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Original Research

Differentially expressed *Secretoglobin 1C1* gene in nasal polyposis

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Abstract: Nasal polyps (NP) are the most common pathological change that occurs in the nasal mucosa and is characterized by mucosal inflammation. Although its etiology and pathogenesis have not been clearly explained, its pathophysiology is arranged by the balance between pro-inflammatory and anti-inflammatory cytokines. The *Secretoglobin 1C1 gene* synthesizes odor molecule binding proteins (OBPs) in the nasal mucosa and regulates some cytokines. The *Secretoglobin 1C1* gene expression could be disrupted by polymorphisms that may act as a possible cause of a disruption in the regulation of the promotor of the gene. Therefore, the main aim of this study was to determine the effects of *Secretoglobin 1C1* gene promotor polymorphisms on the gene expression in NP. In this study, to determine the relationship between the *Secretoglobin 1C1* gene promotor polymorphisms and the gene expression, the levels of 48 subjects were sequenced (24 patients with NP and 22 controls without sinonasal disease). The levels' expression of *Secretoglobin 1C1* in the subjects' nasal mucosa was also detected using RT-PCR. In this study, the level of *Secretoglobin 1C1's* expression increased in NP (P= 0.003). Three polymorphisms were detected in the *Secretoglobin 1C1* gene's promotor. The rs113795008 and rs2280540 variations were significantly high in NP (P= 0.005, P= 0.045). The the rs113795008 homozygous mutant type genotype (G/G) was associated with a high mRNA expression level of *Secretoglobin 1C1* in NP (P= 0.009). A correlation was found between a high level of *Secretoglobin 1C1* expression and its promotor polymorphism, which thus might increase and/or contribute to the susceptibility of developing NP in the Turkish population. These findings suggested that promotor variations in the function of the *Secretoglobin 1C1* gene can alter the gene expression biology in NP.

Key words: Nasal polyps (NP); *Secretoglobin 1C1* gene; Real-Time PCR; Single nucleotide polymorphisms.

Introduction

Nasal polyps (NP) are benign mucosal protrusions that extend toward the nasal cavity and can close the nasal cavity by originating from the epithelium in the middle meatus and ethmoid region. NP constitute the most common pathological change to occur in the nasal mucosa and are characterized by mucosal inflammation (1). NP are frequently accompanied by allergic rhinitis, atopy, aspirin intolerance and asthma (ASA) (2-6).

NP are a clinical indicator for many nested immunological pathways and reflect the local manifestation of systematic disorders (7). Although NP etiology and pathogenesis have not been clearly explained, it is believed that their pathophysiology is arranged by the balance between pro-inflammatory and anti-inflammatory cytokines (8-10). Specifying the factors that affect this balance will provide clarification on the pathogenesis of NP.

It has been stated that NP emerge with genetic diseases, such as cystic fibrosis (CF), primary ciliary dyskinesia, and Youngs and Kartagener's syndrome (11,12). In studies conducted on patients with NP, it has been reported that 13.3-52% of the patients have a positive family history, whereas the mutual interaction of the environment and heritage are significant in rising disease phenotypes, with NP development seen in 52.6% of maternal twins (13-17). Various environmental and genetic factors affect the incidence of the disease phenotype by changing the severity of chronic inflammatory diseases. With the completion of the human genome project, our information about genetic factors has increased and it has been observed that single nucleotide polymorphisms (SNPs) or microsatellite polymorphisms (especially those that take part in the regulatory regions) have a close relationship with disease phenotypes, and genes can change the severity of diseases by changing the expression level of genes (18). The studies conducted on NP improvement revealed a relationship between NP improvement and the SNPs that belong to *HLA* (19,20), *TNF*6 (21), *IL-1A* (22,23), and *AOAH* (13,23) genes. In the studies that investigated gene expression profiles that belong to NP tissue and compared them to a control, it has been stated that there are differentiations in many gene expressions (24-26). Deviations in gene expression are frequently explained by genotypic variations, but epigenetic variations are also responsible. Deviations in gene expression are not only explained by genotypic variations, but also by gene expression biology regulated at various levels, such as post-transcriptional regulation mechanisms involved in the conformation and stability of pre-mRNA, translation initiation, stability and the folding of proteins (27, 28).

The secretoglobin (SCGB) super family involves newfound and rapidly expanding dimeric, biologically active, physiologically and pathophysiologically important proteins (10,25,29). It is a candidate for the creation of a new cytokine family, with its anti-inflammatory and

immunomodulatory functions (24,29,30). The *SCGB* gene super family is generally seen in the organs that include rich secretory epithelium, such as the lungs, breast glands, trachea, prostate, uterus and salivary glands (31). It is an important gene super family whose pathophysiological functions have not been completely understood, have been associated with various disease pathogenesis, and have involved biomarkers that belong to some cancers (29,31). It has been stated that deviations in expression levels on the respiratory tract of the *SCGB* gene super family can be associated with the pathogenesis of chronic respiratory tract illnesses, such as asthma, cystic fibrosis, bronchopulmonary dysplasia (BPD) and chronic obstructive pulmonary illnesses (32). There are eleven genes and five pseudogenes in the human genome in the *SCGB* gene super family (27,30). The *Secretoglobin Family 1C Member 1's* (*Secretoglobin 1C1, SCGB1C, Ligand-binding protein, RYD5*) gene, which belongs to the *Secretoglobin 1C1* sub-family, has been localized on chromosome 11q15.5 (26). As a result of finding a *Secretoglobin 1C1* gene mRNA in the Bowman gland, which is in the olfactory mucosa and secretes mucus, as well as homology studies, it is thought that it binds the hydrophobic odor molecules as odor molecule binding protein (OBP) without a significant specificity (27,34-36).

The previous research has demonstrated that the high IL-5 concentration suppressed the activity of the *Secretoglobin 1C1* in respiratory airway mucosa and caused a significant increase in the *Secretoglobin 1C1* expression level among patients CRS with NP (31,37). Another study has also suggested that the *Secretoglobin 1C1* gene promoter SNPs may increase and/or contribute to the variable risk of development of NP, and emphasized the necessity for functional studies to confirm its potential role (38).

The purposes of the present study were to compare the expression levels of *Secretoglobin 1C1* in normal controls and polyp mucosal samples in patients with NP, and to examine the potential association of *Secretoglobin 1C1* promoter gene polymorphisms with the development of NP.

Materials and Methods

Study population

The study group was chosen from patients who applied to Yenimahalle Education and Research Hospital, and Numune Education and Research Hospital Otolaryngology Clinic. Verbal and written consent was obtained by providing information about the study and methods to the patient and control groups. Ankara Numune Education and Research Hospital Ethics Committee confirmed that the method that was used in the conducted study complied with the human rights and test ethics (ID: 210/2011).

The 24 patients were diagnosed with NP according to the current European Position Paper on Rhinosinusitis and Nasal Polyps and American guideline (EAACI) (39,40). Diseased ethmoid sinus mucosa from the most hypertrophied and hyperemic regions and NP tissues from the apex region of polyps were collected during functional endoscopic sinus surgery (FESC). As controls, the inferior turbinate mucosal samples were obtained during surgery from 22 volunteer patients undergoing septoplasty and/or turbinectomy due to nasal obstruction who did not have any sinus disease or allergic rhinitis. None of the participants had a history of mucopurulent discharge or sinus disease. Freshly obtained tissues and RNA later (Ambion AM7020, Applied Biosystems, USA) were placed in tubes and stored at -20ºC. These surgical samples were processed for reverse transcriptase-polymerase chain reaction (RT-PCR). Peripheral blood samples were also taken from all participants, placed into disodium ethylenediaminetetraacetic acid tubes (EDTA) and kept at -80ºC until DNA genotyping.

Each patient was diagnosed with NP by anamnesis, physical examination, endoscopic examination and computed tomography paranasal sinus. Patients with cystic fibrosis, inverted papilloma, fungal sinusitis and antrochoanal polyps were excluded from the study. Priority was given to ensuring that the patients had not used oral glucocorticoids within the last three months before the surgical operation or used intranasal spray for one month. None of the patients who participated in the study had used antileukotriene and immunotherapy.

mRNA isolation and RT-PCR

A ready kit was used for RNA extraction and cDNA synthesis [respectively, (QIAamp RNA Blood Mini, Quiagen, Germany) (RevertAidTM First Strand cDNA Synthesis Kit-Fermentas)]. To evaluate the expression level of the *Secretoglobin 1C1* gene, the RT-PCR method was utilised. The primer and probes to be used were designed by the software Primer Designer version 2.0 (Scientific & Educational Software) (Table 1). Primers that were synthesized by a commercial firm and TaqMan probes were used (Bio Basic, Mississauga, Ontario, Canada). *Beta Aktin* (*ACTB*) was used as a housekeeping gene for normalization and a 'no template' sample was used as a negative control. For the RT-PCR reaction, a ready kit was used, and it was studied in Rotor-Gene 6 plex equipment [respectively, (SuperHot Master Mix, Bıoron, GmbH, Germany), (Quiagen, Germany)]. The relative expression of gene was calculated by the 'comparative CT' method. The PCR product was confirmed by DNA array analysis (25).

Table 1. Sequences of primers and probes used for PCR amplification.

Secretoglobin 1C1	Sense	5'-GTGCAAGTG-3'
	Antisense	5'-GACACAACGCCCACTACCACCT-3'
	Probe	5'-**HEX**+TCAGCCATAGGACCTGCCACACAAGC+**BHQ**-3'
ACTB	Sense	5'-CCTTCCTTCCTGG-3'
	Antisense	5'-GCATGGAG-3'
	Probe	5'-**FAM**+CCATCATGAAGTGTGACGTGGACATCCG+**BHQ**-3'

Secretoglobin 1C1: Ligand-binding protein gene; ACTB: Beta Aktin gene.

DNA isolation and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a ready kit (NucleoSpin blood DNA, Macherey-Nagel GmbH & Co. Kg, Germany). Genotyping for *Secretoglobin 1C1* was performed by sequencing. First, a polymerase chain reaction (PCR) was performed to enhance the relevant gene region from the isolated genomic DNA (SuperHot Master Mix, Bioron, GmbH, Germany). The primer pairs used in the PCR reaction were designed with the Primer Designer version 2.0 (Scientific & Educational Software). For the promoter, the forward primer used was 5'- AAAGAAAGGC-GTGGGACCAACC-3' and the reverse primer was 5'- CAGGTGGAGTGTTCACTGCAGAGG-3'. A 542-bp fragment was amplified with this primer set. The mixture was denatured at 95ºC for 5 minutes and underwent 35 cycles in a thermocycler PCR system under the following conditions: denaturation at 95ºC for 1 minute, annealing at 60ºC for 45 seconds, extension at 72ºC for 1 minute and a final extension for 10 minutes at 72ºC. A ready kit was used in the purification of the amplified products (NucleoFast 96 PCR, Macherey-Nagel GmbH & Co. Kg, Germany). A sequence reaction was performed on purified PCR products with a ready kit (BigDye Terminator V3.1 Cycle Sequencing, Applied Biosystems, USA). The *Secretoglobin 1C1* was investigated with the data that was obtained from the ABI PRISM 3130 Genetic Analyzer capillary automatic sequence equipment.

Statistical analysis

Data analyses were performed by using SPSS for Windows (Statistıcal Package for Social Sciences, version 16.0, SSPS Inc., Chicago, IL, USA). Results are presented as mean ± SD, or in error-bars, box-plots and dot-plots. Paired sets of data were compared using the Mann-Whitney U-test. Spearman's test was used to determine correlations. Genotypes in patients with NP and in controls were analyzed by the $\chi 2$ test. The level of significance was considered at a P value of less than 0.05.

Results

A total of 24 patients with NP (18 men and 6 women; mean age, 42.08 ± 9.561 years) with 22 healthy controls (15 men and 7 women; mean age, 43 ± 5.947 years) were included in this study. The means were not significantly different between the two groups (P= 0.07). The percentage distribution of gender was also similar in both groups (P= 0.163).

In this study, the relative expression levels of *Secretoglobin 1C1* in sinonasal mucosa from the controls and patients with NP are presented in Figure 1. The data shown in Figure 1 is expressed as the difference in threshold cycles for the target mRNA and *ACTB* (ΔCT). This is a direct reflection of the amount of input of target mRNA, and a change of CT value of one unit is equal to a doubling, or halving, of the level of target mRNA. The higher the ΔCT value, the lower the level of target mRNA. *Secretoglobin 1C1* had the higher expression levels in sinonasal mucosa from patients with NP (for cases; mean ΔCT: 12.1630 ± 2.457, ΔCT range: 8.480-15.912 and for controls: mean ΔCT: 15.176 ± 2.466; ΔCT range: 11.005-20.000 P= 0.001).

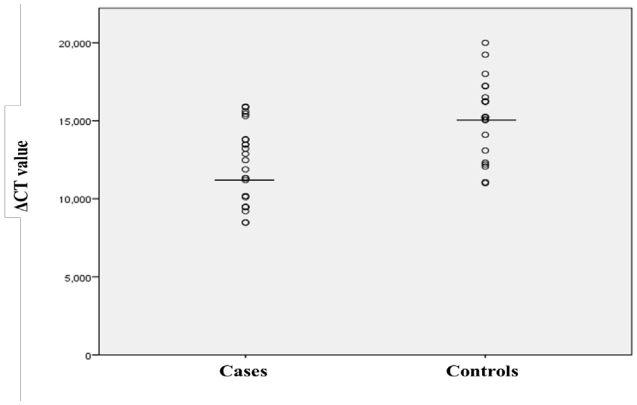

Figure 1. Relative mRNA expression level of *Secretoglobin 1C1* in the nasal mucosa. The relative expression levels are expressed as ΔCT (ΔCT = the difference in threshold cycles for the target). This is a direct reflection of the amount of input of the target mRNA, and a change of CT value of one unit is equal to a doubling (or halving) of the level of target mRNA. The higher the ΔCT value, the lower the level of target mRNA. The control group had 22 patients (15.176 ± 2.466) and the NP group had 24 patients (12.1630 ± 2.457). Data are presented in dot-plots. The result is expressed as mean ± SD and P= 0.001 compared with normal mucosa.

The current study investigated promoter polymorphisms of the *Secretoglobin 1C1* gene in a selected population using direct sequencing. We identified three SNPs [rs113795008 (c.-264A>G), rs144170316 (c.-177C>A) and rs2280540 (c.-103G>A)] that had been previously reported and registered in the dbSNP database (Short Genetic Variations Database at http://www.ncbi.nlm.nih.gov/snp).

The individual SNP analyses revealed that the genotype frequencies of two SNPs [rs113795008 and rs2280540] differed between the cases and controls (P= 0.010 and P= 0.045, respectively). Moreover, in the genotype analysis, we found that the AG and GG in rs113795008 and GA and AA in rs2280540 genotypes were associated with NP (Table 2).

The relative expression level of *Secretoglobin 1C1* according to genotype frequencies of three SNPs in subjects was summarized in Table 3. The patients with NP with the rs113795008 had the lower ΔCT value of *Secretoglobin 1C1* in the nasal mucosa than the controls (P= 0.001). However, the distribution of ΔCT value was similar in control groups and the patients with NP for rs144170316 and rs2280540 (P= 0.181, P= 0.525).

The patients with NP with the rs113795008 GG (9.101 ± 0.492) genotype had significantly higher mRNA expression level of *Secretoglobin 1C1* in the nasal mucosa than those with the AG (12.314 ± 1.767) and AA (14.550 ± 1.294) genotypes (P= 0.001) (Figure 2). The patients with NP with the rs144170316 CA (11.502± 2.328) genotype had higher mRNA expression level of *Secretoglobin 1C1* in the nasal mucosa than those with the CC (12.337 ± 2.521) and genotypes (P= 0.618). The patients with NP with the rs2280540 AA (10.825 ± 2.725) genotype had higher mRNA expression level of *Secretoglobin 1C1* in the nasal mucosa than those with the GA (13.654 ± 2.161) and GG (11.555 ± 2.297) genotypes (P= 0.141) (Table 3).

Table 2. Distribution of genotypic frequency of *Secretoglobin 1C1* gene promoter SNPs in subjects.

SNP ID/SNPs	Genotype/Allele	Controls (n: 22)	Cases (n: 24)	OR (95 CI)	P
	AA	16 (72.7)	7 (29.2)	1.00 (reference)	
rs113795008 (c.-264A>G)	AG	5 (22.7)	11 (48.8)	0.45 (0.09-1.34)	***0.010****
	GG	1 (4.5)	6 (25)	0.34 (0.01-1.23)	
	CC	15 (68.2)	19 (79.2)	1.00 (reference)	
rs144170316 (c.-177C>A)	CA	7 (31.8)	5 (20.8)	1.77 (0.47-6.72)	0.397
	AA	0 (0)	0 (0)	-	
	GG	28 (60.9)	11 (45.8)	1.00 (reference)	
rs2280540 (c.-103G>A)	GA	13 (28.3)	8 (33.3)	0.47 (0.14-2.07)	***0.045***
	AA	5 (10.9)	5 (20.9)	0.32 (0.08-4.01)	

Continuous variables were presented as mean ± standard deviation (SD), and categorical variables were expressed as n (%). Boldface indicates p < 0.05 was considered as statistically significant. SNP ID= Single-nucleotide polymorphism accession number or NCBI dbSNP (http:www.ncbi.nlm.nih.govsnp); SNP= Single nucleotide polymorphism; ΔCT = the difference in threshold cycles for the target; OR= Odds ratio; CI= Confidence interval. * P value according to Pearson χ^2test for case/control

Table 3. Distribution of ΔCT value of *Secretoglobin 1C1* according to gene promoter' SNPs in subjects .

SNP ID/SNPs	Genotype/Allele	ΔCT value for Controls	ΔCT value for Cases	P
	AA	16,263.38 ± 1,850.354	14,550.29 ± 1,294.079	***<0.001****
rs113795008 (c.-264A>G)	AG	12,519.40 ± 1,159.017	12,314.82 ± 1,767.669	***<0.001***
	GG	-	9,101.67 ± 492.764	
	CC	15,934.40 ± 2,087.324	12,337.47 ± 2,521.557	0.181*
rs144170316 (c.-177C>A)	CA	13,552.29 ± 2,572.535	11,502.60 ± 2,328.038	0.618**
	AA	-	-	
	GG	15,229.40 ± 2,264.213	11,555.23 ± 2,297.930	0.525*
rs2280540 (c.-103G>A)	GA	15,322.00 ± 3,388.734	13,654.00 ± 2,161.620	0.141**
	AA	14,415.50 ± 2,962.070	10,825.00 ± 2,725.358	

Continuous variables were presented as mean ± standard deviation (SD), Boldface indicates p < 0.05 was considered as statistically significant. SNP ID= Single-nucleotide polymorphism accession number or NCBI dbSNP (http:www.ncbi.nlm.nih.govsnp); SNP= Single nucleotide polymorphism; ΔCT= the difference in threshold cycles for the target; * P value for cases/controls ** P value for homozygous wildtype-genotype/heterozygous genotype, homozygous mutant-genotype ***0.001****.

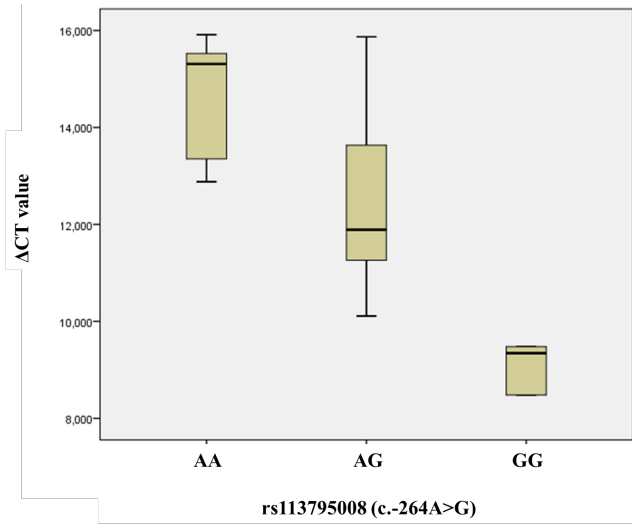

Figure 2. ΔCT variation of *Secretoglobin 1C1* gene among genotypes for rs113795008 (c.-264A>G). The patients with NP with the rs113795008 GG (9.101 ± 0.492) genotype had lower ΔCT value for *Secretoglobin 1C1* in the nasal mucosa than those with the AA (14.550 ± 1.294) and AG (12.314 ± 1.767) genotypes. Data are presented in box-plots. The results are expressed as mean ± SD and P= 0.001, compared with AG and GG in NP.

Discussion

Although many studies have been conducted, NP pathogenesis cannot be explained. Most studies would findigs that cytokine imbalance may contribute to increased inflammation (8-10). Therefore, secretoglobins, which are the candidates for forming a new cytokine family, have gained significance in NP development (29,37-41). It is thought that the *Secretoglobin 1C1* gene is expressed in the nasal mucosa, and the relationship between *RYD5* and NP has been reported in the few studies associated with NP (34-36,38). The cellular location in the sinonasal mucosa is not clear yet, and in-situ hybridization studies are required in this respect (31). In the current study, we studied the promoter SNPs in the *Secretoglobin 1C1* gene and their effects on expression level and NP pathogenesis.

Three polymorphisms were identified in the promoter region of the *Secretoglobin 1C1* gene. The nucleotide substitutions were found at the base pair (bp) positions -264, -177, and -103, respectively. No single rs144170316 and its genotypes were associated with NP. Our data and previous reports demonstrated that the AG and GG in rs113795008 and GA and AA in rs2280540 genotypes were associated with NP (38). The expression of *Secretoglobin 1C1* increased in sinonasal mucosa with polyps, and there was a significant difference between the controls and NP. In addition, the homozygous mutant-type GG in the rs113795008 genotype had higher mRNA expression levels of *Secretoglobin 1C1* in the nasal mucosa and an increased risk of NP. In one study, a relationship was discovered between the SNP (-112 G/A) in the promoter region of the *SCGB3A2* gene and

increased asthma risk (42). Additionally, another study found that *SCGB3A1* genotypes were related to NP (33).

SNPs in the coding regions of genes (cSNPs) or in the regulatory regions are more likely to cause functional differences than SNPs in other regions (43). Accordingly, some SNPs in the promoter region of the gene may contribute to alterations in transcriptional activity. However, further functional studies on the transcriptional activity of the gene polymorphisms are necessary to further elucidate their role in NP etiology.

SCGB1A1 is a prototypical member of the secretoglobin super family in terms of its anti- inflammatory and immunomodulator effects. Previous studies reported a decrease in the expression of *SCGB1A1* in upper and lower respiratory tract inflammatory diseases (10,24,25,30). There was a decrease in the expression of the *SCGB3A2* gene in NP patients (31). Information in the literature about the *Secretoglobin 1C1* gene is very limited. The current study is the first population-based study that has been conducted on the *Secretoglobin 1C1* gene. Therefore, the results of the current study will serve as pre-data for future studies. Additionally, a previous study reported a significant increase in the *Secretoglobin 1C1* expression level among patients with CRS and with NP in comparison to patients with CRS and without NP (31). The results of the current study run parallel to this report, and this difference will be significant in expression studies that involve a greater number of samples.

The similarity between the *Secretoglobin 1C1* in glycoprotein form and Rat prostatein Cl subunitine (RP) amino acid sequences show the steroid-binding ability of *Secretoglobin 1C1* (33,35). The *Secretoglobin 1C1* protein can modulate the inflammation process by binding the steroid ligands as many small epithelial secretion proteins in the family of which they are member. In nasal polyposis, corticosteroids used in the prevention of polyp development can be related to the function of *SCG1BC1* (44).

Secretoglobin 1C1 protein plays a role in the primary defence mechanisms in respiratory airway mucosa; its general expressions are controlled by the local immune system and they can cause inflammation by forming expressions that are cytokine focused (31). On the other hand, it is well known that Th1/Th2 imbalance is important in developing NP (8-10). Pro-inflammatory Th1 and Th2 cytokines in the nasal mucosa can regulate the expression of the *Secretoglobin 1C1* gene; therefore, we believe that this deviation in the expression level of the *Secretoglobin 1C1* gene can be related to the local specific cytokine environment in NP (10,44). Therefore, further studies should focus on the gene-environment interaction of NP.

In our study, the increased expression level of *Secretoglobin 1C1* and some genotypes were found to be related to the tendency toward NP ($P< 0.05$). Knowledge regarding the relationship between SNPs and the clinical features of patients with NP and the effect on expression level might provide clinical data for treating the course of the disease and follow-up care. Although our study was designed to investigate the relationship between the genotypic variations and expression levels in a manner that incorporated array analysis and expression analysis, our results were affected by various limitations. Sample number is an important limitation in population-based studies and the reliability level of data can increase, or results can change in future studies that contain more samples (45).

Our study demonstrated that the presence of some promoter SNPs in the *Secretoglobin 1C1* gene might increase and/or contribute to the susceptibility of developing NP by affecting the expression level of the gene and by changing the severity of inflammation in the Turkish population.

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This study was approved by Ankara Numune Education and Research Hospital Research Ethics Committee (ID: 210/2011).

Interest Conflict
The authors have no affiliations with or involvement in any organization or entity with any financial interest.

Financial Disclosures
The authors have no any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Authors' contributions
Sibel ÖZDAŞ: The idea for research or article/hypothesis generation, planning the methods to generate hypothesis, the manuscript preparation, responsibility for conducting experiments. Talih ÖZDAŞ: biological materials, reagents, referred patients, management of patients, organizing and reporting data, and responsibility for presentation and logical explanation of results. Sibel ÖZDAŞ and Talih ÖZDAŞ: responsibility for conducting literature search, responsibility for creation of an entire or the substantial part of the manuscript, reworking the final, before submission version of the manuscript for intellectual content, not just spelling and grammar check.

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