ORIGINAL ARTICLE

Tumor Necrosis Factor Alpha (TNFα) Gene Promoter Polymorphisms and Haplotypes are Associated with the Febrile Seizure (FS) and TNFα Serum Levels

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Abstract

Objectives

Febrile seizure is a neuroinflammatory disease involving fever-induced seizures affecting children in the early stages of life. TNFα is a pro-inflammatory cytokine reported to be elevated in FS. Specific promoter variants of TNFα could be associated with its elevated cytokine expression and susceptibility to FS. The present study analyzed the association of specific TNFα variants, including TNFα -238 G/A (a genetic variant; G: Guanine, A: Adenine) (rs361525), TNFα -308 G/A (rs1800629), and TNFα -376 G/A (rs1800750) promoter polymorphisms, with FS susceptibility, and TNFα serum levels in an Iranian population.

Materials & Methods

Sixty-eight FS patients and 136 controls were enrolled. The SSP-PCR method was utilized to analyze TNFα promoter genotypes. This research also confirmed the genotyping results by sequencing samples of ten patients and normal controls.

Results

The GG (a genetic sequence; G: Guanine) genotype of -238 SNP was associated with the increased risk of FS [OR = 12.65, 95% CI (2.83-56.60), P-value = 0.0012]. The AA (a genetic sequence; A: Adenine) genotype in the-308 region was increased in patients with FS and associated with the disease [OR = 4.62, 95% CI (1.46-14.56), P-value = 0.028]. The increased occurrence of heterozygous AG in the -376 SNP among control groups has been linked to a decreased risk of FS [OR = 0.22, 95% CI (0.11-0.43), P-value = 0.0001].

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This study revealed that AGA (a genetic sequence; G: Guanine, A: Adenine) (-238/ -308/ -376) haplotype with the highest frequency in controls was associated with a decreased risk of FS, while GAA (a genetic sequence; G: Guanine, A: Adenine) (-238/ -308/ -376) carriers were more susceptible to FS.

Conclusion

The current study suggested that TNFα gene promoter variants at rs361525, rs1800629, and rs1800750 could be associated with the susceptibility to FS and altered serum levels of TNFα.
Keywords: Febrile Seizure; Tumor Necrosis Factor Alpha; Promoter; Polymorphism; Haplotype.
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Introduction

Febrile seizures (FS) or febrile convulsions (FC) are among the most common causes of seizures in approximately 4% of children aged six months to six years (1). FS is generally referred to as convulsions associated with the presence of fever (≥ 38 °C), excluding cases with confirmed intracranial infections, other diagnosed causes of seizures, and identified history of non-febrile seizures(2). FS is classified into two major categories: simple and complex. Simple FS are more prevalent, typically lasting between 10 to 15 minutes, not recurring within 24 hours, comprising primary generalized seizures, not associating with an increased risk of epilepsy(3). The FS that lasts more than 15 minutes, recurs more than once within a 24-hour period, and is associated with focal seizures is complex (4). Although the exact pathogenesis of FS is not well determined, several predisposing factors, including genetic background(5), viral infections(6), and immune response imbalance(7), have been suggested to be involved. Studying the monozygotic twins and the higher rate of FS in patients with a positive family history highlights the significance of genetic factors in FS(8).

Cytokines have been implicated as mediators of the host immune response during inflammatory conditions. Aberrations in the expression of pro-inflammatory cytokines have been reported in various neurological disorders, including epilepsy, encephalitis, neurodegenerative diseases,

neurotrauma, neuropsychological diseases, and FS(9). These alterations could direct environmental inflammation to exacerbate CNS damage (10). The pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are the major molecules involved in increasing the body temperature and causing fever during inflammation(11). Although numerous studies have reported the increased expression of pro-inflammatory cytokines in FS(12), the genetic factors involved in these changes have not been thoroughly evaluated.

TNFα is a pro-inflammatory cytokine involved in the acute phase reaction, specifically in fever(13). Primarily, activated macrophages produce and secrete it. It operates through binding with TNFR1 and TNFR2 receptors. (14). Similar to various inflammatory diseases, the serum levels of TNFα have been reported to be elevated in FS(15, 16). Increased levels of TNFα have been associated with the recurrence of seizures and higher levels of other pro-inflammatory cytokines and proposed as potential disease markers(17, 18). The genetic factors, including polymorphisms in the promoter region, may alter the production of TNFα, determining the differences in disease severity and pathogenicity(19). Several variants have been identified in the promoter region of TNFα, of which TNFα -238 G/A (rs361525 or TNF: NM_000594.3.8: c.-418G>A), TNFα -308 G/A (rs1800629 or TNF: NM_000594.3.8: c.-488G>A), and TNFα -376 G/A (rs1800750 or TNF: NM_000594.3.8: c.-556G>A) polymorphisms are among the most critical sites (20, 21). To our knowledge, no study has examined these three polymorphic loci about TNFα expression of patients with FS in an Iranian population. Therefore, the present study aimed to evaluate the single nucleotide polymorphisms (SNPs) at the positions of -238, -308, and -376 of the TNFα promoter region with TNFα serum levels among patients with FS.

Materials & Methods

Patients, controls, and sample collection

Sixty-eight patients with FS referring to the Department of Pediatrics at Taleghani Children's Hospital, Golestan University of Medical Sciences (GoUMS) from March 2020 to March 2021 were enrolled in this cross-sectional study. As confirmed by an expert specialist following the International League Against Epilepsy (ILAE) classification of epileptic syndromes(22), all of the recruited patients were aged between six months and six years, had a body temperature of 38 °C or more at the time of admission, demonstrated a CRP level of 3 mmol/dL or less, and rolled out for all other possible causes of seizure. All patients with a history of systemic inflammatory disorders were excluded, including infectious or autoimmune diseases, damage to the vital organs (heart, liver, kidney, and the like), malignancies, and metabolic diseases. Besides, this research recruited 136 individuals to the control group referred to the Clinic of Pediatrics with a diagnosed febrile disease and expressed no personal or family history of FS. Following the ethical guidelines provided by the Committee of Ethics at GOUMS, an informed consent was signed by all parents of participating children (23). The clinical and laboratory data of patients with FS (obtained approximately 1 h after the seizure attack) and controls are demonstrated in Table 1. This study also used the Quanto test (http://biostats.usc.edu/cgi-bin/DownloadQuanto.pl) to assess the power post hoc of the study, calculated at the average level of 0.84 (24).

The researchers collected 5 mL of blood samples (2 mL whole blood and 3 mL coagulated) from

all participants within 30 min to 1 h after the seizure attack. These samples were promptly transported to the molecular biology laboratory of the Stem Cell Research Center at GOUMS. The coagulated samples were used to collect serum by centrifugation at 3000 rpm for 5 min and stored at -80 ºC until use.

Isolation of genomic DNA and genotyping

Whole blood samples were used to isolate genomic DNA using the commercial DNA extraction kit (Dena Zist, Mashhad, Iran), following the manufacturer's instructions. The quality and quantity of DNA samples were examined by Nanodrop spectrophotometer (Thermo Scientific, USA) and stored at -20 ºC until use. Approximately 100 ng of isolated DNA was used for genotyping the polymorphic regions using a sequence-specific amplification polymerase chain reaction (SSP-PCR) method. All PCR amplifications were conducted using 2 mM MgCl2, 0.4 mM dNTP mix (Genet Bio, Korea), 0.4 µM of each primer, and 1 U of Taq polymerase (Genet Bio) in a final volume of 25 µL using C1000 thermal cycler (Bio-Rad, USA). The human growth hormone (HGH) gene was amplification's most suitable internal control. The PCR amplification for all experiments was as follows: initial denaturation at 95 ºC for 5 min, 35 cycles of 95 ºC for 10 sec, 62 ºC (-238), 61 ºC (-308), and 58 ºC (-376) for 20 sec, 72 ºC for 30 sec and final extension at 72 ºC for 5 min. The specific primers to the target regions, the product size, and the optimum annealing temperature for each experiment are listed in Table 2. All PCR products were compared using agarose gel electrophoresis (2%) containing SYBR-safe dye (Cinnagen, Tehran, Iran) and visualized under UV trans-illumination. The present study also confirmed the genotyping results of ten samples from patients and normal controls by forward and reverse sequencing of DNA fragments.

TNFα ELISA assay

A commercially available ELISA kit (Invitrogen, UK) was used to assess the serum levels of TNFα in patients with FS in triplicates. The concentration results were as picograms per mL (pg/mL). Stat Fax 2100 ELISA reader (Awareness, USA) was used to obtain the optical density (OD) of all samples at the wavelength of 450 nm.

Data analysis and statistical measures

SPSS 22.0 (IBM SPSS, Chicago, USA) and GraphPad Prism 8 (GraphPad, San Diego, USA) software were utilized to analyze data statistically and depict graphs. Pearson's goodness of fit was used to explore each SNP's Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). SNPstats online web tool (http://bioinfo.iconcologia.net/SNPStats)23 was utilized to reconstruct haplotypes. The odds ratio (OR) and 95% confidence interval (CI) were specified to assess case-control associations. Chi-square goodness of fit or Fisher's exact test was used to compare the frequency of genotypes and haplotypes among groups. The nonparametric Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of multiple groups. P-values lower than 0.05 were considered statistically significant.

Results

TNFα gene promoter alleles and genotypes are associated with FS

Three SNPs, including -238 (rs361525), -308

(rs1800629), and -376 (rs1800750), were included to examine the association of TNFα gene promoter single nucleotide polymorphisms (SNPs) with FS susceptibility. Regarding the alleles distribution and genotypes in patients with FS and controls, the Hardy–Weinberg equilibrium (HWE) was established (Tables 3-5).

Determining the allele frequencies of TNFα -238 (G/A) in patients with FS and controls revealed a significant association between the G allele and FS susceptibility [OR = 3.47, 95% CI (1.62–7.44), P-value = 0.0098]. Moreover, this study evaluated the frequencies of genotypes under various inheritance models. The current study revealed that the GG genotype was significantly associated with the increased risk of FS [OR = 12.65, 95% CI (2.83-56.60), P-value = 0.0012] under the co-dominant model after being adjusted by sex and age covariates. Similarly, GG genotypes were significantly associated with higher FS susceptibility under the recessive model [OR = 6.37, 95% CI (1.95-20.85), P-value = 0.001] (Table 3).

Analyzing the TNFα -308 (G/A) polymorphic region revealed that the A allele with the highest frequency in patients could be introduced as a risk allele [OR = 2.00, 95% CI (1.16-3.45), P-value = 0.01]. Under the co-dominant model, the AA genotype was markedly increased in patients with FS and significantly associated with being subjected to FS [OR = 4.62, 95% CI (1.46-14.56), P-value = 0.028]. In addition, the recessive model analysis approved that the AA genotype was associated with the elevated risk of FS [OR = 3.18, 95% CI (1.15-8.76), P-value = 0.024] (Table 4).

The frequency of the A allele in TNFα -376 (G/A) SNP was significantly higher in controls and could be introduced as a protective allele for FS [OR = 0.55, 95% CI (0.33-0.92), P-value = 0.02]. According to the increased frequency of heterozygous AG among controls, it was related to the decreased risk of FS under co-dominant [OR = 0.22, 95% CI (0.11-0.43), P-value = 0.0001], and over-dominant [OR = 0.20, 95% CI (0.11-0.39), P-value = 0.0003] models. Interestingly, homozygous AA was associated with the increased risk of FS among patients [OR = 3.49, 95% CI (1.10-11.13), P-value = 0.031] (Table 5).

The association of TNFα gene promoter haplotypes with the altered risk of FS

The relative LDs between each pair of TNFα gene promoter SNPs were described in keeping with D′ values. D′ values approaching zero express the lack of LD, while those limiting 1 imply complete LD. Multiple SNP analysis suggested nonhomogeneous LD between TNFα SNPs varying from 0.38 (-308 vs. -376) to 0.51 (-238 vs. -308) and 0.61 (-238 vs. -376) D′ values. Additionally, this study used SNPstats by employing the EM algorithm to construct and analyze all possible haplotypes' interaction with the FS risk. Accordingly, this study explained that the H2 haplotype (AGA: -238/ -308/ -376) with the highest frequency in controls could be associated with the decreased risk of FS [OR = 0.09, 95% CI (0.02–0.31), P-value = 0.0004]. Adversely, the H6 haplotype (GAA: -238/ -308/ -376) was significantly associated with the raised risk of FS [OR = 43.58, 95% CI (3.60–527.76), P-value = 0.0034] (Table 6).

TNFα plasma levels were altered within distinct SNPs

The present research assessed the plasma levels of TNFα compared to the genotypes of studied SNPs among patients with FS. As shown in Figure

Table 1. Clinical characteristics and laboratory parameters of *Febrile Seizure* patients and controls

Characteristics*			**Febrile Seizure (N = 68)**	**Controls (N = 136)**
Age (Weeks)			22.04 ± 1.72	23.19 ± 2.03
Gender	Male		30 (44.1%)	72 (52.9%)
	Female		38 (55.9%)	64 (47.1%)
First Attack			38 (55.9%)	NA
Family History			12 (17.6%)	-
Attack Duration (Seconds)			133.71 ± 49.44	NA
Fever Temperature (°C)			38.63 ± 0.17	NA
TNFα serum level (pg/mL)			91.24 ± 18.54¥	47.60 ± 4.78¥
Type of Seizure		Simple	45 (66.2%)	NA
Complex		23 (33.8%)	NA	
WBC count (× 106/L)			10309.1 ± 1296.0	9796.1 ± 386.0
RBC count (× 106/µL)			4.55 ± 0.11	4.55 ± 0.07
Hb concentration (g/dL)			10.79 ± 0.31	11.33 ± 0.13
Platelet count (per µL)			288409 ± 20086	318761 ± 12488
HCT (%)			32.15 ± 0.79	33.44 ± 0.68
MCH (pg/cell)			26.23 ± 2.42	25.07 ± 0.39
MCHC (g/dL)			33.11 ± 0.47	33.25 ± 0.21
ESR (mm/hour)			12.54 ± 1.96	12.89 ± 0.87

* Data were demonstrated as Mean ± SE (standard Error of Means) or number (percentage). ¥ Independent samples t-Test demonstrated a significant difference. WBC: White Blood Cells, RBC: Red Blood Cells, Hb: Hemoglobin, HCT: Hematocrit, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, ESR: Erythrocyte Sedimentation Rate, NA: Not Applicable.

Table 2. The list of specific primers for target genetic variants

Genetic Variants	**Primer sequences (5' > 3')**	**Method**	**Fragment Length**
-238 (G/A) rs361525	CM: CGGGGTTCAGCCTCCAGGGTC G: ACTCCCCATCCTCCCTGCTCC A: ACTCCCCATCCTCCCTGCTCT	SSP-PCR	87 bp Ta: 62 ºC
-308 (G/A) rs1800629	CM: TCTCGGTTTCTTCTCCATCG G: ATAGGTTTTGAGGGGCATGG A: ATAGGTTTTGAGGGGCATGA	SSP-PCR	184 bp Ta: 61 ºC
-376 (G/A) rs1800750	CM: CCGTCCCCATGCCCCTCAAAA G: TTCCTGCATCCTGTCTGGAAG A: TTCCTGCATCCTGTCTGGAAA	SSP-PCR	95 bp Ta: 58 ºC
HGH internal control	F: GCCTTCCCAACCATTCCCTTA R: TCACGGATTTCTGTTGTGTTTC	PCR	429 bp

* CM: Common; HGH: human growth hormone; bp: base pair; Ta: annealing temperature.

Table 3. The genotype and allele frequencies of *TNFα* -238 (G/A) SNP (rs361525) in FS patients and controls under different inheritance models

Genotypes and alleles Number (%)		**FS patients (n=68)**	**Controls (n=136)**	**OR (95% CI)**	**P-value**
		Number (%)	**Adjusted by covariates***		
TNFα -238 (G/A)	A	62 (46%)	155 (57%)	Reference	
	G	74 (54%)	117 (43%)	3.47 (1.62-7.44)	0.0098
	Co-dominant model				
	AA	5 (7.3%)	23 (16.9%)	Reference	
	AG	52 (76.5%)	109 (80.2%)	2.19 (0.79-6.10)	
	GG	11 (16.2%)	4 (2.9%)	12.65 (2.83-56.60)	0.0012
	Dominant model				
	AA	5 (7.3%)	23 (16.9%)	Reference	
	AG+GG	63 (92.7%)	113 (83.1%)	2.56 (0.93-7.08)	0.05
	Recessive model				
	AA+AG	57 (83.8%)	132 (97.1%)	Reference	
	GG	11 (16.2%)	4 (2.9%)	6.37 (1.95-20.85)	0.001
	Over-dominant model				
	AA+GG	16 (23.5%)	27 (19.9%)	Reference	
	AG	52 (76.5%)	109 (80.2%)	0.81 (0.40-1.62)	0.55
X2 HWE* (P-value)		0.84 (0.81)	1.37 (0.16)		

*P-values lower than 0.05 are considered to be statistically significant. Significant associations are also shown in Bold Red (increased risk in patients) or Green (decreased risk among controls). Sex and age adjustment were performed to standardize the risk assessment. Exact test for Hardy-Weinberg equilibrium was also conducted.

Table 4. The genotype and allele frequencies of *TNFα* -308 (G/A) (rs1800629) SNP in FS patients and controls under different inheritance models

Genotypes and alleles Number (%)		**FS patients (n=68)**	**Controls (n=136)**	**OR (95% CI)**	**P-value**
		Number (%)	**Adjusted by covariates***		
TNFα -308 (G/A)	G	71 (52%)	171 (63%)	Reference	
	A	65 (48%)	101 (37%)	2.00 (1.16-3.45)	0.01
	Co-dominant model				
	GG	13 (19.1%)	42 (30.9%)	Reference	
	AG	45 (66.2%)	87 (64.0%)	1.67 (0.81-3.43)	
	AA	10 (14.7%)	7 (5.2%)	4.62 (1.46-14.56)	0.028
	Dominant model				
	GG	13 (19.1%)	42 (30.9%)	Reference	
	AG+AA	55 (80.9%)	94 (69.1%)	1.89 (0.93-3.83)	0.069
	Recessive model				
	GG+AG	58 (85.3%)	129 (94.8%)	Reference	
	AA	10 (14.7%)	7 (5.2%)	3.18 (1.15-8.76)	0.024
	Over-dominant model				
	GG+AA	23 (33.8%)	49 (36.0%)	Reference	
	AG	45 (66.2%)	87 (64.0%)	1.10 (0.60-2.03)	0.76
X2 HWE* (P-value)		0.98 (0.46)	1.30 (0.26)		

*P-values lower than 0.05 are considered to be statistically significant. Significant associations are also shown in Bold Red (increased risk in patients) or Green (decreased risk among controls). Sex and age adjustment were performed to standardize the risk assessment. Exact test for Hardy-Weinberg equilibrium was also conducted.

Table 5. The genotype and allele frequencies of *TNFα* -376 (G/A) (rs1800750) SNP in FS patients and controls under different inheritance models.

Genotypes and alleles Number (%)		FS patients (n=68) Number (%)	Controls n=136) Adjusted by covariates*	OR (95% CI)	P-value
TNFα -376 (G/A)	G	104 (76%)	180 (66%)	Reference	
	A	32 (24%)	92 (34%)	0.55 (0.33-0.92)	0.02
	Co-dominant model				
	GG	44 (64.7%)	49 (36.0%)	Reference	
	AG	16 (23.5%)	82 (60.3%)	0.22 (0.11-0.43)	0.0001
	AA	8 (11.8%)	5 (3.7%)	1.78 (0.54-5.85)	
	Dominant model				
	GG	44 (64.7%)	49 (36.0%)	Reference	
	AG+AA	24 (35.3%)	87 (64.0%)	0.31 (0.17-0.56)	0.004
	Recessive model				
	GG+AG	60 (88.2%)	131 (96.3%)	Reference	
	AA	8 (11.8%)	5 (3.7%)	3.49 (1.10-11.13)	0.031
	Over-dominant model				
	GG+AA	52 (76.5%)	54 (39.7%)	Reference	
	AG	16 (23.5%)	82 (60.3%)	0.20 (0.11-0.39)	0.0003
X2 HWE* (P-value)		0.79 (0.86)	1.85 (0.34)		

*P-values lower than 0.05 are considered to be statistically significant. Significant associations are also shown in Bold Red (increased risk in patients) or Green (decreased risk among controls). Sex and age adjustment were performed to standardize the risk assessment. Exact test for Hardy-Weinberg equilibrium was also conducted.

Table 6. Analysis of haplotype frequencies of *TNFα* promoter SNPs and their association with FS susceptibility; Adjusted by covariates (Sex and Age).

Haplotype	-238 (G/A)	-308 (G/A)	-376 (G/A)	FS patients (n=68)	Controls (n=136)	OR (95% CI)	P-value
H1	G	A	G	19.58%	26.53%	Reference	
H2	A	G	A	12.06%	30.3%	0.09 (0.02 - 0.31)	0.0004
H3	A	G	G	13.15%	18.82%	0.60 (0.17 - 2.11)	0.42
H4	G	G	G	27%	12.96%	4.28 (0.77 - 23.77)	0.098
H5	A	A	G	16.74%	7.86%	1.89 (0.28 - 12.59)	0.51
H6	G	A	A	7.83%	2.74%	43.58 (3.60 - 527.76)	0.0034
H7	A	A	A	3.64%	0%	10.36 (0.19 - 568.92)	0.25
Global haplotype association p-value: 0.0001							

*Data are expressed as (%). Haplotype frequencies and P-values were estimated using the implementation of the EM-algorithm. The most frequent haplotype was assumed as the reference haplotype. P-values lower than 0.05 are considered to be statistically significant. Significant associations are also shown in Bold Red (increased risk in patients) or Green (decreased risk among controls).

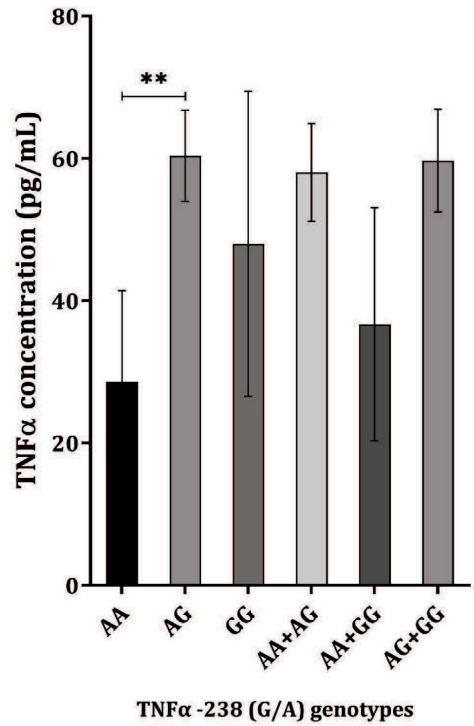

***Figure1**. **Association assessment of TNFα -238 (G/A) genotypes with TNFα plasma levels according to various inheritance models**; TNFα plasma levels were significantly lower in AA genotype carriers compared to AG. Statistical analysis was performed using the non-parametric independent samples Kruskal Wallis test followed by Dunn-Bonferroni post hoc. Each bar represents means±SEM. P-values lower than 0.05 considered as statistically significant.*

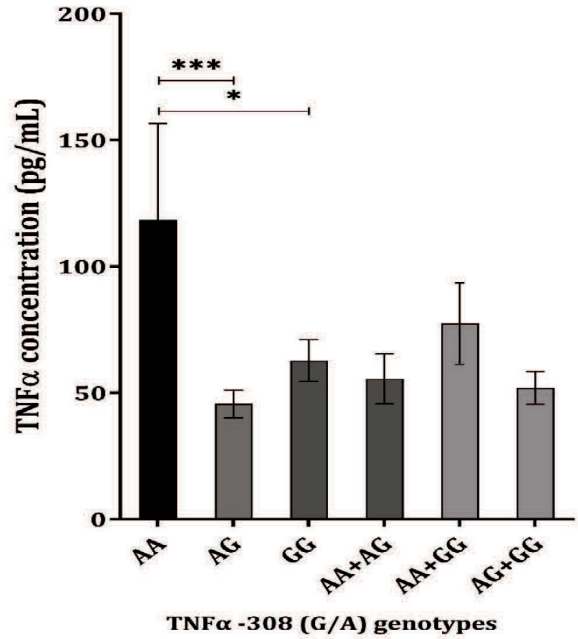

***Figure 2**. **Association assessment of TNFα -308 (G/A) genotypes with TNFα plasma levels according to various inheritance models**; TNFα plasma levels were significantly higher in AA genotype carriers compared to both AG and GG genotypes. Statistical analysis was performed using the non-parametric independent samples Kruskal Wallis test followed by Dunn-Bonferroni post hoc. Each bar represents means±SEM. P-values lower than 0.05 considered as statistically significant.*

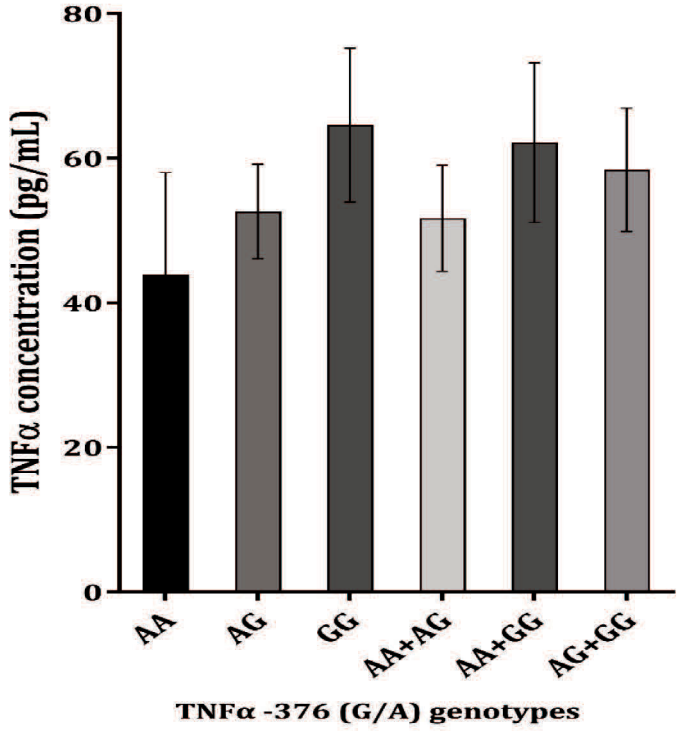

Figure 3. Association assessment of TNFα -376 (G/A) genotypes with TNFα plasma levels according to various inheritance models*; TNFα plasma levels were lower in AA genotype carriers but not significantly different compared to other genotypes. Statistical analysis was performed using the non-parametric independent samples Kruskal Wallis test followed by Dunn-Bonferroni post hoc. Each bar represents means±SEM. P-values lower than 0.05 considered as statistically significant*

1, the TNFα plasma level was significantly lower in AA genotype carriers of TNFα -238 (G/A) SNP (P-value < 0.01). Considering TNFα concentration levels in the genotypes of TNFα -308 (G/A), SNP determined that TNFα was markedly raised among AA genotype carriers in comparison to AG (P-value < 0.001) and GG (P-value < 0.05) (Figure 2). Furthermore, this study analyzed the plasma levels of TNFα about TNFα -376 (G/A) SNP and determined no significant difference despite the lower expression of examined cytokine among AA genotype carriers (Figure 3).

Discussion

FS (or FC) are acute neuroinflammatory conditions involving fever-induced seizures affecting children in the early stages of life(1). Similar to other neuroinflammatory disorders, the expression of pro-inflammatory cytokines is dysregulated. The genetic factors, including variants in these genes' promoter regions, have been suggested to be involved in FS pathogenesis (25-28). TNFα is a pro-inflammatory cytokine reported to be elevated in FS(13, 15, 16), mainly produced and secreted by activated macrophages(14). TNFα higher levels have been associated with the severity and complexity of seizures and proposed as potential disease markers in FS(17, 18). As demonstrated in previous studies, specific variants in the promoter region of TNFα could be associated with the elevated expression of its cytokine and increased susceptibility to several neuroinflammatory disorders, including Ischemic Stroke(29), Alzheimer's disease (30), Multiple Sclerosis(31), Parkinson's disease(32) and FS(19,

33). The current study analyzed the association of genotype and haplotype frequencies in three distinguished TNFα variants, including -238 G/A (rs361525), -308 G/A (rs1800629), and -376 G/A (rs1800750) gene promoter polymorphisms, with susceptibility to FS, and TNFα serum levels in an Iranian population.

Although limited studies have investigated the SNPs in the promoter region of the TNFα gene, these reports have been controversial. Considering TNFα -238 G/A SNP, this study demonstrated that the GG genotype and G allele were significantly associated with the increased risk of FS. The obtained findings on Iranian patients agree that Zare-Shahabadi et al. showed that the G allele and GG genotype at -238 were the most significant variants in increasing children's susceptibility to FS(33). Moreover, Choi et al. demonstrated no significant association between the TNFα gene promoter SNPs and FS in a Korean population, contrasting with our results(19). The dissimilarities in the results could be due to the differences in studied populations and their ethnicities. At the same time, it has been highlighted that ethnicity may significantly influence the cytokine gene polymorphism distribution(34). Analyzing the TNFα -308 G/A variant showed that the A allele with the highest frequency in patients could be introduced as a risk allele. At the same time, the AA genotype was markedly increased in patients with FS. The obtained results were inconsistent with the findings of Choi et al. and Zare-Shahabadi et al., who reported a higher frequency of G the allele in patients with FS, which was insignificant (19, 33). Moreover, Khoshdel et al. demonstrated no significant association between TNFα -308 G/A SNP and FS(35), turning our study into the first one reporting the considerable association in TNFα -308 G/A variants with FS. The frequency of the A allele in TNFα -376 G/A SNP was significantly higher in control subjects of our study and could be introduced as a protective allele for FS. To the best of our knowledge, this was the first study investigating the association of TNFα -376 G/A variants with FS. Evaluating the interaction of analyzed SNPs revealed heterogeneous LD in the TNFα SNPs with the most substantial LD between -238 and -376 variants.

Since previous studies have not assessed the linkage of these variants in the Iranian population, and Choi et al. showed no connection between these variants in their findings on Korean patients(19), further studies should be performed to approve or decline the present results. The association of all haplotypes with FS susceptibility revealed that AGA (-238/ -308/ -376) haplotype with the highest frequency in controls was associated with a decreased risk of FS, while GAA (-238/ -308/ -376) carriers were more susceptible to FS. The obtained findings were inconsistent with those of Choi et al.(19) and Zare-Shahabadi et al.(33). They demonstrated no significant association between haplotypes of TNFα gene promoter polymorphisms and FS. Besides, this study demonstrated that TNFα plasma levels were significantly lower in AA genotype carriers of the TNFα -238 G/A variant and increased in AA genotype carriers of TNFα -308 G/A SNP. Although several studies have reported elevated serum levels of TNFα in patients with FS (16, 36), no study has examined these three polymorphic loci concerning TNFα serum levels in FS. To the best of our knowledge, this was the first report that should be investigated more in future studies. The current study also accompanied various limitations, including the lack of subgroup analysis and HWE in the rs361525 control group. This was

due to limited samples and infrequent previous publications to compare these findings and enrich the discussion. Some discrepancies exist between the obtained findings about rs361525 and Iranome (http://www.iranome.ir/variant/6-31543101-G-A), which might also be due to the limited sample size in our study.

In Conclusion

This research's findings demonstrated that the TNFα gene promoter variants at the positions of -238 (rs361525), -308 (rs1800629), and -376 (rs1800750) could be associated with the susceptibility to FS and altered serum levels of TNFα in an Iranian population. Since no previous reports existed on the association of TNFα gene promoter SNPs, their cytokine expression, and FS in Iran, the present study strengthens our understanding of the genetic susceptibility of FS. In contrast, further clinical and molecular studies are needed to elucidate better the role of TNFα gene promoter polymorphisms in FS pathogenesis.

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This research project was approved in advance by the ethics committee at the Golestan University of Medical Sciences (GoUMS) (Code of ethics: IR.GOUMS.REC.1399.315).

Authors' Contribution

Study conception or design: S A H, and S M; Data Processing, Collection, Perform Experiment: B G, E D, S M, and M Z E; Data analyzing and draft manuscript preparation: S M, M R, N B, P H, and S M; Critical revision of the paper: S M and S A H; Supervision of the research: S M and S A H; Final approval of the version to be published: B G, E D, M Z E, M R, N B, P H, S M, S A H.

Conflict of interest

The authors declared no conflict of interest regarding the publication of this paper.

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