Detection of TNF-α as a cofactor in the pathogenesis of nasal polypi

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Background
Nasal polyposis (NP) is a multifactorial disorder that is correlated with multiple inflammatory, environmental, as well as genetic factors. Moreover, tumor necrosis factor (TNF)-alpha is regarded as the most prominent pro-inflammatory cytokine that contributes to the pathogenesis of NP. Multiple polymorphisms of that gene can influence its function.

Aim
The aim of this study was to detect TNF-α polymorphism in patients with nasal polypi to determine its contribution to the pathogenesis of nasal polypi in the Egyptian population.

Methods
This is a case–control study, in which 25 patients with NP as well as 25 healthy participants were included, who attended Ain Shams University hospital. Participants were examined and prepared for the study. Participants were subjected to DNA extraction, and restriction fragment length polymorphism–polymerase chain reaction was administrated to detect polymorphism. The comparison of the genotype frequency distribution and the TNF-alpha gene alleles with NP was done using χ^2 test.

Results
The results showed a statistically marked difference between the G/G genotype in the two groups (P=0.0001). In addition, the presence of allele A in the patient group and control group was 10 and 1%, respectively, which is statistically significant (P=0.0001). The genotype G/G, A/A, as well as G/A frequency in the NP group was 8, 40, and 52%, and it was 76, 1, and 5% in the control group, respectively. The findings of the present study showed that the polymorphism in TNF-alpha gene is probably an important NP risk agent in the Egyptian population. The presence of the TNF-alpha G308A allele in our study was to some extent higher compared with the other populations in other previous studies.

Conclusion
According to the scientific evidence for G/A 308 polymorphism related to the promoter of TNF-α gene in Egypt, the genotypic pattern distribution in all regions appears to be the same. Nonetheless, the amount of allele A was higher in the current research than in the control group, in addition to the incidence of the NP-associated G/A genotype. However, to obtain accurate findings, larger samples are required. Regarding the results of the present study, this polymorphism may be determined as a risk agent for NP susceptibility in relation to the Egyptian population.

Keywords:
chronic rhinosinusitis, eosinophil cationic protein, intercellular adhesion molecule-1, nasal polypi

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Introduction

Nasal polyposis (NP) appears to be one of the most prevailing chronic inflammatory diseases of the nasal mucosa as well as the sinuses that causes nasal obstruction. Most patients complain of nasal obstruction, breathing difficulty, nasal discharge, posterior nasal drip, nasal congestion, sinus pain, anosmia, and hyposmia. It is not clear why some people develop chronic inflammation that causes nasal polyps, whereas others do not. People with chronic sinus infections, allergic rhinitis, asthma, and cystic fibrosis are more likely to develop nasal polyps [1].

During the past 10 years, multiple studies have been performed to detect the susceptible genes that correlate with nasal polypi-related traits. Despite the multiple contributions to the identification of candidate genes as well as their association with formation of nasal polypi, the genetic and molecular modifications that are required for their evolution and development remain unclear [2].

Even though several inflammatory cytokines have been detected in the tissues of the nasal polypi, the primary trigger, which results in the inflammation associated

with edema, eosinophilia, as well as lymphocytosis, remains unidentified [3].

Tumor necrosis factor (TNF-α) is a strong immune mediator as well as a pro-inflammatory cytokine that is involved in the pathogenesis of several famous diseases. The presence of this particular gene on the short arm of chromosome 6 (with major histocompatibility complex (MHC) genes in addition to complement) resulted in an increase in the possibility of polymorphism to contribute to the genetic correlation of this region of the genome with the nose polyps [4].

Rostkowska *et al*. [5] investigated the gene-coding expression of TNF-α as well as its receptors (TNF-R1 and TNF-R2) in patients with NP. TNF-R1 was identified as the predominant form of the TNF-α receptor in nasal polyps, thus indicating the evident prevalence of this type in TNF-α signaling. These findings increase the probability that eosinophils induced by NP can affect the biological responses via the dependent mechanism of TNF-α. They proposed that the differences between eosinophilic nasal polyp and NP associated with TNFα, and the expression of its receptors is probably a reflection of the special feature of this disease.

Materials and methods

In our case–control study, 50 participants underwent blood sampling (25 healthy individuals and 25 patients with NP). The patients had a referral to an academic specialist at Ain Shams university, Cairo, between February 2016 and July 2020. The control group had no known nasal diseases, whereas the NP group patients had a history of rhino-sinusitis and a family history of NP and allergy. The exclusion criteria were patients with fungal sinusitis. The study was approved by the Ethics Committee of Ain Shams University.

Genotyping

The genotyping performed on the specimens involved the following stages:

DNA extraction

(1) DNA extraction from 250 μl of blood by the Chemagic DNA Blood 250 Kit and Chemagic magnetic stand 2 × 12 (Art. No. CMG-300) (Chemagen Technology, PerkinElmer, Baesweiler, Germany) was carried out as follows:
(2) Lysis buffer in a volume of 350 μl was added to 250 μl of whole blood. It was vortexed until a uniform solution was obtained.
(3) The sample was incubated for 5 min at room temperature.
(4) 50 μl of resuspended magnetic beads was added, followed immediately by 950 μl of binding buffer 2, and mixed well. Then, the mixture was incubated at room temperature for 5 min.
(5) The magnetic bead/DNA complex was separated for 2 min, the supernatant was discarded, and then the tube was removed from the chemagic stand 2 × 12 (magnet position).
(6) The bead pellet was completely resuspended in 800 μl of wash buffer 3.
(7) The separation of the magnetic bead/DNA complex lasted for one minute, and then the supernatant was left behind.
(8) The tube was then removed from the magnetic separator, and the washing procedure was repeated (Steps 5 and 6) with 800 μl of Wash Buffer 4, and then with 800 μl of Wash Buffer 5.
(9) The tube was left in the magnet position.
(10) When the beads were attracted to the magnet, 1.5 ml of wash buffer number 6 was added and left for 90 s without resuspending the bead pellet. The supernatant was carefully removed and discarded.
(11) Overall, 200 μl of elution buffer number 7 was added, and the magnetic bead/DNA complex was resuspended. It was incubated for ten minutes at room temperature, with occasional agitation.
(12) The magnetic beads were separated and transferred to a clean tube.
(13) The DNA was stored at a temperature of –20°C till it is used.

Selection and synthesis of oligonucleotides

TNF-α gene lies in the greatly polymorphic MHC area on chromosome 6p21.3. The synthetization of the following primers was done:

Forward primer, 5'-AGGCAATAGGTTTTGA GGGCCAT-3', and reverse primer, 5'-TCCTCCCT GCTCCGATTCCG-3' [6].

Genotyping was performed using ASO-PCR method after the expansion of nearly 200 ng genomic DNA template to a PCR reaction volume of 25 μl, using two various alleles for one nucleotide polymorphism in one PCR reaction.

The PCR program (S24 thermal cycler™, Quanta Biotech®, Surrey, England, UK) consisted of PCR conditions as follows: 5 min at 95°C, then 30 denaturation cycles at 94°C for 30 s, annealing at 60°C for 30 s, as well as expansion for 45 s at 72°C.

The last extension procedure was carried out for 8 min at 72°C.

The genotypes of the PCR products for TNF-α–308 (G/A) polymorphism (107 bp) were defined via electrophoresis on 2% high-resolution agarose gels, and stained with ethidium bromide in 1 × Tris-EDTA (ethylenediaminetetraacetic acid).

Then the amplification products were digested with *Nco*I restriction enzyme. The existence of a *Nco*I restriction site in the TNF-α (A allele) mutant allele was demonstrated by the dissent of 107 bp amplicon to produce 2 fragments of 87 bp as well as 20 bp, and subsequently was electrophoresed on a 3% agarose gel stained with ethidium bromide in 1 × Tris-EDTA.

Comparison of the distribution of the genotypes as well as frequencies of allele was statistically performed in healthy controls against the patient group.

Results

With respect to the NP group, there were 11 males and 14 females, and their mean age was 41.8±10.2 years (minimum and maximum 30 and 60 years), whereas in the control group, there were 15 males and 10 females, with a mean age of 30.7±5.9 years (minimum 26 and maximum 69 years). No statistically significant differences were detected regarding sex (*P*=0.258) between the control and the study groups. In addition, there was no significant difference concerning age in both groups (*P*<0. 247), as shown in Table 1.

A total of 19 controls (76%) had the GG genotype, whereas just two cases (8%) had this genotype in the NP group (*P*=0.0001). Moreover, there was a marked frequency difference with respect to alleles in both of the polyps as well as the control groups (*P*=0.0001). A total of 13 participants (52%) had the GA genotype, whereas only five controls (20%) had the GA genotype. Overall, 10 (40%) had the AA genotype, whereas only one control (4%) had the AA genotype, as displayed in Table 2.

According to the relationship between the TNF-α-308G/A promoter single-nucleotide polymorphism (SNP) genotype as well as the NP phenotype, 33.3% of the GG genotype cases were antrochoanal, whereas 0% of the GG were unilateral nasal polyp. Overall, 5.6% had GG bilateral nasal polyps. Moreover, 0% had an antrochoanal GA, whereas 75% had a unilateral GA nasal polyp. In addition, 55.6% had GA bilateral nasal polyps. Overall, 66.7% had AA antrochoanal, whereas 25% had AA unilateral nasal polyps. Additionally, 38.9% had AA bilateral nasal polyps. These results are not statistically significant (*P* value=0.201), as displayed in Table 3.

Table 1 Demographic features of controls as well as cases

Variable	Cases (*n*=25)	Controls (*n*=25)	*P*
Age (years)	41.8±10.2	30.7±5.9	<0.247*
Sex (M/F)	11/14	15/10	0.258**

Data are mean±SD or ratio. F, female; M, male. *Unpaired *t*-test. **Pearson χ^2 test.

According to the relationship between TNF-α-308G/A promoter SNP allele segregation and the NP phenotype, 33.3% of G allele cases were antrochoanal, whereas 37.5% of G allele cases were unilateral nasal polyp. Overall, 33.3% of G allele cases were bilateral nasal polyps. Moreover, 66.7% of A allele cases were antrochoanal, whereas 62.8% of A allele cases were unilateral nasal polyp. In addition, 66.7% of A allele cases were bilateral nasal polyps. These results are not statistically significant (*P* value<0.999), as presented in Table 4.

According to the relationship between sex and TNF-α-308G/A promoter SNP genotype, there was no statistically significant differences between males and females regarding genotype in both the case and the control groups. Overall, there was no significant differences with respect to alleles, as shown in Tables 5 and 6.

Discussion

NP is regarded as an inherent inflammatory disease that affects paranasal sinuses as well as nasal mucosa. There was an increase in tissue eosinophilia, edema, and surface epithelial structure [7]. Despite hypothesizing some etiologic factors such as allergies and fungal and bacterial infections, as well as some genetic factors, the actual pathogen of this disease remains unelucidated [8,9].

TNF-α is responsible for regulating the receptors as well as molecules of the endothelial cells of nasal polyps [7]. The polymorphism of TNF gene can induce increased risk or severity of occurrence of NP [10,11]. TNF-α gene is particularly important in respiratory tract inflammation, and affects the permeability of epithelial cell [12]. TNF-α represents a member of the pro-inflammatory cytokine family and has a synergistic role in the chronic inflammation process. The expression of adhesion molecules in nasal polyps is regulated by TNF-alpha gene by eradicating eosinophils in lamina propria [13].

Table 2 Comparison of TNF-α-308G/A promoter SNP genotype in cases and controls

TNF-α-308G/A promoter SNP genotype	Cases (*n*=25)	Controls (*n*=25)	*P*
Homozygote wild (GG)	2 (8.0%)	19 (76.0%)	<0.0001*
Heterozygote (GA)	13 (52.0%)	5 (20.0%)	
Homozygote mutant (AA)	10 (40.0%)	1 (4.0%)	

Data are number (%). SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor. *Pearson χ^2 test.

Table 3 The correlation between the TNF-α-308G/A promoter SNP genotype as well as the nasal polyposis phenotype

	Phenotype of cases			
TNF-α-308G/A promoter SNP genotype	Antrochoanal polyp (*n*=3)	Unilateral nasal polyp (*n*=4)	Bilateral nasal polyps (*n*=18)	*P*
Homozygote wild (GG)	1 (33.3%)	0 (0.0%)	1 (5.6%)	0.201*
Heterozygote (GA)	0 (0.0%)	3 (75.0%)	10 (55.6%)	
Homozygote mutant (AA)	2 (66.7%)	1 (25.0%)	7 (38.9%)	

Data are number (%). SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor. *Fisher's exact test.

Table 4 Relation between TNF-α-308G/A promoter SNP allele segregation and phenotype of nasal polyposis

Allele	Phenotype of cases			*P*
TNF-α-308G/A promoter SNP allele	Bilateral	Unilateral	Antrochoanal	>0.999*
G	12 (33.3%)	3 (37.5%)	2 (33.3%)	
A	24 (66.7%)	5 (62.8%)	4 (66.7%)	
Total	36	8	6	

Data are number (%). SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor. *Fisher's exact test.

Table 5 Relation between sex and TNF-α-308G/A promoter SNP genotype

Group	TNF-α308 G/A promoter SNP genotype	Sex		Total	*P*
		M	F		
Cases (*n*=25)	Homozygote wild (GG)	2 (18.2%)	0	2 (8.0%)	0.188*
	Heterozygote (GA)	4 (36.4%)	9 (64.3%)	13 (52.0%)	
	Homozygote mutant (AA)	5 (45.5%)	5 (35.7%)	10 (40.0%)	
	Group total	11	14	25	
Controls (*n*=25)	Homozygote wild (GG)	12 (80.0%)	7 (70.0%)	19 (76.0%)	0.769*
	Heterozygote (GA)	3 (20.0%)	2 (20.0%)	5 (20.0%)	
	Homozygote mutant (AA)	0 (0.0%)	1 (10.0%)	1 (4.0%)	
	Group total	15	10	25	
All subjects (*n*=50)	Homozygote wild (GG)	14 (53.8%)	7 (29.2%)	21 (42.0%)	0.198**
	Heterozygote (GA)	7 (26.9%)	11 (45.8%)	18 (36.0%)	
	Homozygote mutant (AA)	5 (19.2%)	6 (25.0%)	11 (22.0%)	
	Grand total	26	24	50	

Data are number (%). SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor. *Fisher's exact test. **Pearson Chi-squared test.

Table 6 Relation between sex and TNF-α-308G/A promoter SNP allele segregation

Group	TNF-α-308G/A promoter SNP allele	Sex		Total	*P*
		M	F		
Cases (*n*=25)	G	8 (36.4%)	9 (32.1%)	17	0.773*
	A	14 (63.6%)	19 (67.9%)	33	
	Group total	22	28	50	
Controls (*n*=25)	G	27 (90.0%)	16 (80.0%)	43	0.416*
	A	3 (10.0%)	4 (20.0%)	7	
	Group total	30	20	50	
All subjects (*n*=50)	G	35 (67.3%)	25 (52.1%)	60	0.122**
	A	17 (32.7%)	23 (48.9%)	40	
	Grand total	52	48	100	

Data are number (%). SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor. *Fisher's exact test. **Pearson χ^2 test.

Baikhan *et al*. [14] illustrated that the existence of TNF-α-308 G/A SNP is a self-reliant risk agent for nasal polypi development as well as gene-transcriptional activity. In conjunction with the study by Batikhan *et al*., there was a statistically marked correlation between TNF-α G/A 308 polymorphism and NP (*P*=0.0001).

The findings indicated that G/A genotype 308 patients experienced 2.6 times greater risk to develop NP, whereas G/G 308 patients in the TNF-alpha gene in the promoter gene experienced a 54% lower risk to develop NP. Furthermore, the findings of the current study are consistent with previous studies that investigated the correlation between TNF-α G/A 308 gene polymorphisms and development of diabetes, NP, and acne [4,15]. Regarding the various frequencies of SNP marker in different populations, researchers who have attributed a certain disease or a specific disorder association with the same SNP are argumentative. Consequently, every population study has value in relation to the field of personalized medicine; nonetheless, the G308A allele frequency in the Egyptian population is somewhat contrasting when compared with other populations.

When comparing the frequency of G308A minor allele in 5 various populations, their data are available via databases of Ensemble; there is a lower frequency of allele G308A variant in European, African, South Asian, American, as well as East Asian is 13, 12, 5, 7, and 6%, respectively. Although this frequency is 20% in the current study, there is no significant deviation from Hardy–Weinberg equilibrium in such populations. As illustrated earlier, the lower frequency of G308A allele in this study is slightly higher compared with other major populations.

In the current study, the male patients were roughly equal to females and patients' age ranged from 16 to 69 years in all age groups; this can be attributed to the small sample size (25 patients).

The occurrence of NP in males was approximately equal in females (51% and 49%, respectively). Bernstein and his research team did not confirm these results [4,16]. They demonstrated that chemicals, occupational exposure, as well as smoking in males are probably responsible for such differences. Furthermore, the study results were inconsistent with Busaba and his research team, as they stated marked differences regarding the association between chronic rhinosinusitis and NP and sex. Moreover, those males had more tendency to experience chronic rhinosinusitis with NP [17]. With respect to the examination, eosinophils are estimated to be approximately 80% of inflammatory cells; moreover, they proliferate in the nasal polyps [7].

TNF-α stimulates the production of vascular cell adhesion molecule 1 (VCAM-1); moreover, increasing the VCAM-1 production results in more eosinophil migration into nasal polyps. In contrast, the increase of eosinophils in the tissues of nasal polyp produces more TNF-α, which consequently brings large amounts of eosinophils into the polyp mucosa [4,18]. Based on the current results, it was found that A allele TNF-α 308 gene was greater in patients with NP compared with the control group (P=0.0001), and the A allele was known to be mutated at 107 bp, which is associated with the TNF-α gene transcription [19]. Berghea and Szabo found no correlation between the allelic frequency of TNF-α 308 G/A among Romanians [3] as well as Hungarians [9], which contradicts the present study, owing to the reason that their research is concerned with patients with chronic rhino-sinusitis, asthmatics, as well as patients with aspirin sensitivity; nonetheless, their research was not concerned with patients with nasal polypi.

Furthermore, Berghea *et al.* [3] only enrolled 45 patients, and they proposed multicenter studies as well as collaborative studies for more precise interpretation. After accurate stratification of the patient group based on observing the clinical symptoms, Szabo concluded that there is a marked higher rate of transmitting rare A allele that contain genotypes in patients with chronic rhinosinusitis with NP, who were sensitive to aspirin as well [9].

The results of the current study also indicated that age is regarded as a risk factor for NP in TNF-α promoter gene G/A 308 genotype patients, which aligns with the findings of previous studies that demonstrated that the occurrence as well as the prevalence of nasal polyps increases with age, particularly between the ages of 40 and 60 years [20,21].

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Conflicts of interest
There are no conflicts of interest.

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