TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2016) 46: 1449-1458

doi:10.3906/sag-1511-16

The common genetic variants of *toll-like receptor* and susceptibility to adenoid hypertrophy: a hospital-based cohort study

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Received: 03.11.2015 • **Accepted/Published Online:** 14.01.2016 • **Final Version:** 17.11.2016

Background/aim: Adenoid hypertrophy (AH) is one of the most frequent pediatric disorders. The aim of this study was to investigate the effects of *TLR2-R753Q*, *TLR4-T399I*, and *TLR4-D299G* polymorphisms in children with AH.

Materials and methods: The variants of the *TLR* gene were determined by restriction fragment length polymorphism (PCR-RFLP) analysis in 60 patients with AH and in 50 healthy children. Data were analyzed with SNPStats and multifactor dimensionality reduction (MDR) software.

Results: We found that the presence of the G allele, the AG+GG and AG genotypes at *TLR4-D299G*, and the GGT haplotype were associated with AH in children (P = 0.013, P = 0.02, P = 0.038, and P = 0.001, respectively). On the contrary, no association was found between *TLR2-R753Q* and predisposition to AH. The CT genotype at *TLR4-T399I* showed a sex-specific association with AH, occurring only in boys with allergies (P = 0.0048). In addition, MDR analysis indicated a strong synergy between *TLR* gene markers contributing to AH. Allergic children with the diplotypes that included minor alleles of *TLR4-D299G* or *TLR4-T399I* had about a 4-fold increased risk for AH.

Conclusion: Common genetic variants of the gene encoding the TLR4 protein may have differential effects on AH and the presence of sex-specific allergy.

Key words: Adenoid hypertrophy, allergy, *TLR2-R753Q*, *TLR4-D299G*, *TLR4-T399I*, polymorphism, PCR-RFLP

1. Introduction

Adenoid hypertrophy (AH) is one of the most common pediatric otorhinolaryngological disorders and it is usually accompanied by recurrent acute tonsillitis and tonsillar hypertrophy (1,2). Adenoids are immune organs active at mainly between 3 and 10 years of age (1,3). Recurrent or chronic inflammation of the adenoids leads to chronic activation of the cell-mediated and humoral immune response resulting in AH. AH causes significant clinical symptoms related to the obstruction of the upper airway, including snoring, sleep apnea, nasal congestion, and hyponasal speech (4,5). Identifying the factors that affect the immunological response would provide important data to explain the pathogenesis of AH.

Toll-like receptors (TLRs) play an integral role in the regulation of the immune system through the recognition of pathogen-associated molecular patterns and activation of immune response genes (6). *TLR2* and *TLR4* have been well studied (7,8). They initiate intracellular signal cascades that involve adaptor proteins and they lead to interferon production (9).

Three single nucleotide polymorphisms (SNPs), *TLR2* (*2258* A>G) (corresponding to an Arg753Gln substitution mutation; SNP database [dbSNP] accession number rs5743708), *TLR4* (*896 A>G*) (corresponding to an Asp299Gly substitution mutation; dbSNP accession number rs4986790), and *TLR4* (*1196 C>T*) (corresponding to a Thr399Ile substitution mutation; dbSNP accession number rs4986791), have been shown to be associated with receptor hyporesponsiveness and immunopathology (10). There has been great interest regarding the association of these SNPs with susceptibility to infectious and noninfectious disease states (11–13). However, the possible contribution of those SNPs to AH is not yet known.

In this study, we aimed to reassess the association of *TLR2-R753Q*, *TLR4-D299G*, and *TLR4-T399I* and their combinations with susceptibility to AH using odds ratios

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(ORs), which can elucidate the magnitude and association of individual genotypes.

2. Materials and methods

2.1. Study population

One hundred and ten subjects aged between 3 and 10 years (24 girls and 36 boys; age range 3–7 years; mean ± standard deviation [SD] 4.81 ± 1.2) who were admitted to the ear, nose, and throat clinic of the Yenimahalle Education and Research Hospital, Ankara, Turkey, between January and August 2015 were included in the study. The child's parents or accompanying relatives Figure provided their verbal and written informed consent for inclusion in the study. The study protocol was approved by the Dışkapı Education and Research Hospital's Ethics Committee (registration number 2015-008), and the study was conducted in accordance with human rights and experimental ethics.

2.1.1. Children with AH

Sixty children with symptoms of sleeping with their mouths open, mouth breathing, snoring, witnessed apnea, hyponasal speech, malnutrition, and midfacial developmental disorders who were admitted to the hospital, diagnosed with AH, and scheduled for adenoidectomy were included in the study. AH diagnosis was made with a characteristic history and physical examination findings, including nasal endoscopy, and confirmed with lateral cephalometric X-ray findings (13,14). AH was graded according to Parikh's classification: grade 1 indicated adenoid tissue not in contact with adjacent structures; grade 2 indicated adenoid tissue in contact with the tubal orifice; grade 3 indicated adenoid tissue in contact with the vomer; and grade 4 indicated adenoid tissue in contact with the soft palate (13).

The diagnosis of sleep-disordered breathing (SDB) was primarily based on physical examination and medical history of the patients obtained from the parents or the guardians of the child (15,16). They filled in an established and validated asthma and allergy questionnaire (ISAAC) (17), and they were asked whether they had a diagnosis of asthma or allergic bronchitis by a physician, or a history of wheezing.

2.1.2. Healthy children

The control group consisted of 50 healthy children who had presented to the outpatient clinic for regular follow-up or minor trauma. An endoscopic examination was performed, and the diagnosis of AH was ruled out in these children. All children and their parents were evaluated for the presence of asthma, allergies, and SDB using questionnaires.

A personal and family history of immune deficiency or diseases with known immune-related etiologies, craniofacial abnormalities, congenital defects, or mental retardation; cardiovascular, pulmonary, or metabolic diseases; and genetic and neuromuscular diseases served as exclusion criteria in this study (18).

2.2. Identification of genetic variants of the *TLR*

Blood samples were obtained from the participants after they provided their informed consent. The blood samples were put into tubes containing EDTA. A standard kit was used in accordance with the manufacturer's instructions for genomic DNA extraction from the peripheral blood samples (NucleoSpin blood DNA; Macherey-Nagel GmbH & Co. Kg, Düren, Germany). Polymorphisms were detected using allele-specific PCR, followed by restriction fragment length polymorphism (PCR-RFLP) analysis. All primers are summarized in Table 1. Primers for the detection of the *TLR4-D299G* SNP and *TLR4-T399I* SNPs were forward 3' and cut point 5'. The nucleotides in parentheses were modified and changed to the underlined ones.

For detection of the *TLR2-R753Q* polymorphism, the protocol was designed on the basis of the fact that the polymorphism results in the creation of a DNA sequence recognized by the restriction enzyme SfcI (New England

Table 1. The primers used for PCR-RFLP.

Polymorphism	Location	Primer nucleotide sequence (5-3)	Product size (bp)
TLR2-R753Q	2258 G>A	F- ATGGTCCAGGAGCTGGAGA	430
		R- TGACATAAAGATCCCAACTAGACAA	
TLR4-D299G	896 A>G	F- ATTAGCATACTTAGACTACTACCTC(G)CATG	249
		R- GATCAACTTCTGAAAAAGCATTCCCAC	
TLR4-T399I	1196 C>T	F- GGTTGCTGTTCTCAAAGTGATTTTGGGA(C)GAA	408
		R- GATCAACTTCTGAAAAAGCATTCCCAC	

F = Forward primer; R = reverse primer, bp = base pairs.

BioLabs, Ipswich, MA, USA) (19). The PCR conditions were 2 min at 94 °C, followed by 38 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 45 s with 5 min at 72 °C after the last cycle. The presence of undigested PCR products (430 bp) was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 307-bp and 123-bp fragments.

Molecular detection of the *TLR4-D299G* and *TLR4-T399I* SNPs was done using PCR-RFLP (20,21). In brief, the forward primers were modified at the 5' end in two reactions, creating restriction enzyme recognition sites (NcoI for the *TLR4-D299G* polymorphism and HinfI for the *TLR4-T399I* polymorphism), so that if a polymorphism was present, PCR-RFLP analysis would create digestion fragments visible on agarose gels (20,21). The conditions of both PCRs were 5 min at 94 °C, followed by 38 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s with 5 min at 72 °C after the last cycle. For the *TLR4-D299G* SNP, a 249-bp fragment was amplified by PCR and subjected to NcoI digestion (Invitrogen, Carlsbad, CA, USA) overnight at 37 °C. The presence of undigested PCR products was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 226-bp and 23-bp fragments. For the *TLR4-T399I* SNP, a 408-bp fragment was amplified by PCR and subjected to HinfI digestion (FastDigest; Thermo Fisher Scientific, Waltham, MA, USA) for 4 h at 37 °C. The presence of undigested PCR products was indicative of wild-type samples, whereas the presence of the polymorphism resulted in the digestion of the PCR products to 378-bp and 29-bp fragments.

For all PCRs described here, a total of 2 µL (20–50 ng/µL) of DNA was amplified in a 25-µL reaction mixture using, 2.5 µL of 10X buffer (Complete; Bioron Inc., Ludwigshafen, Germany), 0.5 µL of 10 mM deoxynucleoside triphosphate, 0.5 µL of 20 pmol of each primer, 2.5 µL of 25 mM $MgCl_2$, 0.2 µL (1.0 U) of Taq polymerase (Invitrogen), and 16 µL of dH_2O in a buffer supplied by the manufacturer. All PCR and digestion procedures were carried out in a PCR thermal cycler (T100 Thermal Cycler; Bio-Rad, Hercules, CA, USA), and the PCR and digestion products were analyzed in 2% Tris-borate-EDTA agarose gels. For confirmation of the PCR-RFLP analysis results, randomly chosen PCR products positive and negative for the *TLR* polymorphisms were purified by use of a kit (NucleoFast 96 PCR; Macherey-Nagel GmbH & Co.), and they were directly sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a sequencing kit (BigDye Terminator V3.1 Cycle Sequencing; Applied Biosystems).

2.3. Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A nonparametric Mann–Whitney U test was used to compare age. Pearson's chi-square test was used to assess the differences for the alleles and genotypes.

The frequency of each genotype was evaluated using SNPStats software (http://bioinfo.iconcologia.net/index.php?module=Snpstats) for concordance with Hardy–Weinberg equilibrium (HWE) (22). Moreover, the degree of pair-wise linkage disequilibrium (LD) and the genotype and haplotype analyses were conducted using regression in this logistic model and expressed as the OR and 95% confidence interval (95% CI) (23). We used the Multifactor Dimensionality Reduction (MDR) software package (version 1.0.0, available at www.epistasis.org) to construct all possible interactions among different genotype variants belonging to the *TLR* gene (24). MDR is a novel computational tool described by Hahn et al., and it is a nonparametric method (i.e. no parameters are estimated). It can detect SNP × SNP and SNP × phenotype interactions (25,26). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of subjects

A total of 60 children with AH (24 girls, 36 boys; age range 3–7 years) and 50 healthy children (22 girls, 28 boys; age range 3–10 years) without AH were included in this study. The demographic characteristics of the children with AH and the controls are shown in Table 2. The mean age of the children with AH was 4.79 ± 1.097 years and the mean age of the control children was 4.84 ± 1.394 years ($P = 0.680$). Sex distribution was similar in the two groups ($P = 0.065$).

SDB, asthma, and allergy prevalences were higher in children with AH than in the controls (62%, 27%, 37% and $P = 0.001$, $P = 0.042$, $P = 0.045$, respectively). In addition, 68 (71%) patients had an AH grade of 3, and 27 (29%) had an AH grade greater than 3. AH grades of 4 and 3 were more frequently seen than AH grades of 2 and 1 (25%, 55%, 12%, 8% for AH grades 4, 3, 2, and 1, respectively; $P = 0.001$).

3.2. Association of the *TLR* genotypes with AH and phenotypes

All genotype distributions of the control subjects were consistent with those expected from HWE (all $P = 1$) (Table 3). Three SNPs were screened in healthy children and the children with AH. Genotype analysis revealed that *TLR2-R753Q* GA (0.03 vs. 0.02) and *TLR4-T399I* CT (0.12 vs. 0.06) genotypes were more frequent in children with AH than in healthy children in the codominant model ($P = 0.06$ and $P = 0.30$). In addition, the difference between children with AH and controls were significant in the G-dominant all-inheritance model (AG + GG vs. GG) of the *TLR4-D299G* genotype (all $P > 0.05$). When the minor allele frequency of the *TLR4-D299G* was considered,

Table 2. The demographic characteristics of the children.

Variables		Children with AH (n: 60) n (%)	Controls (n: 50) n (%)	P-value
Age (years) (mean ± SD)		4.79 [0.993]	4.84 [1.419]	0.68*
Sex M/F		36 (60) / 24 (40)	28 (56) / 22 (44)	0.654**
AH grade	1	5 (8)	0 (0)	<0.0001**
	2	7 (12)	0 (0)	
	3	15 (25)	0 (0)	
	4	33 (55)	0 (0)	
SDB (+)		37 (62)	5 (10)	0.001**
Asthma (+)		16 (27)	9 (18)	0.042**
Allergy (+)		22 (37)	10 (20)	0.045**

SD = Standard deviation; n (%) = frequency; AH = adenoid hypertrophy; SDB = sleep-disordered breathing.
The data are presented as mean ± SD or numbers (%) unless otherwise specified.
*Mann–Whitney U test. ** χ^2 test.

Table 3. The *TLR* gene polymorphisms.

Polymorphism	SNP ID	Locus	Region	Allele change	Allele	MAF				***P for HWE	Genotyped (%)
						AH	Control	*Database	**Turkey		
TLR2-R753Q	rs5743708	2258	Exon3	[G/A]	A	0.02	0.01	0.00–0.052	0.05[39]–0.02[40]	1	97
TLR4-D299G	rs4986790	896	Exon3	[A/G]	G	0.08	0.01	0.00–0.076	0.027[36]	1	92
TLR4-T399I	rs4986791	1196	Exon3	[C/T]	T	0.06	0.03	0.00–0.152	0.027[36]	1	91

AH: Adenoid hypertrophy; SNP ID = single-nucleotide polymorphism accession number or NCBI dbSNP (http:www.ncbi.nlm.nih.govsnp); MAF = minor allele frequencies; HWE = Hardy–Weinberg equilibrium.
*Minimum–maximum MAF values from the HapMap databases (http:www.hapmap.org) or NCBI dbSNP (http:www.ncbi.nlm.nih.govsnp).
**MAF from healthy control groups in independent studies on Turkish population.
***HWE P-value in the controls.

significant differences were observed between children with AH and the controls (for *TLR4-D299G*: 7.5% and 1%, P = 0.013; for *TLR2-R753Q*: 1.7% and 1%, P = 0.67; for *TLR4-T399I*: 5.8% and 3%, P = 0.30) (Table 4).

Allergic children within the G-log additive model for *TLR4-D299G* and allergic boys within the T-dominant inheritance codominant model for *TLR4-T399I* were more frequent among children with AH than those with other genotypes (adjusted OR, 3.87; 95% CI, 1.07–13.95; P = 0.0034 and adjusted OR, 7.86; 95% CI, 1.77–34.89; P = 0.0048, respectively).

Moreover, SDB in the G-dominant all-inheritance models (except for recessive *TLR4-D299G*) and the T-dominant inheritance codominant model for *TLR4-T399I* were associated with a significantly increased risk of AH when compared to those with the other genotypes (for codominant: adjusted OR, 5.66; 95% CI, 1.08–29.51; P = 0.031; for dominant: adjusted OR, 6.60; 95% CI, 1.30–33.49; P = 0.011; for overdominant: adjusted OR, 5.66; 95% CI, 1.08–29.51; P = 0.031; for log-addictive: adjusted OR, 5.50; 95% CI, 1.06–28.67; P = 0.028; adjusted OR, 6.06; 95% CI, 1.25–29.42; P = 0.0089; and for codominant at *TLR4-T399I*: adjusted OR, 18.27; 95% CI, 2.22–150.34; P = 0.0089).

There was a tendency towards a higher AH grade in AH patients carrying the AG genotype at *TLR4-D299G* and the CT genotype at *TLR4-T399I* (OR, 3.29; 95% CI, 1.83–4.74; P = 0.55 and OR, 0.67; 95% CI, 0.11–1.23; P = 0.190, respectively).

The frequencies of GGT and AAC haplotypes were significantly higher in the AH cases than in the controls (OR, ∞; 95% CI, 0.00–∞, P = 0.0001) (*TLR2* and *TLR4*, three SNPs were ordered from 5' to 3'). The frequencies of

Table 4. Frequencies of *TLR* SNP genotype and alleles.

SNPs	Genotype/allele	Children with AH (n: 60)	Controls (n: 50)	Models	Adjusted OR (95% CI)*	P-value**
		n (%)	n (%)			
TLR2-R753Q	GG	58 (97)	49 (98)	Codominant	1.00 (reference)	0.066
	GA	2 (3)	1 (2)		0.58 (0.05–6.66)	
	AA	0 (0)	0 (0)		0.00 (0.00–NA)	
	A‡	0.017	0.010		1.68 (0.15–18.78)	0.67
TLR4-D299G	AA	52 (87)	49 (98)	Codominant	1.00 (reference)	0.05
	AG	7 (11)	1 (2)		0.15 (0.02–1.28)	
	GG	1 (2)	0 (0)		0.00 (0.00–NA)	
	AA	52 (87)	49 (98)	Dominant	1.00 (reference)	0.02
	AG-GG	8 (13)	1 (2)		0.13 (0.02–1.10)	
	AA-AG	59 (98)	50 (100)	Recessive	1.00 (reference)	0.27
	GG	1 (2)	0 (0)		0.00 (0.00–NA)	
	AA-GG	53 (88)	49 (98)	Overdominant	1.00 (reference)	0.038
	AG	7 (11)	1 (2)		0.15 (0.02–1.30)	
	---	---	---	Log-addictive	0.14 (0.02–1.15)	0.017
	G‡	0.075	0.010		8.03 (1.00–64.48)	0.013
TLR4-T399I	CC	53 (88)	47 (94)	Codominant	1.00 (reference)	0.30
	CT	7 (12)	3 (6)		0.48 (0.12–1.98)	
	TT	0 (0)	0 (0)		0.00 (0.00–NA)	
	T‡	0.058	0.030		2.00 (0.50–7.96)	0.30

n (%) = Frequency; NA = not analyzed; SNP = single nucleotide polymorphism; AH = adenoid hypertrophy; OR = odds ratio; CI = confidence interval.
‡ Assumed risk alleles; *Adjusted for age and sex; ** χ^2 test.

Table 5. Associations between AH risk and frequencies of haplotypes based on *TLR* variants.

No	Haplotypes*			Haplotype frequencies		Cure OR (95% CI)**	***P-value
	TLR2-R753Q	*TLR4-D299G*	*TLR4-T399I*	Patients	Controls		
1	G	A	C	0.8821	0.96	1.00 (reference)	---
2	G	G	C	0.0429	0.01	0.27 (0.03–2.13)	0.22
3	G	A	T	0.0263	0.02	0.68 (0.11–4.29)	0.69
4	G	G	T	0.0321	0.00	0.00 (–Inf to Inf)	<0.001
5	A	A	C	0.0167	0.00	0.00 (–Inf to Inf)	<0.001

AH = Adenoid hypertrophy; OR = odds ratio; CI = confidence interval; Inf = infinity.
*The alleles of haplotypes were genotyped as the location of in the SNPs *TLR*2 and *TLR4*.
**In logistic regression model.
***Global haplotype association P-value = 0.12

GGT in cases with SDB were significantly higher than in the controls (OR, 2.11; 95% CI, 0.32–3.90; P = 0.023). The frequency of the AAC haplotype was significantly higher in the allergic AH cases than in the allergic controls (OR, 14.73; 95% CI, 1.56–139.23; P = 0.001). Moreover, *TLR4-D299G* and *TLR4-T399I* had a high level of LD (D = 0.88, $r^2 = 0.35$).

3.3. MDR analyses

MDR software was used to analyze the interaction of the SNPs and phenotypes that might affect AH. At the end of the analysis, we found four predictive models for AH. *TLR4-D299G* was the best single-locus predictive model, and the AG and GG genotypes were significantly associated with the diagnosis of AH [testing balance accuracy (TBA): 0.556 and cross-validation consistency (CVC): 10/10] (for AG: OR, 7.5 and for GG: OR, ∞, 95% CI, 0.90–62.50; P = 0.0308).

According to the best two-locus predictive model (*TLR2-R753Q_TLR4-D299G*), children carrying the GA + AA, GG + AG, and GG + GG diplotypes showed a TBA of 56.5% and a CVC of 9/10 for prediction of risk of AH (for GA + AA: OR, 7.5; for GG + AG: OR, 7; and for GG + GG: OR, ∞, 95% CI, 0.99–23.04; P = 0.0338).

The three-locus model (*TLR2-R753Q_TLR4-D299G_ TLR4-T399I*) was the third best attribute for prediction of AH risk, having a TBA of 64.4% and a CVC of 10/10, which were statistically significant (for GG + AA + CC: OR, 1.02; for GG + AA + CT: OR, 1.5; for GG + AG + CC: OR, 3; for GG + AG + CT and GA + GG + CC: OR, ∞, 95% CI, 1.15–16.20; P = 0.020).

In addition, the individuals with the combination of *TLR4-D299G* and *TLR4-T399I* as well as allergies were in the high-risk group. The dendrograms provided by MDR were examined to assist in the visualization and interpretation of potential genotype × genotype and genotype × phenotype interactions (Figure 1A) (27). The dendrogram demonstrated the nature of the interactions between *TLR4-D299G* and *TLR4-T399I*, and the synergistic association of allergies with those three factors for predicting susceptibility to AH. As observed in the dendrogram, *TLR4-D299G* and allergy fit in one cluster, while *T399I* fits in another cluster (Figure 1B) (TBA 0.573, CVC 9/10, OR 4.88, CI 1.80–13.25, P = 0.001).

4. Discussion

In this study, we investigated the potential associations of the frequent polymorphisms of the *TLR* gene with AH, and their relationships with phenotypes. To the best of our knowledge, this is the first study presenting associations between the *TLR* gene and AH in Turkey. Data analysis demonstrated that *TLR4* polymorphisms were associated with an increased risk of AH, but it appeared that there was no association between *TLR2-R753Q* and a predisposition to AH. However, the presence of the CT genotype at *TLR4-T399I* seemed to cause susceptibility to allergies in boys with AH.

TLRs, together with the interleukin-1 receptors, are a class of proteins known as the interleukin-1 receptor/TLR superfamily and play important roles in the initiation and maintenance of immune responses (6). Numerous studies have implicated TLRs in the pathogenesis of infectious and noninfectious disease states (10–12).

Lorenz et al. (28) were the first to report that a genetic variant at the C terminus of the *TLR2* gene was associated with infection. The SNP *TLR2-R753Q* sequence change causes an arginine-to-glutamine substitution at residue 753 of the protein. The amino acid is conserved among species, and it is a part of highly conserved stretch of amino acids at *TLR2*. In addition, in vitro functional analysis demonstrated that the SNP affected the signaling function of the molecule. This missense mutation decreased the ability to respond to infectious agents (28). Thus, *TLR2-R753Q* leads to decreased NF-kB and cellular activation (28,29). However, in the present study, the minor allele and all genotypes of *TLR2-R753Q* were not associated with AH.

The *TLR4*-knockout mouse model was shown to be lipopolysaccharide-hyporesponsive (8). Similarly, two common mutations identified in the *TLR4* gene receptor at residues 299 and 399 affected the extracellular domain of the protein and were shown to be associated with receptor hyporesponsiveness in macrophages, epithelial cells, and peripheral blood mononuclear cells (30). *TLR4* missense mutations might act in concert with other genetic changes to influence the complex immunologic response; they may also be modifying factors for AH and other phenotypes, and particularly for allergies and asthma (4). Recently it was shown that *TLR4* mutations are linked to an increased risk of recurrent tonsillitis and/or tonsillar hypertrophy (31). The adenoids are similar to tonsils, and they are important components of the lymphoid tissue situated in the upper airways, known as Waldeyer's ring (1). Therefore, tonsillar hypertrophy is usually accompanied by hypertrophy of the adenoids (2). In our study, children carrying the G allele at *TLR4-D299G* had a significantly increased risk for AH. On the contrary, we found no association between *TLR4-T399I* and a predisposition to AH. As in vitro studies have shown, *TLR4-D299G* might have a greater functional impact on the receptor rather than *TLR4-T399I*, and this may be a possible reason for this result (30).

Factors such as obesity, asthma, and allergies are etiologic contributors and comorbidities of pediatric SDB (32). However, AH is the most pervasive primary etiology of SDB (33). In the present study, children with SDB carrying the minor alleles of *TLR4-D299G* and *TLR4-T399I* had about 5.50- to 18.27-fold increased risk for

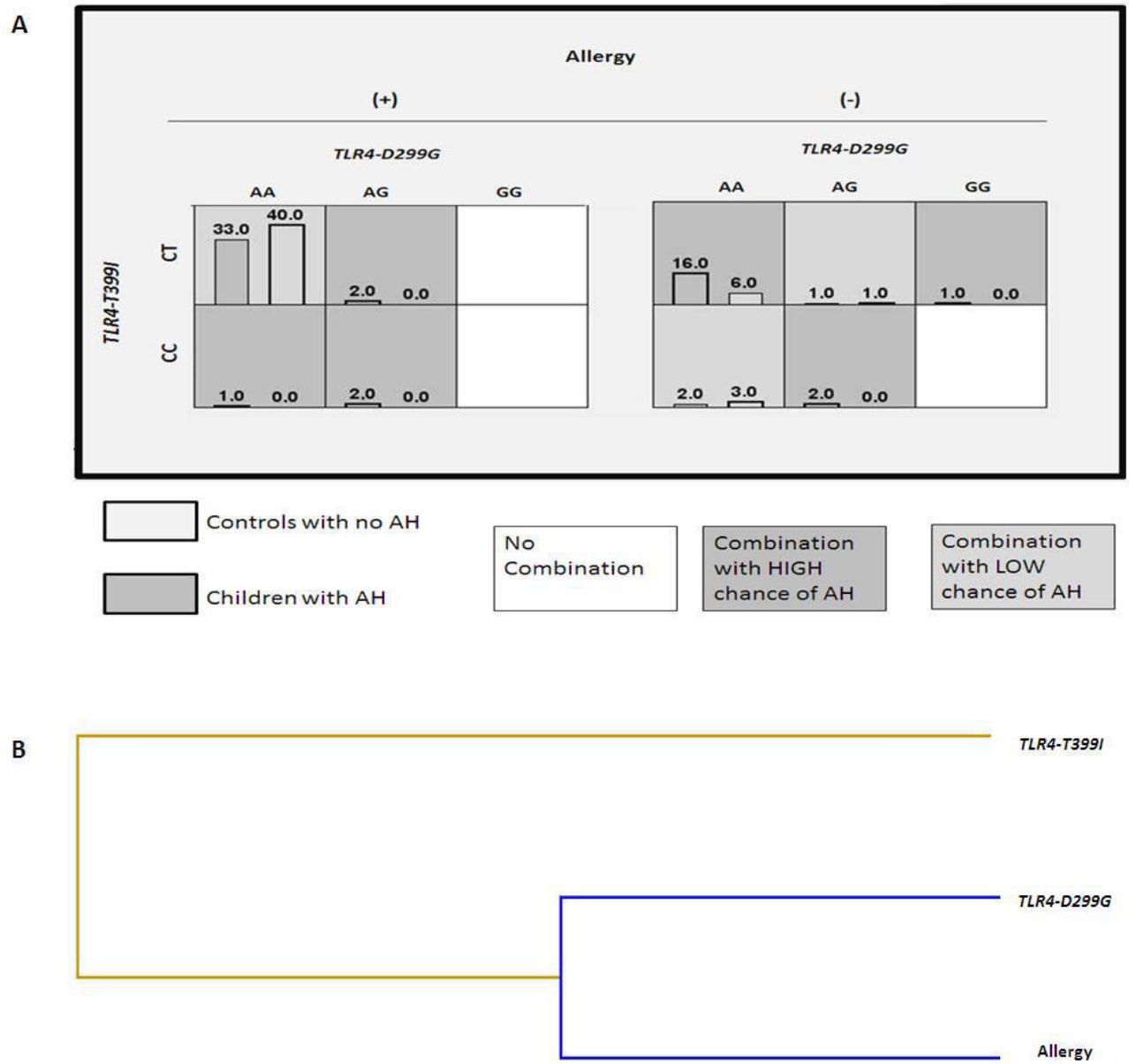

Figure 1. Distribution of the *TLR4-D299G* and *TLR4-T399I* genotype and allergy combinations in cases and controls according to MDR. A) The *TLR4-D299G* and *TLR4-T399I* genotype and allergy combinations that are able to correctly predict AH with an accuracy of 64.3%. The *TLR4* GG+CC, AA+CC, and AG+CT genotypes had ∞-fold, 2.6-fold, and ∞-fold increased risk for allergic AH, respectively. For each genotype combination, the numbers of allergic or nonallergic cases are displayed in the histogram on the left in each cell, while the numbers of allergic or nonallergic controls are displayed in the histogram on the right. A darker shade indicates the high-risk group. The pattern of high- and low-risk for the presence of AH differs depending on the presence of the allergy and value of *TLR4-D299G* and *TLR4-T399I* (TBA 0.573, CVC 9/10, OR 4.88, CI 1.80–13.25, P = 0.001). B) The dendrogram demonstrates the nature of the interactions between the *TLR4-D299G/TLR4-T399I* genotype and allergy included in the genetic classifier obtained by MDR. There was a synergistic interaction, with the strongest interaction between *TLR4-D299G* and allergy.

AH. Therefore, similar genotypes and haplotypes were associated with AH and SDB and unsurprisingly showed the role of the *TLR* gene in the molecular mechanisms underlying AH.

The role of allergies in promoting chronic inflammation associated with AH remains unclear in children (5,33). A number of studies showed that *TLR4* SNPs were associated with childhood allergies and/or asthma (34,35). Similarly, recent studies conducted on Turkish children harboring heterozygous *TLR4* polymorphisms reported a relation of those polymorphisms with asthma and atopic asthma (36,37). In the present study, we showed that presence of

the minor alleles of *TLR4-D299G* and *TLR4-T399I* and the AAC haplotype were associated with an increased (3.87-, 7.86-, and 15-fold) risk of allergic AH. We also showed that this association was sex-specific, as it was stronger in boys with the *TLR4-T399I* CT genotype. Interestingly, the allergic children carrying minor alleles *TLR4-D299G* and *TLR4-T399I* had about a 4-fold increased risk of AH, as indicated with MDR analysis. However, we did not find any significant association between asthma and *TLR* gene polymorphisms in the Turkish population studied. These findings indicated complex interactions between *TLR4* gene variations and susceptibility to allergic AH. They also indicated that AH and allergies probably have a shared genetic background, since they have common pathways with several immunological processes that could be further modified by genetic variations in other innate immune response genes (38).

In the haplotype analysis, we found that the GGT haplotype was frequent in AH cases, and it was associated with AH in the studied population. Notably, the LD between *TLR4-D299G* and *TLR4-T399I* was stronger, consistent with previously reported data (HapMap, accessible at http://hapmap.ncbi.nlm.nih.gov/). In the current study, we also used a data mining approach: MDR reduces data dimensionality by pooling genotypes from multiple SNPs into either high-risk or low-risk groups for a disease, thereby circumventing the problem of high-order genotype combinations with a low number of observations. It has successfully identified combinations of multilocus genotypes and discrete environmental factors that are associated with some complex diseases (24–26). Via the interaction information analysis, we have shown for the first time a strong synergism between the *TLR* gene markers contributing to AH in a Turkish population. Furthermore, our MDR results showed that the main effects of the logistic regression model or other traditional approaches might not be observed (the 1 + 1 = 3 principle) (25). Thus, the haplotype findings, as well as LD and MDR analysis, are consistent with each other in the present study, providing an insight into possible interactions between *TLR* gene polymorphisms and susceptibility to AH.

The minor allele frequencies of *TLR* gene polymorphisms have been reported in international web-based databases among different populations (http:www.hapmap.org and http:www.ncbi.nlm.nih.govsnp). The minor allele frequencies of the *TLR* gene found in healthy controls in the present study were similar to previous studies performed in Turkish populations, but the prevalence rates were different from the ones reported in different regions of the world (36,37,39,40). Those discrepancies may be due to sample size, ethnic background, or geographic region, and they may affect the results of the genetic association studies (41).

One of the potential limitations of our study is that it is a hospital-based cohort including only Turkish children. Another important limitation is the relatively small sample size, which may notably affect the results (42). For this reason, our findings need to be replicated in further studies with larger-scale population-based studies in different populations.

In conclusion, our findings indicated that *TLR4* gene polymorphisms predisposed individuals to AH and allergic AH. Those genetic variations might lead to an alteration in the inflammatory cascade that could regulate AH pathophysiology. Further studies with a larger sample size and a more representative population should be performed to verify our results.

Acknowledgment

The study protocol was approved by the Dışkapı Education and Research Hospital Ethics Committee (registration number 2015-008), and financially supported by the Yıldırım Beyazıt University Scientific Research Project Coordination Unit (Project No: 898).

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