertiary pediatric allergy specialty clinic, these were strengths of the our study. However, there were some limitations. First, the sample size was smaller than previous studies have referred to; additionally, it does not represent all Turkish children. Second, this was a case-control study; thus, we have no longitudinal data about the progress and bronchodilator or ICS responses of patients with a homozygote CC genotype of the ARG1 rs2781667T>C and/or ARG1 haplotype 4. Third, we included no information about the functionality of the homozygote CC genotype of the ARG1rs2781667T>C and/or ARG 1 haplotype 4 in lung and nasal mucosa.

In conclusion, our study revealed that ARG1rs2781667T>C and/or ARG 1 haplotype 4 were more frequent in patients with MW and asthma than in the EW group. The frequencies of the ARG1 haplotype 2 and haplotype 5 were significantly higher in the EW group, while haplotype 9 was more frequent in the healthy controls. Taken together, we conclude that variations in ARG1 gene may be potentially important for differentiating PSW phenotypes. Our findings need to be confirmed in larger, different PSW populations, preferably with different ethnic backgrounds.

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11-OMIM: ARGINASE 1 http://omim.org/entry/608313


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