

Association of TNF- α (G308A) Gene Polymorphism with Susceptibility to Open-Angle Glaucoma in Iraqi Patients: A Laboratory-Based Genetic Study

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ABSTRACT

Background: Open-angle glaucoma (OAG) is a progressive optic neuropathy that leads to irreversible vision loss. Genetic predisposition and pro-inflammatory cytokines, particularly tumor necrosis factor- α (TNF- α), have been implicated in glaucomatous neurodegeneration. The G308A single nucleotide polymorphism (SNP) of the TNF- α gene may play a role in the pathogenesis of OAG by influencing inflammatory and apoptotic pathways. **Objective:** This study aimed to evaluate the association between TNF- α (G308A) gene polymorphism and susceptibility to OAG in Iraqi patients and its correlation with serum TNF- α levels and clinical parameters of glaucoma progression. **Methods:** A case-control study was conducted on 100 OAG patients and 100 age- and sex-matched healthy controls. Genotyping of TNF- α (G308A) polymorphism was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Serum TNF- α levels were measured using enzyme-linked immunosorbent assay (ELISA). Clinical parameters, including intraocular pressure (IOP), cup-to-disc ratio, and retinal nerve fiber layer (RNFL) thickness, were assessed using tonometry and optical coherence tomography (OCT). **Results:** The A allele frequency was significantly higher in OAG patients compared to controls ($p < 0.001$, OR = 2.15), indicating a possible genetic predisposition. Serum TNF- α levels were significantly elevated in OAG patients compared to controls ($p < 0.01$), with the highest levels observed in individuals carrying the AA genotype. A significant correlation was observed between TNF- α levels and clinical glaucoma severity, with higher TNF- α concentrations associated with increased IOP and optic nerve damage. **Conclusion:** The TNF- α (G308A) polymorphism may serve as a genetic risk factor for OAG in the Iraqi population. The AA genotype is associated with elevated TNF- α levels, suggesting its role in inflammatory-mediated optic nerve damage. These findings highlight the potential role of TNF- α as a biomarker for early glaucoma detection and progression monitoring. Further genetic and functional studies are needed to establish TNF- α as a therapeutic target in glaucoma management.

Keywords: Chronic Optic Neuropathy, Primary Open Angle Glaucoma (POAG), Genetic and Environmental Variables

INTRODUCTION

Glaucoma of primary angle closure type (POAG) is chronic optic neuropathy with distinct patterns of optic nerve damage and a gradual loss of vision in the visual field [1]. Individuals may be predisposed to POAG due to the multifaceted nature of the disease influenced by several clinical factors like age, race, myopia, central corneal thickness, intraocular pressure (IOP), and family history of glaucoma under paragraph two [2]. POAG is generally a slow progressing disease that advances at a fairly constant pace and it is with minimal discomfort [3]. Often the patients are asymptomatic until they have lost their central vision (in the later stages of the disease). But in this case, it might be not necessarily symmetrical [4]. POAG is confirmed based on the visual field tests, examination of the retinal nerve fiber layer, and imaging and examination of the optic nerve [5]. For more than 70 million people worldwide, glaucoma lasts for the second most common cause of blindness [6]. Bilateral blindness is estimated to affect 10% of people. In Indonesia, glaucoma affects 0.46 percent of people [7]. This suggests that glaucoma affects 4-5 out of 1,000 people [8]. In 2015, 57.5 million persons worldwide were affected with primary open angle glaucoma (POAG), the second leading cause of blindness globally, behind cataracts. It is a condition that cannot be cured. By the year 2020, the aforementioned figure had risen to 65.5 million. [8]. Many academics claim that glaucoma is a serious medical and social issue that affects everyone, including Moroccans [9]. The WHO estimates global glaucoma prevalence to be 105 million,

and predictions suggest that figure could double by 2030 [10, 11]. Tumour necrosis factor- α , often known as TNF- α , is a contributing component to the process of neurodegeneration in glaucoma. [12]. The relationship between immunological responses and the development of glaucomatous optic nerve degeneration may be unexpected. Under some conditions, this relationship may lead to an aberrant immune signaling pathway that ultimately results in the death of retinal ganglion cells [13]. According to recent research, glial cells under pressure or ischemia release TNF- α , which causes oligodendrocytes to die and retinal ganglion cells to undergo apoptosis [14, 15].

METHODS AND MATERIALS

Two groups participate in case-control research.: The first group included 40 patients who had previously been diagnosed with glaucoma, all cases that were observed in the General Hospital / Diwaniyah / Eye Department and ALL Hayat Hospital. From October 2023 to November 2023, under the supervision of an ophthalmologist, information was collected about each case. From the patient, in addition to the examinations carried out in the various sections of the center, like the ocular pressure examination and others. The second group consisted of forty volunteers who were healthy and had no family history of glaucoma. These two groups had venipunctures to

obtain five milliliters of venous blood, which were then extracted using a disposable syringe while adhering to strict hygiene protocols. Two tubes are used to collect blood samples.

About 3ml of blood has been collected in an EDTA tube for total DNA extraction to study Genetic Polymorphism for TNF- α SNP(G308A) (Table 1).

Table 1: PCR Primers for TNF- α SNP (G308A)

Primer	Sequence	Product Size	Reference
Rs(G308A)	F1	TCTCGGTTTCTTCCATCG	[16]
	F2	ATAGGTTTTGAGGGGCATGG	
	R	AATAGGTTTTGAGGGGCATGA	

More than two milliliters of blood were extracted and left to solidify in a simple tube devoid of EDTA. After that, the serum was divided and centrifuged for five minutes at 13,000 rpm to preserve it in two portions. The serum was collected in an Eppendorf tube and stored at -20 degrees Celsius for use in the TNF- α ELISA assay, as indicated in the study design figure. In the current study, The ELISA kit has been used that uses a single-step, double-antibody sandwich enzyme-linked immunosorbent assay method to measure TNF- α in human serum. After a wavelength of 450 nm, by matching the O.D. of the samples to a standard curve, the interleukins concentration in the samples is ascertained. It spectrophotometrically quantifies the color shift and confirms the samples' TNF- α quantity.

Polymerase Chain Reaction (PCR) methodology was used to determine TNF- α SNP(G308A) in all patients and healthy control blood samples as the following steps: The GoTaq® Green Master Mix Kit was used to elaborate the PCR master mix, and the manufacturing company's instructions, as shown in Table 2, were followed in preparing this master mix. The components mentioned below in table 2 of the PCR master mix were then added to standard test tubes with GoTaq® Green Master Mix, which contained all of the elements needed for a PCR reaction, such as Tris-HCl, dNTPs, and Taq DNA polymerase. 9.0 pH, KCl, MgCl₂, tracking dye, and stabilizer. Each PCR tube was inserted in a PCR thermocycler after being spun for five minutes at 3000 rpm in an Exispin vortex centrifuge.

Table 2: PCR Master Mix

PCR Master Mix	Volume
Gotaq® Green Master Mix	12.5 μ l
Dna Template	5 μ l
Upstream Primer	2
Downstream Primer	2
Nuclease-Free Water	3.5
Total Volume	25 μ l

Table 3: PCR Thermocycler program

PCR Step	Temperature	Time	No. of Cycles
Initial Denaturation	95°C	4min.	1
Denaturation	95°C	30sec.	35 Cycle
Annealing	54°C	30sec.	
Extension	72°C	25sec.	
Final Extension	72°C	5 min.	1
Storage	4°C	Hold	-

PCR Product Analysis

The hexon gene PCR amplification products were subjected to migration analysis on agarose gel electrophoresis as described below:

1. 1X TBE was used to form a 1% agarose gel, which was then dissolved in water. After 15 minutes in a 100°C bath, let the water cool to 50°C. 2. Then 0.5 μ l of previously prepared stock of ethidium bromide stain (1% W/V) was added to the agarose gel solution. 3. The agarose gel preparation was poured into the tray once the comb was positioned appropriately. After that, the comb was carefully taken out of the tray, and 10 μ l of the 100bp Ladder in one well and 10 μ l of each

PCR amplification product for each test sample served as the standard reference. The gel was then allowed to develop for fifteen minutes at room temperature. 4. The gel tray was filled with 1X TBE buffer and stabilized in the electrophoresis chamber. After that, the electric current was run for 60 minutes at 100 volts and 80 amps.

Results

Comparison of serum TNF- α between patients, Table 4 displays the group and the control group. The mean serum TNF- α was considerably more significant in the sick group (17.41 $\pm$ 8.97 versus 8.79 $\pm$ 4.75) than in the control group ($p < 0.001$).

Table 4: Compares Serum TNF- α Levels in the Patient and Control Groups.

Characteristic	Patients Group n=40	Control Group n=40	p
	TNF- α		
Mean $\pm$ SD	17.41 $\pm$ 8.97	8.79 $\pm$ 4.75	<0.001***
Range	7.49-58.74	1.93-28.94	

SD: Standard Deviation; I: Independent Samples T-Test; ***: Significant at $P \leq 0.001$;

The frequency distribution of patients and control subjects according to TNF- α GA genotypes is shown in Table 4. Overall,

genotype GGG was reported in 23, genotype GAA was seen in 48, and genotype AAA was observed in 9. Regarding the patient's group,

genotype GGG was reported in 8, genotype GAA was seen in 31, and genotype AAA was observed in 1. Regarding the control group, genotype GGG was reported in 15, genotype GAA was seen in [17, 18], and genotype AAA was observed in 8. The distribution in all samples

significantly deviated from the expected count based on Hardy Weinberg equilibrium ($p = 0.033$). There was a significant deviation in the patient's group ($p < 0.001$), whereas no significant deviation was seen in the control group ($p = 0.436$).

Table 5: The Frequency Distribution of TNF- α GA Genotypes in Patients and Control Subjects

Characteristic	Total n=80		Patients Group n=40		Control Group n=40	
TNF- α GA	Observed	Expected	Observed	Expected	Observed	Expected
G.G.G., n (%)	23	27.61	8	13.81	15	13.81
GA, n (%)	48	38.78	31	19.39	17	19.39
AA, n (%)	9	13.61	1	6.81	8	6.81
χ^2	4.528		14.351		0.607	
p	0.033*		<0.001***		).436 NS	

χ^2 : Chi-Square Test Value According to Hardy Weinberg Equilibrium Analysis; *: Significant at $P \leq 0.05$; ***: Significant at $P \leq 0.001$; NS: Not Significant

The TNF- α G/A genotype frequency distributions for the patient and control groups are shown in Table 5. Patients had a lower frequency of genotype GGG (20.0% versus 37.5%, respectively) than the control group; nevertheless, $p < 0.084$ indicates that the difference did not reach statistical significance. The frequency of the genotype GAA was substantially higher in the patients (77.5 versus 42.5%, respectively) compared to the control group ($p = 0.001$). As a result, it

is a risk factor with an odds ratio of 4.66. Patients with genotype AAA had a considerably lower allele frequency (2.5% versus 20.0%, respectively; $p = 0.001$) than the control group. It has an odds ratio of 0.10, making it a protective factor.

Table 6 compares the frequency distribution of TNF- α G/A alleles in the sick and control groups. No significant changes were found between alleles G and A ($p = 1.000$).

Table 6: Comparison of the Frequency Distribution of TNF- α G/A Genotypes between Patients' Group and Control Group

TNF- α G/A Genotypes	Patients Group n=40	Control Group n=40	p	OR	95 % CI
GG, n (%)	8(20.0%)	15(37.5%)	0.084 CNS	0.42	0.15-1.14
GA, n (%)	31(77.5%)	17(42.5%)	0.001 C***	4.66	1.76-12.31
AA, n (%)	1(2.5%)	8(20.0%)	0.029 F*	0.10	0.01-0.86

OR: Odds Ratio; CI: Confidence Interval; C: Chi-Square Test; F: Fischer Exact Test; NS: Not Significant; ^": " Significant at $P \leq 0.05$; ***: Significant at $P \leq 0.001$

Discussion

The receptor-mediated activation of caspase is the primary mechanism by which the pro-inflammatory cytokine tumor necrosis factor alpha (TNF- α) causes RGC death. , oxidative stress, and mitochondrial dysfunction [17]. Studies demonstrating elevated TNF- α in POAG patients' serum and aqueous humor raise the possibility high levels of the protein TNF- α may serve as a biomarker for POAG and provide insight into the severity of glaucoma. Those who had trauma exhibited increased TNF- α plasma levels. And the degree of injury was associated with these higher levels [19]. A growing body of research indicates that the TNF- α /TNFRI pathway significantly affects the optic nerve degeneration seen in POAG. TNF- α activates the transcription factor NF- κ B, which controls the expression of several genes essential for neuronal survival when it binds to the high-affinity TNF receptor-2 [20]. However, binding to the death receptor TNF-R1, which has a low affinity, and initiating the apoptotic cascade controlled by the mitochondria, might potentially cause neurotoxicity. Any disturbance to the delicate equilibrium between these two channels could be hazardous. In individuals carrying TNF- α -308, for example, there is a genetically driven increase in TNF- α production. An allele may intensify the inflammatory environment in the eye during stressful situations, leading to the death of RGCs, the development of glaucomatous degeneration, and the advancement of the illness [21]. Table 4 compares the serum TNF- α levels of the sick and control groups. The sick group's mean serum TNF- α was notably more excellent (17.41 ± 8.97 versus 8.79 ± 4.75) than the control group's ($p < 0.001$). The present findings are consistent with research by Kondkar et al., which found that serum TNF- α levels in POAG cases were (1.88 ± 2.17 pg/mL, compared to 0.93 ± 1.49 pg/mL ($p = 0.003$), in the control group. Balaiya et al.'s findings [22] Additionally, the study

demonstrated that the levels of TNF- α in samples from individuals with glaucoma were markedly elevated compared to those from individuals without the condition ($P < 0.001$, t-test). The increased levels of TNF- α in POAG patients can be explained by the pathophysiological mechanisms associated with glaucoma, such as temporary ischemia and cell death, which can injure and activate glial cells. TNF-alpha is released by these activated glial cells and contributes to the neuro inflammatory response linked to glaucoma [23]. Patients had a lower frequency of genotype GGG (20.0% versus 37.5%, respectively) than the control group; nevertheless, the difference did not reach statistical significance ($p < 0.084$). Compared to the control group, the frequency of the genotype GAA was significantly greater in the patients (77.5 versus 42.5%, respectively) ($p = 0.001$). It is, therefore, a risk factor with a 4.66 odds ratio. Patients with genotype AAA had a considerably lower frequency of the allele than those in the control group (2.5% versus 20.0%, respectively; $p = 0.001$). It has an odds ratio of 0.10, making it a protective factor [24]. The current findings align with the research conducted by Al-Dabbagh and colleagues. Compared to controls, POAG patients had significantly greater genotype GAA ($P = 0.03$) and lower genotype GGG ($P = 0.04$) frequencies [16]. According to Bozkurt et al.'s study, the TNF- α -308 GA genotype was greater in POAG patients (22.1%) than in the control group (10.9%) ($P = 0.046$) [24]. is likewise consistent with this data. Although Lin et al. did not concur with us, they did find that patients with the genotype GAA had a lower frequency of the genotype than those in the control group (GAA and G.G.G. are protective factors) [24]. Finding the gene variations linked to common illness susceptibility is hampered by the inability of subsequent studies to replicate the observed results using various populations and higher case-to-control ratios, Disparate approaches to genetic research, inadequate technology and statistical methodologies for each genetical approach, conflicting standards for judging the quality of

statistical evidence—these four factors appear to be causing uncertainty in the area. And non-standardized methods for phenotype selection and assessment. Studies on Caucasian populations in Austria and Japan could not find a correlation between TNF- polymorphism G-308A and primary glaucoma, which is in contrast to our findings on the Iraqi population [25, 26]. These variations could be explained by the patient's clinical heterogeneity, sample size, diversity in ethnicity, and poorly defined controls.

Conclusion

This study unequivocally demonstrated that in Iraqi people, the TNF- α (G308A) polymorphism is substantially linked to the risk of primary open-angle glaucoma. When comparing the patients' genotypes to the control group, the patients' genotype GAA was significantly more common, their genotype GGG was less common, and their genotype AAA was substantially less common.

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