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Single-Nucleotide Polymorphisms on the *RYD5* Gene in Nasal Polyposis

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Nasal polyposis (NP) is a chronic inflammatory disease. Several genes play major roles in the pathophysiology of the disease. We analyzed *RYD5* gene polymorphisms to determine the effect of these variants or their genetic combinations on NP. We genotyped the *RYD5* gene in 434 participants (196 patients with NP and 238 controls). Data were analyzed with SPSS, SNPStats, and multifactor dimensionality reduction (MDR) software. We genotyped 10 single-nucleotide polymorphisms (SNPs) in the *RYD5* gene. *RYD5* (+152G > T) (p.Gly51Va) has not been reported previously. The PolyPhen and PROVEAN predicted the missense mutation as deleterious, but sorting intolerant from tolerant (SIFT) did not. In the genotype analysis, we found that four SNPs (*RYD5* [−264A > G], [−103G > A], [+57-14C > T], and [+66A > G]) were significantly associated with NP. The individuals with combined genotypes of six risk alleles (*RYD5* −264G, −103A, +13C, +57-14T, +66G, and +279T) had significantly higher risks for NP compared with the ones with one or four risk alleles. Haplotype analysis revealed that the two haplotypes were associated with risk of NP. As indicated by MDR analysis, *RYD5* (−264A > G and −103G > A) and *RYD5* (−264A > G, −177C > A, and −103G > A) were the best predictive combinations and they had the highest synergistic interaction on NP. In addition, *RYD5* (+13C > T) was significantly associated with increased risk of both NP with asthma and NP with allergy and asthma. Some SNPs and their combinations in the *RYD5* gene are associated with increased probability for developing NP. We emphasize the importance of genetic factors on NP and NP-related clinical phenotypes.

Introduction

Nasal polyposis (NP) appears as a result of chronic inflammation of the sinonasal mucosa. The prevalence of NP in the general population has been estimated as 1–4%, although evidence is insufficient (Fokkens *et al.*, 2012). NP is a multifactorial disease and its etiopathogenesis has been known to be associated with conditions such as allergic rhinitis, allergy, asthma, and aspirin intolerance (Van Zele *et al.*, 2006; Stankovic *et al.*, 2008).

Increased synthesis of proinflammatory leukotrienes and decreased synthesis of anti-inflammatory prostaglandins (PGE2) have been proposed as mechanisms not only for aspirin-sensitive nasal polyps but also aspirin-tolerant chronic rhinosinusitis (CRS) with nasal polyps (Fokkens *et al.*, 2012). Identifying the factors that affect the balance between proinflammatory leukotrienes and anti-inflammatory prostaglandins would contribute significantly to the understanding of the pathogenesis of NP.

Inheritance has been proposed as a possible etiology of NP (Cohen *et al.*, 2006). The human genome project has shown that single-nucleotide polymorphisms (SNPs), microsatellite polymorphisms (particularly those within the regulatory regions of genes), and their combinations have close relationships with disease phenotypes and that genes can serve as disease modifiers by altering expression levels (Collins *et al.*, 1998).

Secretoglobins (SCGBs) represent an interesting family of biologically active small proteins (~10 kDa in humans) that dimerize following their secretion (Taylor *et al.*, 2006). They have been indicated as candidates for a new cytokine family owing to their anti-inflammatory and immunomodulatory functions. The *SCGB* superfamily has been rapidly expanding with the discovery of many new human genes (Mukherjee *et al.*, 1999; Jackson *et al.*, 2011; Lu *et al.*, 2011). Some SCGBs have been associated with a number of disease states involving airways, including asthma, cystic fibrosis, bronchopulmonary dysplasia, and chronic obstructive pulmonary disease, either as contributing agents or biomarkers (Reynolds *et al.*, 2002).

The *RYD5* gene, also known as *SCGB1C1* (secretoglobin, family 1C, member 1), encodes a 95 amino acid secretory protein that belongs to the SCGB family. The *RYD5* gene is located on the human chromosome 11p15.5 (Taylor *et al.*,

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2006) and is expressed in Bowman's glands in the rat nasal olfactory mucosa (Dear *et al.*, 1991). Bowman's glands, also known as olfactory glands, are branched tubuloalveolar serous glands that secrete through ducts to the olfactory surface and their serous secretion serves as a trap and solvent for odoriferous substances (Hayran, 2013). In a study conducted on CRS patients with or without NP, increased *RYD5* expression was only observed in CRS patients with NP. The authors concluded that increased expression of *RYD5* might contribute to the polyp formation (Lu *et al.*, 2011). Those findings indicated that *RYD5* could play a role in NP formation.

The aim of this study was to analyze SNPs of the *RYD5* gene, and to determine the effects of those individual variants, or their genetic combinations on NP.

Materials and Methods

Study population, patient selection, radiological imaging, and laboratory tests

Blood samples were obtained from 434 participants (196 patients with NP and 238 control subjects). There were 112 males and 84 females in the study group with a mean age of 40.99 ± 11.02 years (range: 21 and 65 years). The mean age of the control subjects was 41.69 ± 11.51 years (range: 17–66 years), and there were 140 males and 98 females (Table 1). There were no differences between NP patients and the controls for age or gender ($p = 0.180$ and $p = 0.320$, respectively). The patients with NP were the consecutive patients who were admitted to the Ankara Numune Education and Research Hospital and Yenimahalle State Hospital Otorhinolaryngology clinics due to nasal obstruction, diagnosed with having nasal polyps, and agreed to participate in the study. NP was clinically diagnosed according to the criteria of European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS), with the presence of two or more symptoms, and the visualization of the polyps bilaterally in the middle meatus on nasal endoscopic examination (Fokkens *et al.*, 2012). Among 196 patients with NP, 67 had a history of previous surgery for nasal polyps.

The control subjects were healthy volunteers and they did not have any history of sinonasal diseases, chronic periodontal disease, inflammatory bowel disease, cancer, sepsis, or any other chronic inflammatory disorders. Absence of NP was considered when symptoms neither suggested rhinosinusitis nor were nasal polyps seen on nasal endoscopy.

The paranasal sinus CT scans were obtained and scored according to the Lund-Mackay system (Lund and Mackay, 1993). This system scores the opacification of each paranasal sinus as follows: 0: no abnormalities; 1: partial opacification; or 2: total opacification. The ostiomeatal complex is scored as 0 (no occlusion) or 2 (occlusion).

The polyp size was classified following Lildholdt's classification: 0: no polyps; 1: polyps only in the middle meatus (small polyps not reaching the upper edge of the inferior turbinate); 2: polyps that reached the upper surface of the inferior turbinate; and 3: severe polyposis or polyps that completely obstructed the nasal cavity (large polyps reaching the lower edge of the inferior turbinate) (Lildhold *et al.*, 1997; Fokkens *et al.*, 2012).

The patient was regarded as asthmatic in case of a positive history for asthma or if he/she was diagnosed with having asthma after consultation with the Pulmonology Department.

Skin prick tests to determine allergy were performed according to the recommendations of the European Academy of Allergy and Clinical Immunology (EAACI, 1993), using the Quintest multiple skin prick test device (Hollister-Stier Laboratories LLC, Spokane, United Kingdom) in all patients diagnosed with having NP. The patients were tested for sensitivity to 18 allergens (ALK Abello, Madrid, Spain) commonly seen in our geographic area. Skin prick tests were considered positive if at least one allergen elicited a wheal reaction >3 mm in diameter after subtraction of the diameter of the wheal produced by the negative control. The patient was considered allergic if he/she had at least one positive skin prick test result. Total serum immunoglobulin (Ig) E concentration was determined with the nephelometric assays method (Dade Behring/Siemens) (Wittig *et al.*, 1980).

The exclusion criteria were the presence of an antrochoanal polyp, cystic fibrosis, inverted papilloma, and fungal sinusitis.

All participants were informed about the study and their written and verbal informed consents were obtained. The study was approved by the Ethics Committee of the Ankara Numune Education and Research Hospital, Ankara, Turkey.

Genotyping

Genomic DNA was extracted from the blood samples of 217 participants using the NucleoSpin blood DNA kit (Macherey-Nagel GmbH & Co. Kg). For direct sequencing, genomic DNA was amplified using polymerase chain reaction (PCR) (SuperHot Master Mix; Bioron GmbH). The

Table 1. Clinical Features of Subjects

Clinical features	*Patients with nasal polyposis* (n = *196*)	*Controls* (n = *238*)	p
Age, years	40.99 ± 11.02	41.69 ± 11.51	0.180
Gender, M/F	112/84	140/98	0.320
IgE, μg/L	15.29 ± 10.01	9.0550 ± 6.33587	< **0.001**
Computed tomography score	9.49 ± 4.68 (3–19)	—	NA
Asthma (+), *n* (%)	70 (36)	0 (0)	< **0.001**
Allergy (+), *n* (%)	74 (38)	0 (0)	< **0.001**
Polyp size, *n* (%)			
1	74 (38)	0 (0)	< **0.001**
2	70 (36)	0 (0)	< **0.001**
3	52 (27)	0 (0)	< **0.001**

Boldface indicates $p < 0.05$ was considered as statistically significant.
NA, not analyzed.

Table 2. Used Primers for Polymerase Chain Reaction Amplification of the *RYD5* Gene

Primers	*Nucleotide sequence (5′-3′)*	*Region*	*Product size (bp)*
F	AAAGAAAGGCGTGGGACCAACC	Exon1	542
R	CAGGTGGAGTGTTCACTGCAGAGG		
F	GAGGAGAGGTGGGCATTGAAGG	Exon2	446
R	GTGCAATGTCTGTGGGTGGTGG		
F	CCACTGAGGGCCTTGCTTGC	Exon3	264
R	CAGAGACAGGAGCCTGAGCTGC		

bp, base pairs; F, forward primer; R, reverse primer.

primers are summarized in Table 2. A commercial kit was used for purification of PCR products (NucleoFast 96 PCR; Macherey-Nagel GmbH & Co. Kg). The PCR products were sequenced with an ABI PRISM 3130 genetic analyzer (Applied Biosystems), and sequence data were analyzed using SeqManII software (Applied Biosystems).

In silico *analyses*

We selected an exonic variant that caused amino acid alterations due to the important role of nonsynonymous SNPs (nsSNPs) in protein function and to be able to predict the functional role of the SNP by using a web-based software. Sorting intolerant from tolerant (SIFT) (Ng and Henikoff, 2003) algorithm, Polymorphism Phenotyping (PolyPhen) (Adzhubei *et al.*, 2010), and the Protein Variation Effect Analyzer (PROVEAN) (Choi *et al.*, 2012) programs were used to predict the functional effect of the identified single-nucleotide change.

Since SNPs in the promoter region can affect promoter activity as nucleotide change may alter the binding affinity of the transcriptional factor involved in the regulation of gene expression (Garcia-Barcelo *et al.*, 2005), *in silico* search for putative transcription factor-binding elements harbored by the *RYD5* promoter polymorphisms was done using the software TFSEARCH (V1.3) as the *in silico* predictions program with a default threshold score of 85.0 (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer *et al.*, 1998).

Statistical analyses

Statistical Package for Social Sciences, version 11.0 (SSPS, Inc.), was used for statistical analysis. The frequency of each *RYD5* genotype was tested for concordance with Hardy–Weinberg equilibrium (HWE) using χ^2 (Trikalinos *et al.*, 2006).

SNPStats (http://bioinfo.iconcologia.net/index.php?module=Snpstats) was used to determine the degree of pairwise linkage disequilibrium (LD) for SNPs and for haplotype analysis (Sole *et al.*, 2006). This software was regressed in a logistic model, assuming the codominant (major homozygotes versus heterozygotes versus minor homozygotes), the dominant (major homozygotes versus heterozygotes plus minor homozygotes), and the recessive (major homozygotes plus heterozygotes versus minor homozygotes) models of inheritance with covariates. Risk estimates were expressed as the odds ratio (OR) and 95% confidence interval (95% CI).

A promising data mining analytical approach, the multifactor dimensionality reduction (MDR) software package (version 1.0.0, available at www.epistasis.org), was employed in all possible interactions among *RYD5* genotypes and adjusted for sex, allergy, IgE level, CT, asthma, and polyp size as covariates. MDR has been applied for the identification of gene–gene and SNP-SNP interactions that are well recognized as playing important roles in understanding complex traits, such as disease susceptibility (Yang *et al.*, 2010; Naushad *et al.*, 2011). This software is a nonparametric (no parameters are estimated) and model-free (no genetic model is assumed) method designed to detect interactions in case–control studies in the absence of significant main effects and has emerged as one of the powerful methods for detecting statistical interactions in genetic association studies (Ritchie *et al.*, 2001; Hahn *et al.*, 2003). This approach aims to construct all possible combinations of examined polymorphisms and selects the overall best model. The accuracy of each model is evaluated by a Bayes classifier in the context of 10-fold cross-validation. A single best model simultaneously has the maximum testing accuracy and cross-validation consistency (CVC) (a measure of the number of times of 10 divisions of the data set that the best model is extracted). Statistical significance was evaluated using a 1000-fold permutation test to compare the observed testing accuracy with the expected one under the null hypothesis of null association. Permutation testing corrects for multiple testing by repeating the entire analysis on 1000 data sets that are consistent with the null hypothesis (Ritchie *et al.*, 2003). For all analyses, $p < 0.05$ was considered as statistically significant.

Results

Genetic analyses

We identified 10 polymorphisms, which are summarized in Table 3. Five SNPs were identified at positions −264, −177, −103, −49, and −35 in the promoter region of the *RYD5* gene. The other SNPs were identified in the +13 position of the exon1 region, +57-14 position of the intron1 region, +152 position of the exon2, +66 position of the exon2 region, and +279 position of the exon3 region of the *RYD5* gene. These SNPs were previously reported and registered in the dbSNP database (Short Genetic Variations Database, http://www.ncbi.nlm.nih.gov/snp), except *RYD*5 (+152G > T).

Association between individual SNPs, genotypes, and haplotypes of the RYD5 *and risk of NP*

The primary information and allele frequencies observed are listed in Table 3. All genotype distributions of control subjects were consistent with the ones expected from the HWE (all $p > 0.05$).

On individual SNP analysis, there were significant differences between NP patients and the controls for the genotype

Table 3. Results from Genotyping for the *RYD5* Gene

Locus	*SNP ID*	*Region*	*Allele change*	*Amino acid change*	*Allele*	*MAF*			p^2 *for HWE*[b]	*Genotyped (%)*
						Case	*Control*	*Database*[a]		
−264	rs113795008	Promoter	C[A/G]T	—	G	0.25	0.11	0.11	0.0051	72
−177	rs535294582	Promoter	C[C/A]A	—	A	0.01	0.4	0.0018	1	94
−103	rs2280540	Promoter	C[G/A]G	—	A	0.36	0.22	0.24	0.79	54
−49	rs144999256	Promoter	G[G/A]C	—	A	0.01	0.00	0.0166	1	99
−35	rs148962288	Promoter	G[G/A]A	—	A	0.01	0.4	NA	1	95
+13	rs7951297	Exon 1	C[C/T]G	Arg5Cys	T	0.46	0.53	0.2662	0.58	29
+57–14	rs2294083	Intron 1	T[C/T]G	—	T	0.32	0.22	0.24	0.79	57
+66	rs2294082	Exon 2	C[A/G]G	Thr22Thr	G	0.29	0.2	0.24	0.39	61
+152	NA	Exon 2	G[G/T]C	Gly51Vln	T	0.025	0.00	NA	1	99
+279	rs61997072	Exon 3	A[C/T]G	Asp93Asp	T	0.21	0.16	0,12	0.16	69

[a]MAF from the HapMap databases (http://www.hapmap.org) or NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp).
[b]HWE *p*-value in the control.
HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequencies; NA, not available; SNP ID, single-nucleotide polymorphism accession number or NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp).

frequencies of four SNPs (*RYD5* [−264A>G], [−103G>A], [+57-14C>T], and [+66A>G]) ($p=0.001$, $p=0.002$, $p=0.031$, and $p=0.023$, respectively) (Table 4). The *RYD5*−264GG and AG genotypes were associated with a significantly higher risk for NP (OR=3.65, 95% CI=1.21–11.04; and OR=2.91, 95% CI=1.45–5.82). Similar associations were found in the genotypes of *RYD5*−103AA, +57-14TT, and +66GG (OR=5.14, 95% CI=1.91–13.83; OR=3.65, 95% CI=1.33–10.05; and OR=3.13, 95% CI=1.12–8.75, respectively). We found that the dominant models of *RYD5*−264 (AG+GG/AA) and *RYD5*−103 (GA+AA/GG) and the recessive models of *RYD5*−103 (GG+GA/GG), +57-14 (CC+CT/TT), and +66 (AA+AG/GG) showed significant associations with NP (OR=3.08, 95% CI=1.66–5.74; and OR=1.87, 95% CI=1.09–3.21; OR=4.53, 95% CI=1.73–11.85; OR=3.40, 95% CI=1.27–9.14; and OR=2.88, 95% CI=1.05–7.89, respectively).

The frequencies of the *RYD5*−264G (0.25 vs. 0.11), −103A (0.36 vs. 0.22), +13C (0.54 vs. 0.47), +57-14T (0.32 vs. 0.22), +66 G (0.29 vs. 0.20), and +279 T (0.21 vs. 0.16) alleles were significantly different in NP patients when compared with the controls, but other SNPs were not ($p=0.001$ for all) (Table 4).

Considering the potential interactions of these six SNPs on the risk of NP, we combined them based on the numbers of variant (risk) alleles (*RYD5*−264G, −103A, +13C, +57-14T, +66G, and +279T). The combined genotypes with these six variant (risk) alleles (GACTGT, respectively) had high risks for NP (OR=17.63, 95% CI=17.10–19.16), the ones with one variant (risk) allele (G——, respectively) and four variant (risk) alleles (-ACTG-, respectively) had lower risks for NP (OR=15.03, 95% CI=1.71–131.85; and OR=3.06, 95% CI=1.17–8.02, respectively). In our study, the distributions of these combined genotypes differed significantly between the NP cases and controls ($p=0.0001$).

The analysis revealed that *RYD5*−103, +13, +57-14, +66, and +279 had high pairwise LD (all $D'>0.75$). A haplotype analysis was performed, including 10 SNPs, and it was found that there were >100 possible haplotypes derived from the known genotypes. Haplotypes with a frequency of <0.01 in the cases and the controls were pooled into a single group, and the remaining 10 haplotypes were analyzed (Table 5). The frequencies of GCAGGCTGGT and ACAGGCTGGC haplotypes in NP cases and GCGGGTCAGC haplotype in controls were significantly higher ($p=0.0001$, OR=30, 95% CI=20.74–40.96; $p=0.028$, OR=3.06, 95% CI=1.13–8.27, and $p=0.021$, OR=13.42, 95% CI=1.51–119.50) than the common haplotype ACGGGTCAGC.

Association between genotypes and haplotypes of the RYD5 *and NP-related phenotypes*

The frequencies of the *RYD5*−103GA, +13CC genotype, and ACAGGCTGGT haplotype were higher in asthmatic patients compared with those without (OR=2.75, 95% CI=1.06–7.15; OR=0.20, 95% CI=0.06–0.66; OR=3.19, 95% CI=1.23–8.27; $p=0.002$, respectively).

The frequencies of *RYD5*−264AG, +279TT genotype, and GCGGGTCAGC haplotype were significantly higher in allergic patients compared with nonallergic patients (OR=10.43, 95% CI=1.18–92.15; OR=3.29, 95% CI=1.29–8.41; OR=5.66, 95% CI=1.06–30.28; $p=0.0046$, respectively).

No significant association was shown between total IgE levels, polyp size, CT, and *RYD5* genotypes and haplotypes in NP cases (all $p>0.05$; data not shown).

SNP-SNP interactions

In light of the significant findings in the haplotype analysis, it is of great interest to explore the potential interaction of 10 examined polymorphisms in the *RYD5* gene. To achieve this goal, a promising data mining analytical approach, MDR, was employed. Each best model across all possible combinations is assessed by the testing balanced accuracy (TBA), CVC, and significance level.

MDR analysis found two predictive models for NP. A two-SNP interaction between *RYD5* (−264A>G) and *RYD5* (−103G>A) was detected with a CVC of 10/10 and a TBA of 63.19%. The GG+GA and GG+AA genotypes were more common in patients with NP (OR=3.56, 95% CI=2.00–6.32, $p=0.001$) (represented as dark gray boxes in Fig. 1). We detected a three-SNP interaction between *RYD5* (−264A>G), *RYD5* (−177C>A), and *RYD5* (−103G>A) (TBA=0.606, CV=8/10, OR=3.85, 95% CI=2.16–6.85, $p=0.001$). The

Table 4. Frequencies of *RYD5* Single-Nucleotide Polymorphism Genotype and Alleles

SNPs	*Genotype/ Allele*	*Cases (*n = *196),* n *(%)*	*Controls (*n = *238),* n *(%)*	*Models*	*OR (95 CI)*	p-*Value*
RYD5 (−264A > G)	AA	118 (60)	196 (82)	Codominant	1.00 (reference)	**0.001**
	AG	56 (29)	32 (14)		**2.91 (1.45–5.82)**	
	GG	22 (11)	10 (4)		**3.65 (1.21–11.04)**	
	AA	118 (60)	196 (82)	Dominant	1.00 (reference)	**0.04**
	AG-GG	78 (40)	42 (18)		**3.08 (1.66–5.74)**	
	AA-AG	174 (89)	228 (96)	Recessive	1.00 (reference)	**0.048**
	GG	22 (11)	10 (4)		**2.88 (0.97–8.60)**	
	G[a]	0.25	0.11			**0.001**
RYD5 (−177C > A)	CC	192 (98)	218 (92)	Codominant	1.00 (reference)	0.070
	CA	4 (2)	20 (8)		0.23 (0.05–1.06)	
	AA	0 (0)	0 (0)			
	A[a]	0.01	0.04			0.150
RYD5 (−103G > A)	GG	90 (46)	146 (61)	Codominant	1.00 (reference)	**0.002**
	GA	68 (35)	80 (34)		1.38 (0.77–2.49)	
	AA	38 (19)	12 (5)		**5.14 (1.91–13.83)**	
	GG	90 (46)	146 (61)	Dominant	1.00 (reference)	**0.023**
	GA-AA	106 (54)	94 (39)		**1.87 (1.09–3.21)**	
	GG-GA	158 (81)	226 (95)	Recessive	1.00 (reference)	**0.003**
	AA	38 (19)	12 (5)		**4.53 (1.73–11.85)**	
	A[a]	0.36	0.22			0.**001**
RYD5 (−49G > A)	GG	192 (98)	238 (100)	Codominant	1.00 (reference)	0.74
	GA	4 (2)	0 (0)		0.98 (0.01–4.01)	
	AA	0 (0)	0 (0)			
	A[a]	0.01	0			0.203
RYD5 (− 35G > A)	GG	192 (98)	220 (92)	Codominant	1.00 (reference)	0.053
	GA	4 (2)	18 (8)		0.25 (0.05–1.21)	
	AA	0 (0)	0 (0)			
	A[a]	0.01	0.04			0.073
RYD5 (+13C > T)	CC	68 (35)	56 (24)	Codominant	1.00 (reference)	0.17
	CT	74 (38)	112 (47)		0.54 (0.28–1.04)	
	TT	54 (28)	70 (29)		0.64 (0.31–1.29)	
	CC	68 (35)	56 (24)	Dominant	1.00 (reference)	0.07
	CT-TT	128 (65)	182 (77)		0.58 (0.32–1.05)	
	CC-CT	142 (73)	168 (71)	Recessive	1.00 (reference)	0.76
	TT	54 (28)	70 (29)		0.91 (0.50–1.65	
	T[a]	0.46	0.53			**0.001**
RYD5 (+57-14C > T)	CC	100 (51)	146 (61)	Codominant	1.00 (reference)	**0.031**
	CT	66 (34)	80 (34)		**1.20 (0.67–2.16)**	
	TT	30 (15)	12 (5)		**3.65 (1.33–10.05)**	
	CC	100 (51)	146 (61)	Dominant	1.00 (reference)	0.13
	CT-TT	96 (49)	92 (39)		1.52 (0.89–2.62)	
	CC-CT	166 (85)	226 (95)	Recessive	1.00 (reference)	**0.01**
	TT	30 (15)	12 (5)		**3.40 (1.27–9.14)**	
	T[a]	0.32	0.22			**0.001**
RYD5 (+66A > G)	AA	108 (55)	156 (66)	Codominant	1.00 (reference)	**0. 023**
	AG	62 (32)	70 (29)		1.28 (0.71–2.32)	
	GG	26 (13)	12 (5)		**3.13 (1.12–8.75)**	
	AA	108 (55)	156 (65)	Dominant	1.00 (reference)	0.12
	AG-GG	88 (45)	82 (35)		1.55 (0.90–2.68)	
	AA-AG	170 (87)	226 (95)	Recessive	1.00 (reference)	**0.032**
	GG	26 (13)	12 (5)		**2.88 (1.05–7.89)**	
	G[a]	0.29	0.20			**0.001**
RYD5 (+152G > T)	GG	195 (99)	238 (100)		1.00 (reference)	0.21
	GT	1 (1)	0 (0)	Codominant	0.99 (0.01–4.5)	
	T[a]	0.025	0			0.452
RYD5 (+279C > T)	CC	126 (64)	174 (73)		1.00 (reference)	0.34
	CT	56 (29)	54 (23)	Codominant	1.43 (0.77–2.66)	
	TT	14 (7)	10 (4)		1.93 (0.59–6.37)	
	CC	126 (64)	174 (73)	Dominant	1.00 (reference)	0.16
	CT-TT	70 (36)	64 (27)		1.51 (0.85–2.69)	
	CC-CT	182 (93)	228 (96)	Recessive	1.00 (reference)	0.35
	TT	14 (7)	10 (4)		1.75 (0.54–5.71)	
	T[a]	0.21	0.16			**0.001**

Boldface indicates $p < 0.05$ was considered as statistically significant.
[a]Assumed risk alleles.
CI, confidence interval; *n* (%), frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.

Table 5. Associations Between Risk of Nasal Polyposis and Frequencies of Haplotypes on the Basis of the Observed *RYD5* Genotypes Among Cases and Controls

Haplotypes[a]	*Haplotype frequencies*		*OR (95% CI)*[b]	p-*Value*
	Cases	*Controls*		
A-C-G-G-G-T-C-A-G-C	0.4282	0.4824	1.00	—
A-C-A-G-G-C-T-G-G-T	0.1573	0.1555	1.28 (0.73–2.24)	0.38
A-C-G-G-G—C-C-A-G-C	0.117	0.1602	0.45 (0.20–1.02)	0.057
G-C-G-G-G-C-C-A-G-C	0.1096	0.0919	1.52 (0.83–2.76)	0.17
A-C-A-G-G-C-T-G-G-C	0.0464	0.0297	**3.06 (1.13–8.27)**	**0.028**
G-C-G-G-G-T-C-A-G-C	0.0253	0.0378	**13.42 (1.51–119.50)**	**0.021**
A-A-G-G-A-T-C-A-G-C	0.025	0.005	0.00 (–Inf–Inf)	1
A-C-A-G-G-C-T-A-G-C	0.0204	0.021	1.32 (0.30–5.69)	0.71
G-C-A-G-G-C-T-G-G-T	0.0174	0.090	**30 (20.74–40.96)**	**< 0.001**
G-C-A-G-G-C-T-G-G-C	0.0161	0.0123	1.95 (0.26–14.75)	0.52

Boldface indicates $p < 0.05$ was considered as statistically significant.
[a]The alleles of haplotypes were arrayed as the location of the SNPs in *RYD5*.
[b]In logistic regression model.
Inf, indefinite.

differences between cases and controls were significant in *RYD5* GG + CC + AA, GG + AA + GG, and GG + AA + GG genotypes.

MDR analyses showed that *RYD5* +13CC and CT genotypes were higher in asthmatic females with NP when compared with NP patients without asthma and they were significantly associated with the diagnosis of asthma (TBA = 0.703, CVC = 5/10, OR = 15.87, 95% CI = 8.26–30.50, $p = 0.001$). Additionally, the patients with NP with the presence of *RYD5* + 13CT and allergy were associated with asthma (TBA = 0.686 and CVC = 8/10, OR = 9.01, 95% CI = 4.88–16.64, $p = 0.001$; Fig. 2).

There were no associations between the extra combinations of other SNPs and gender, serum total IgE value, CT score, or polyp size. The other SNP combinations had lesser synergistic effects compared with their single main effects.

RYD5 *(+152G > T) (pGly51Val) mutation analysis*

In this study, direct sequencing of the *RYD5* gene showed a heterozygous point mutation *RYD5* (+152G > T) in exon2 (using GenBank X60661 as reference sequence and starting with +1 at the A of the ATG translation initiation codon), which leads to an amino acid change, GGC(Gly) to GGT (Val) at position 5: p.Gly51Val (the amino acid residues are numbered starting with the amino-terminal glycine acid residue of the mature RYD5 as number +1), in 1 of 196 NP patients (Fig. 3).

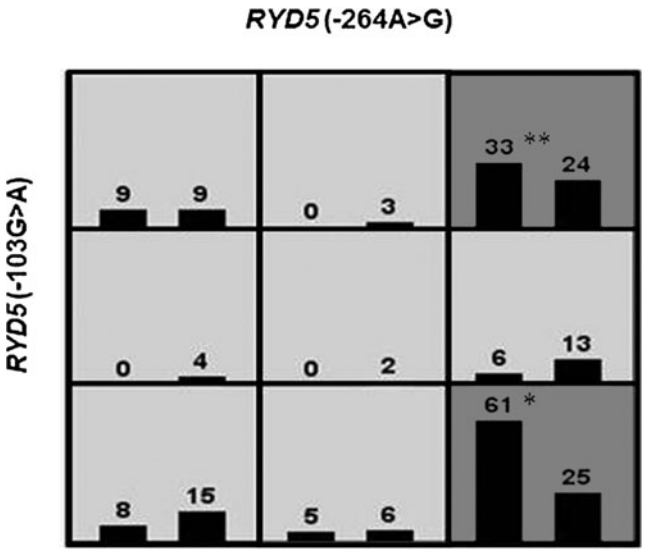

FIG. 1. The two-locus *RYD5* (–264A > G) and *RYD5* (–103G > A) genotype combinations associated with high risk and low risk for NP. The *RYD5* GG + GA* and GG + AA** genotypes had a 2.4-fold and 1.3-fold increased risk for NP. For each genotype combination, the number of cases is displayed in the *left bar*, while the number of controls is displayed in the *right box*. *Darker shade* indicates the high-risk group. Note that the pattern of high and low risk for the *RYD5* (–103G > A) differs depending on the value of the *RYD5* (–264A > G) (TBA = 0.631, CVC = 10/10, $p = 0.001$, OR = 3.52). CVC, cross-validation consistency; NP, nasal polyposis; OR, odds ratio; TBA, testing balanced accuracy.

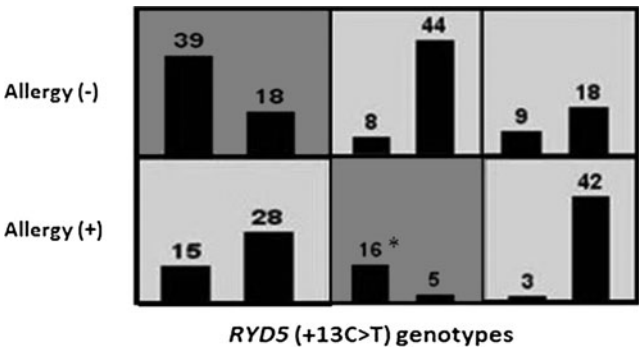

FIG. 2. The *RYD5* (+13C > T) genotypes and allergy combinations associated with high risk and low risk for asthma. The *RYD5* +13CT* genotype had a 3.2-fold increased risk of NP and allergy and asthma. For each genotype combination, the number of patients with asthma is displayed in the *left bar*, while the number of patients without asthma is displayed in the *right box*. *Darker shade* indicates the high-risk group. The pattern of high and low risk for the *RYD5* (–103G > A) differs depending on the presence of the allergy (negative or positive skin prick test) (TBA = 0.686, CVC = 8/10, $p = 0.001$, OR = 9.01).

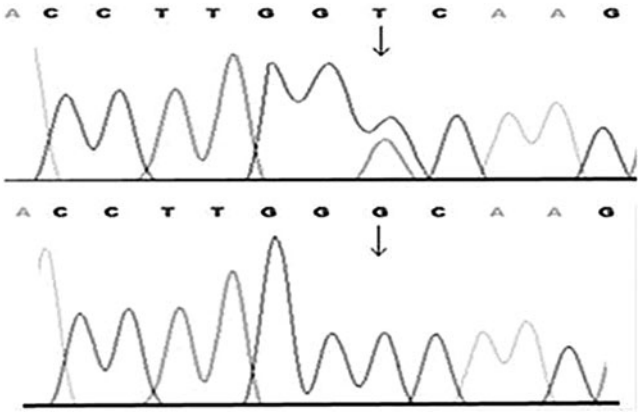

FIG. 3. Electropherogram of the direct sequence of the exon2 *RYD5* gene. Heterozygous novel mutation (c.152 G > T) in DNA from the patient's blood sample (*top*). Wild-type sequence corresponding to a healthy control DNA (*bottom*).

Clinical–pathological characteristic of the NP Case with RYD5 *mutation*

The *RYD5* (+152G > T) (pGly51Val) variant was detected in one 51-year-old NP patient and not detected in the 238 controls. The patient's medical data showed that the patient had a positive prick test, polyp size 3, and underwent two operations (revision surgeries). This variant has not been reported before in the open access mutation database or literature, and the parents of the probands are not available for mutation analysis.

In silico *predictions of functional impact of* RYD5 *SNPs*

The *RYD5* (+152G > T) polymorphism is a missense mutation that causes a residue change in the *RYD5* gene product (pGly51Vln) and it might impair RYD5 protein function. The PolyPhen and PROVEAN analyses indicated that this variant is a possibly damaging protein function, but the SIFT analysis predicted this variant to be tolerant (score 0.08). In addition, the RYD5 Gly51 position throughout the orthologs was conserved.

Three of five SNPs were identified in the promoter region of the *RYD5* gene and were located in sequences with high homology to transcription factor-binding motifs by TFSEARCH, and *RYD5* (−264A > G) (p300, score 85.1), (−177C > A) (MyoD, score 100), and (−103G > A) (Skn-1, score 86.3) were suggested to form new binding sites. However, *RYD5* (−35G > A) and (−49G > A) had no predicted effect on transcription factor-binding sites.

Discussion

NP is a chronic inflammatory disease (Van Zele *et al.*, 2006; Stankovic *et al.*, 2008; Fokkens *et al.*, 2012). It has been known that the cytokine-driven regulation of expression in the SCGB superfamily in the airways plays a role in the pathogenesis of some diseases, such as asthma, rhinitis, and NP (Dear *et al.*, 1991; Mukherjee *et al.*, 1999; Jackson *et al.*, 2011; Lu *et al.*, 2011; Pala *et al.*, 2014). Similar to other secretory proteins, RYD5 protein is a member of the SCGB superfamily and it has a hydrophobic N-terminal region with a 14 amino acid signal peptide sequence, which is required for its channeling into the target organelle (Dear *et al.*, 1991; Lu *et al.*, 2011). As it leads to the signal peptide domain, it probably affects the function of the signal peptide motifs of RYD5 protein that is expressed in nasal mucosa (Arg5Cys) (Dear *et al.*, 1991). In addition, it was shown that IFN-gamma downregulated and TH2 cytokines, namely IL-4 and IL-13, upregulated mRNA expression of *RYD5* in patients with CRS with NP, but not in CRS patients without NP (Lu *et al.*, 2011). Therefore, it may be postulated that that *RYD5* may be a modifying factor for NP. Our study is the first one in the literature that investigated SNPs of the *RYD5* gene in patients with NP.

In this study, we found that the allele distributions of six SNPs and genotype frequencies of four SNPs in the *RYD5* gene were significantly associated with NP. Moreover, we observed associations of *RYD5* (+13C > T) in patients with allergy, asthma, and NP.

Five polymorphisms were previously identified in the promoter region of the *RYD5* gene. A > G, C > A, G > A, G > A, and A > G substitutions were found at the base pair (bp) positions −264, −177, −103, −49, and −35, respectively (Kim *et al.*, 2009; The 1000 Genome Consortium, 2010). We found that no single RYD5 SNP, (−177C > A), (−49G > A), or (−35G > A) was associated with NP. The RYD5 − 264 (AG, GG, G-dominant) and −103 (AA and A-dominant or recessive) genotypes were associated with NP. In addition, RYD5 − 264G and −103A allele and the combined genotypes with other four variant (risk) alleles increased the risk for NP.

SNPs in the coding regions of genes (cSNPs) or in the regulatory regions are more likely to cause functional differences when compared with SNPs in other regions. Therefore, the potentially functional *RYD5* promoter polymorphisms could alter transcriptional activity, affecting susceptibility to develop NP (Garcia-Barcelo *et al.*, 2005). To date, no studies have investigated the *RYD5* promoter SNPs for their functional roles or their contribution for developing NP or any other diseases. In this study, we reported *RYD5* promoter polymorphisms for the first time and showed that the *RYD5* (−264A > G), (−177C > A), and (−103G > A) *in silico* analysis identified prediction of putative transcription factor-binding sites. Therefore, we suggested that these promoter polymorphisms could alter the binding affinity and affect promoter activity. Whether any of these or other transcription factors/repressors bind and regulate the activity of the *RYD5* promoter *in vivo* needs further investigation.

We identified one polymorphism at position +57-14C > T in intron1 of the *RYD5* gene. We found that the *RYD5* +57-14 TT and +57-14 (CC + CT/TT) genotype in the recessive model had an increased risk for NP. In addition, the frequency of +57-14T allele and the combined genotypes with other five variant (risk) alleles had increased risks for NP. Approximately, 15% of disease-causing SNPs directly affect pre-mRNA splicing. Single base substitutions localized at the exon–intron boundaries can impair one of the cis-transcriptional elements known as exonic splicing enhancers and thereby affect normal pre-mRNA splicing. Splice site nucleotide changes may also result in exon skipping, in the activation of cryptic splice sites, in the creation of a pseudoexon within an intron, or in intron retention (Krawczak *et al.*, 1992). However, proper interpretation of the effects of polymorphisms might be difficult, especially when they result in noncoding variants (Hirschhorn and Daly, 2005). We suggest that the *RYD5* +57-14 located in the exon–intron

boundaries may affect the exon splicing ability and mRNA transcription and contribute to the risk of NP. Further functional analysis with RNA splicing assay should be carried out to verify the effect of the variants at the mRNA level.

In exon1 of the *RYD5* gene, we identified an nsSNP at position +13C > T (p.Arg5Cys). This polymorphism corresponds to the residue 5 of the RYD5 protein's sequence and leads to an arginine–cysteine substitution (PolyPhen score: 0.093 and SIFT score: 0.18) (Wheeler, 2008). We found that *RYD5* +13C allele and the combined genotypes with other five variant (risk) alleles increased the risk for NP, and *RYD5* − 103GA and +13CC genotypes had risk for asthma. On the other hand, *RYD5* − 264AG and +279TT genotypes were found to be associated with allergy in patients with NP. This association was also detected in the haplotype analysis.

It was previously reported that allergy and asthma affect upper and lower airways where mucosa shows similarities, they might share a common genetic background, and the phenotypes of NP are well documented in both diseases (Barnes, 2000; Ober and Hoffjan, 2006; Fokkens *et al.*, 2012). In our study, we found that some SNPs in the regulatory regions of *RYD5* increased risk of NP, asthma, and allergies and they may be responsible for the molecular mechanisms underlying these phenotypes. The MDR analysis showed that *RYD5* (+13C > T) and its combinations had close relationships with NP and asthma or allergies. The patients with NP carrying *RYD5* +13CC or CT genotypes were associated with a ∞-fold increased risk for asthma and this association was stronger in females. In addition, the *RYD5* +13CT genotype and allergy had a 3.2-fold increased risk of asthma. It also seemed that the association between *RYD5* +13CT and asthma with NP resulted from the allergic component in these patients. This finding indicates that the effect of *RYD5* (+13C > T) genotype on allergic inflammation may show different patterns based on gender and allergic status in asthmatic patients with NP.

In exon2 and exon3 of the *RYD5* gene, the A > G and C > T substitutions were found at +66 and +279 bp positions, respectively. *RYD5* (+66A > G) (p.Thr22Thr) polymorphism alters the 22nd codon from CAG to CGG, both of which code for the amino acid, threonine. In addition, *RYD5* (+279C > T) (p.Asp93Asp) causes a change in the 93rd codon from ACG to ATG, both of which code for the amino acid, aspartate (Kim *et al.*, 2009). We found that the frequency of *RYD5* +66 (AA and G-recessive) genotypes was higher in patients with NP, but the +279 genotype did not differ between the study and the control groups +66G and +279T, and the combined genotypes with other four variant (risk) alleles may be at higher risk for NP. These polymorphisms are synonymous SNPs, which do not change the amino acid sequence or missense, and they may alter the *RYD5* mRNA folding, mRNA stability, and translation ([p.Thr22Thr] [p.Asp93Asp]) (Duan *et al.*, 2003). Those SNPs located in the functional domain of the *RYD5* gene may influence the function of RYD5 protein in the pathogenesis of NP and allergy.

Rare variants often have functional effects on protein–protein interactions (Duan *et al.*, 2003). Furthermore, rare variants that have been reported in several diseases might confer a stronger increase in disease risk compared with common variants and may make a substantial contribution to the multifactorial inheritance of common chronic diseases (Stenson *et al.*, 2003; Bodmer and Bonilla, 2008). In our study, the sequencing of exon2 of the *RYD5* gene revealed a heterozygous missense mutation +152G > T (p.Gly51Vln). This variant changes glycine to valine at position 51 and it was only identified in a 51-year-old patient with NP who had a positive prick test, polyp size 3, and had two previous surgeries. We are not certain whether it is a *de novo* mutation since the parents of the probands are not available for mutation analysis. This variant seems to be a rare variant with an allele frequency of 0.02% among NP patients as it has been detected in only one patient with NP in our study. However, the variants observed in a particular ethnicity may vary significantly, just as it may vary from one population to the other. Certain changes identified as mutations with high allele frequencies in a given population (or considered as high nucleotide polymorphism changes) may have very low frequencies in other populations or may not be detected at all.

A number of missense mutations alter the amino acid sequence of expressed proteins and have been associated with disease states, such as cancer, diabetes, and cystic fibrosis (Stenson *et al.*, 2003). According to *in silico* analysis, the PolyPhen and PROVEAN predicted these exonic variants as deleterious, but the SIFT predicted it as being tolerant. In our study, the amino acid glycine at position 51 is important and has been conserved throughout the orthologs. The RYD5 dimer forms an internal hydrophobic cavity; therefore, this residue may be critical to conserve this form and cannot be replaced by a branched-chain amino acid. Consequently, this amino acid change is likely to affect the ligand activity of RYD5 through the modification of the protein structure and may contribute to the susceptibility for NP. These bioinformatic tools are useful to narrow down the candidate mutations. PolyPhen-PROVEAN (63%) and SIFT (79%) have correct prediction rates (Choi *et al.*, 2012; Gray *et al.*, 2012). On the other hand, the low frequency of this variant suggests that this may be due to the small number of patients enrolled in the current study. Therefore, further *in vitro* functional analyses should be conducted to elucidate the pathogenic role of this polymorphism in larger study cohorts.

Haplotype analysis may be more informative regarding the effect of a genetic interaction on a disease phenotype when compared with SNP analysis (Collins *et al.*, 1998). In the current study, we found that two haplotypes carrying mutant alleles of *RYD5* − 264, −103, +13, +66, +57-14, and +279 might account for susceptibility to NP. However, the GCGGGTCAGC haplotype carrying the −264G allele may have the potential to protect against NP. Haplotypes carrying *RYD5* − 103A, +13C, +57-14T, and +66G alleles were found to have significantly increased risks for NP. In addition, these findings are consistent with our LD and genotype analysis.

In this study, we found that complex allelic interaction in haplotypes and haplotype analysis can reveal relevant but simple interactions between SNPs; therefore, we used a data mining approach, MDR, for detecting and characterizing combinations of attributes that interact to influence NP and phenotypes (the 1 + 1 = 3 principle). Moreover, this method may be able to detect interactions in the absence of main effects where LD and other approaches cannot (Ritchie *et al.*, 2001; Hahn *et al.*, 2003). According to MDR, we analyzed the best two (−264 and −103) and three (−264, −177 and −103) locus models, which were both significant at $p = 0.001$, and they were regarded as the overall best MDR models in this study. The combinations of *RYD5* − 264GG and −103 A-dominant genotypes had increased risk for NP (2.4-fold and

1.3-fold). The *RYD5* −264, −177, and −103 (GG + AA + AA, GG + AA + GG, and GG + CC + AA) genotypes had a 2-, 1.5-, and ∞-fold increased risk for NP. Analyzed by MDR, these findings are consistent with our genotype analysis. Individual SNPs or the interactions of SNPs were not associated with serum total IgE, polyp size, or CT scores.

On the other hand, our association analyses and MDR provide important additional information on more specific NP and NP-related phenotypes. Furthermore, literature on *RYD5* is somewhat scarce, and the *RYD5* SNP potential relationship with NP and NP-related clinical phenotypes has been studied for the first time in the current study. Our results support previously reported association between *RYD5* and NP formation and the polygenic etiology of this common and complex disease.

Our study has some limitations. Histopathological classification of polyps as eosinophilic and neutrophilic and investigation of allele frequencies in those populations could have yielded more clear results. Our sample size might not be large enough, and the findings may be aleatoric and should be interpreted with caution. In addition, a lack of association between *RYD5* SNPs and some of the NP-related phenotypes may be due to the small number of our patients. As reported before, since the allele and genotype frequencies in small samples might be notably affected, large study cohorts are important to find genetic risk factors (B-Rao, 2001). Moreover, replicating the findings is difficult in different populations in association studies, and thus using phenotypic classifications is important. There may be possible extensions of this study, and we intend to collect representative data and consider using them in our future work.

In conclusion, our study demonstrated that the presence of some SNPs and their combinations in the *RYD5* gene might increase and/or contribute to the susceptibility of developing NP, NP with allergic asthma, and asthma in a Turkish population. Furthermore, *in silico* analyses have shown that a rare variant nsSNP in *RYD5* (+152G > T) might potentially alter the RYD5 protein structure. However, functional studies are needed to elucidate the role of *RYD5* SNPs in the molecular mechanisms underlying NP, allergic asthma, and asthma with NP, and more detailed environmental exposure data are needed to confirm the effect of the genetic basis of NP pathogenesis. In addition, environmental effects may be crucial factors in the progression of NP and NP-related clinical phenotypes, and there is need for further studies focusing on the gene–environment interactions in NP, allergies, and asthma.

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Authors' Contributions

Sibel Özdaş, Afife İzbirak, and Talih Özdaş directly participated in the execution of the study, molecular biology, analysis of gene polymorphism, experiment, and the writing of the manuscript. Kürşat Murat Özcan and Selim S. Erbek participated in study planning, subjects screened, and analysis of the study. Sabri Köseoğlu and Hüseyin Dere participated in blood sample collection.

Disclosure Statement

No competing financial interests exist.

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