Toll Like Receptor-4 896A/G Gene Variation, a Risk Factor for Migraine Headaches

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ABSTRACT

Background: The pathogenesis of migraine involves immune-mediated mechanisms in the vascular endothelium. Toll like receptor 4 (TLR-4) is a signaling receptor of innate immunity which plays a role in various neuropathologies related to neuron inflammation. **Objective:** This case/control study is aimed to investigate whether TLR-4 896A/G variation is related to migraine headaches in an Iranian population. **Methods:** A total of 170 migraine patients (130 females, mean age 33.24 ± 11 years) and 170 age, sex, and ethnicity matched healthy controls (118 females, mean age of 31 ± 10 years) were recruited. Genotyping was carried out using the tetra primer amplification refractory mutation system (ARMS)-PCR. **Results:** The frequency of G allele was higher in migraine patients than the controls (15% vs. 4.7%; $p<0.0001$). Interestingly, the distribution of heterozygous 896A/G genotype statistically differed between migraineurs and controls (25.3% vs. 8.2%, $p=0.00002$, OR 3.87, 95% CI; 2.02-7.4). Multivariate logistic regression analysis indicated that G allele in affected female migraineurs is an independent factor associated with increased risk of migraine (OR 3.2, 95% CI 1.23-8.24, $p=0.01$). **Conclusion:** Our results showed TLR-4 polymorphism as a genetic risk factor for migraine. However, further studies in different populations are required to elucidate the precise role of TLR-4 896A/G mutation in susceptibility to migraine.

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Keywords: Female, Genetic Polymorphism, Inflammation, Migraine, Toll like Receptor

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INTRODUCTION

Migraine is a chronic neurovascular syndrome which is defined by paroxysmal attacks of severe, pulsating and unilateral or bilateral headache accompanied by autonomic symptoms such as nausea, vomiting, photo- and Phonophobia (1). Migraine attacks are categorized into two subtypes: migraine with aura (MA) and migraine without aura (MoA) (2). In one third of patients who suffer from MA, the headache phase is preceded or associated with temporary focal symptoms of neurological aura. These are usually visual but may also involve sensory disturbances, speech difficulties, and motor symptoms (3). The prevalence of migraine has been reported as high as 10% in general population worldwide (4) involving 6% of males and 18% of females (5). In spite of a significant increase in understanding of several important aspects of migraine pathophysiology during the last two decades, it has not been completely understood (6). Influence of diverse risk factors on induction and severity of migraine may result in the determination of clinically and genetically subgroups which may be helpful in development of new therapeutic strategies (6). Although genetic factors play important roles in migraine, the etiology of this disease is complex and involves both multiple genetic and environmental factors (7).

The role of immune inflammation in persistent neuropathic pain has been previously studied. It has been suggested that cytokines and chemokines may stimulate the activation of trigeminal nerves leading to inflammation (8,9). Moreover, infection with *Helicobacter pylori* has been recently reported as a risk factor for this disease (10). *H. pylori* contains a lipopolysaccharide which stimulates the production of inflammatory cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (11). However, the exact mechanism by which infections trigger the neurovascular changes leading to migraine attacks has not been revealed.

Single nucleotide polymorphisms (SNPs) leading to an amino acid substitution have been shown to be related with the pathology of migraine (12). In this regard, a candidate gene association study of 77 SNPs suggested that genetic variants in TNF, CCR2, TGFB1, NOS3, and IL-9 are associated with migraine (12). TLR-4, a leading receptor of innate immunity and inflammation, is expressed in microglia and astrocytes after inflammation in the central nervous system (13).

The role of TLR-4 in brain ischemia and stroke has been previously reported (13,14). Endogenous and exogenous mediators due to brain ischemia have been introduced as the legends of TLR-4. Furthermore, it has been demonstrated that the necrotic neurons induce a proinflammatory response in microglia which is dependent on MyD88, the adapter protein involved in TLR-4 signaling pathway (15). Mutations in the TLR-4 gene have been associated with the risk of ischemic stroke and atherothrombosis (16). The 896A/G (rs4986790) is the most common exonic polymorphism in TLR-4, resulting in amino acid substitution in the extracellular domain of the receptor (Asp299Gly), has been found to have functional consequences. This variant down regulates the TLR-4 signaling pathway and leads to a blunted inflammatory response (17). It was also suggested that this SNP plays a role in various inflammatory conditions such as gram-negative bacterial infections, arteriosclerosis, and Crohn disease (18). To the best of our knowledge, this polymorphism has not been studied in migraineurs, yet. Therefore, we attempted to evaluate if this polymorphism is related to MA and MoA migraines.

MATERIALS AND METHODS

Study Population. We studied 170 migraine patients (130 females and 40 males with a mean age of 33.24 ± 10.57 years) recruited consecutively from our out-patient headache center, and 170 healthy subjects (118 females and 52 males with a mean age of 31.11 ± 9.83 years) as control. All study participants were unrelated Caucasian subjects from Mazandarn Province, north of Iran. Diagnosis was made according to the International Headache Society (HIS) criteria (1) by two neurologists after administration of a structured questionnaire and direct interview and examination. Detailed questions about the presence of headache, severity, frequency, duration, site, type, aura symptoms and associated symptoms (nausea, vomiting, sensitivity to light and sound) were obtained. Age- and sex-matched unrelated healthy controls selected from blood donors, medical and non medical staff of the same geographic area. Individuals with a positive family history of migraine or any type of severe or recurrent headache in their first-degree relatives were excluded from the control group. Patients having known inflammatory disorders, infectious or immune diseases and abnormal plasma C-reactive protein levels were excluded. Patients experiencing daily headaches and those whose radiological tests, including computer-assisted tomography, revealed any pathology were also excluded from the study. Blood samples were taken after obtaining a written informed consent. The research protocol was approved by The Ethics Committee of Mazandaran University of Medical Sciences.

Genotyping. Venous blood was collected from each subject into tubes containing 50 mMol/L of EDTA, genomic DNA was isolated from anti-coagulated peripheral blood buffy coat using Miller's salting out method (19), and stored at -20°C at a final concentration of 200 µg/ml. TLR-4 genotyping was carried out using tetra- primer amplification refractory mutation system (ARMS)-PCR using the following primers (20,21).

Outer upper: 5′-TGAACCCTATGAACTTTATCC-3′,
Outer lower: 5′-GTTAACTAATTCTAAATGTTGCCATC-3′,
Inner upper (A allele): 5′-GCATACTTAGACTACTACCTCGAAGA-3′,
Inner lower (G allele): 5′-CAAACAATTAAATAAGTCAATAATAC-3′.

The Polymerase Chain Reaction (PCR) for specific allele amplification was carried out in a final 12.5 µl volume containing 100 ng DNA, 2 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Fermatas, Germany), 1 µM of each primer, 250 µM dNTPs mixed and 1x PCR buffer. The PCR amplification was carried out in 35 cycles including an initial hot start denaturation at 95°C for 5 min, followed by 9 cycles of 94°C for 30 s, X°C for 30 s (where X was initially 72°C, and was decreased 1°C per cycle to 64°C), 72°C for 30 s; and then 30 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 10 min. A common PCR control product (385 bp) and allele-specific products (G 292 bp, A 147 bp) were identified by electrophoresis on a 2% agarose gel against a 100-bp ladder and stained with 0.5 µg/ml ethidium bromide. Genotypes were scored independently by two individuals and ambiguous genotypes were repeated or omitted.

Statistical Analysis. Normal distribution of the data were done using Kolmogrov-Smirnov test and the the quantitative data were evaluated using independent Student's t-test, while the qualitative data were assessed applying chi square or Fisher Exact tests. Chi-square analysis was used to test for the deviation of genotype frequencies from

Hardy-Weinberg equilibrium. Association between genotypes or alleles of TLR-4 polymorphism and its disease risk factors was investigated using multivariate logistic regression analysis. The strength of association between the presence of the disease and the constitutionally determined alleles was estimated by the odds ratio (OR). OR with a confidence interval of 95% was obtained. p values less than 0.05 were considered to be statistically significant. All data were analyzed using Statistical Package for Social Sciences (SPSS) software version 11.

RESULTS

Table 1 shows the demographic characteristics of the patients and the healthy controls. Women were more likely to have migraine than men (76.5% vs. 23.5%), however, the sex distribution in controls subjects were almost similar to the patients (p=0.18). About 103 (60.6%) of migraineurs had a known family history of migraine, or at least one affected first degree relative. Clinically, the affected group had an average age of approximately 16 years at disease onset; the average duration of attacks was 2 to 4 days, with a mean frequency of 3.8 attacks per month. In addition, comparison of socioeconomic parameters revealed that migraineurs were less educated than the controls (70% vs 88.8%, p<0.0001) and were less employed at the time of sampling (34.1% vs. 44.1%, p=0.03).

Table 1. Characteristics of patients with migraine and the healthy control group.

Characteristic	Controls (n=170)	Patients (n=170)	P Value
Age (year)	31.64 ± 9.89	33.24 ± 10.6	0.15
Female	118 (69.4)	130 (76.5)	0.18
Male	52 (30.6)	40 (23.5)	
Pregnancy	18 (10.6)	23 (13.5)	0.51
History of oral contraceptive use *			
Yes	52 (44.1)	70 (53.8)	0.15
No	66 (55.9)	60 (46.2)	
Frequency of attack episodes (per month)	-	3.8 ± 2.6	
Family history of migraine (%)	-	103 (60.6)	
Currently employed -n (%)	75 (44.1)	58 (34.1)	0.03
≥ 12 years education -n (%)	151 (88.8)	119 (70.0%)	<0.0001
Disease duration (year)**	-	4 ± 4	

Data are expressed as mean ± S.D or number (percentage) unless otherwise stated.
P values for $\chi 2$ test for categorical variables and Student's t-test for continuous variables. * Results and p-values obtained in female subjects. ** Median was evaluated.

Genotype and allele frequencies for the 896A/G SNP of TLR-4 gene were analyzed for 170 healthy controls and 170 migraineurs. The genotype distributions in controls and patients with migraine did not deviate from that expected based on Hardy-Weinberg equilibrium (p=0.29 and p=0.92, respectively). As shown in Table 2, the frequency of G allele was higher in migraine patients than the controls (15% vs. 4.7%; p<0.0001). On

the other hand, the presence of G allele was associated with the risk of migraine (OR 3.5, 95% CI 1.9-6.4, p=0.000007). Interestingly, the frequency of heterozygous 896A/G genotype increased significantly in migraineurs compared with the controls (25.3% vs. 8.2%, p=0.00002, OR 3.87, 95% CI 2.02-7.4). Multivariate logistic regression analysis when adjusted for covariates of sex, age, and pregnancy demonstrated that patients who carried the G allele (G/G + A/G vs. A/A) of TLR-4 had a significantly higher risk for migraine with an OR of 3.95 (95% CI 2.1-4.7, p= 0.00006).

Table 2. Genotype and allele frequencies of 896A/G of TLR-4 between patients with migraine and healthy controls.

TLR-4 (rs4986790)	Controls n=170	Patients n=170	OR	95% CI	P Value
A/A	155 (91.2)	123 (77.4)			
A/G	14 (8.2)	43 (25.3)	3.87	2.02-7.4	0.00002
G/G	1 (0.6)	4 (2.4)	5.04	0.6-45.7	0.11
A allele	324 (95.3)	289 (85.0)			
G allele	16 (4.7)	51 (15.0)	3.57	1.9-6.4	0.000007
G dominant genotypes**	15 (8.8)	47 (27.6)	3.95	2.11-7.4	0.00006

Values are the numbers (percentages) of patients or controls positive for each allele or genotype. *The p values were calculated by the $\chi 2$ test from 3×2 or 2×2 contingency tables for genotypes and alleles, respectively.
** Gly299 dominant genotypes including; A/G + G/G

Furthermore, stratification of migraineurs into MoA and MA, showed no significant differences regarding to age, sex, family history of migraine and also frequency of migraine episodes during a month. As shown in Table 3, the frequency of G allele was more prevalent in MA than in MoA, but it was not statistically significant (17.2% vs. 13.8% p=0.39).

Table 3. 896A/G genotype and allele frequencies of patients with MoA and MA.

	MoA n=109	MA n=61	OR	95% CI	P Value
Age	32.26 ± 10.05	35 ± 11.33			0.105
Female	79 (72.5)	51 (83.6)	0.52	0.23-1.15	0.132
Male	30 (27.5)	10 (16.4)			
Positive family history of migraine (%)	64 (58.5)	39 (63.9)	1.25	0.65-2.4	0.52
Monthly frequency of migraine attacks	3.4 ± 2.7	4.6 ± 2.8			0.16
TLR-4 genotype *(rs4986790)*					
A/A	81 (74.3)	42 (68.9)			
A/G	26 (23.9)	17 (27.9)	1.26	0.62-2.58	0.4
G/G	2 (1.8)	2 (3.3)	1.93	0.26-14.2	0.51
A allele	188 (86.2)	101 (82.8)			
G allele	30 (13.8)	21 (17.2)	1.3	0.71-2.4	0.39
G allele dominant model	28 (25.7)	19 (31.1)	1.31	0.65-2.61	0.44

Values are the numbers (percentages) or mean ± SD of patients with MoA or MA positive for each parameter.
MoA, migraineurs without aura; MA, migraineurs with aura
The p values were calculated by the $\chi 2$ test and Student's t-test,as appropriate.

In addition, there was no significant difference in the distribution of TLR-4 896A/G SNP genotype between MoA and MA (p=0.51). Due to a very low frequency of the variant homozygous genotype, the heterozygous A/G and homozygous G/G genotypes were grouped together in a dominant model for G allele. The frequencies of G/G + A/G genotypes were 25.7% and 31.1% that are not significantly different in MoA patients when compared with the MA patients.

As it had previously been reported, the prevalence of migraine was more prominent in females than in males, further analysis was done between the female and male migraineurs. As shown in Table 4, heterozygous A/G genotype was significantly higher in female patients than in the male patients (29.2% vs. 12.5% p=0.03). On the other hand, multivariate logistic regression analysis when fixed for covariates of age and history of migraine, indicated that G allele in affected female migraineurs is an independent factor associated with an increased risk of migraine (OR 3.2, 95% CI 1.23-8.24, p=0.01).

Table 4. Effect of gender on 896A/G genotype and allele frequencies of patients with migraine.

TLR-4 (rs4986790)	Female patients n=130	Male patients n=40	OR	95% CI	P Value
A/A	88 (67.7)	35 (87.5)			
A/G	38 (29.2)	5 (12.5)	3.02	1.1-8.3	0.03
G/G	4 (3.1)	0 (0)	3.6	0.19-48.82	0.2
A allele	214 (82.3)	75 (93.75)			
G allele	46 (17.7)	5 (6.25)	3.2	1.23-8.24	0.01
G allele dominant model	42 (32.3)	5 (12.5)	3.34	1.22-9.14	0.014

Values are the numbers (percentages) of patients or controls positive for each allele or genotype. The p values were calculated by the $\chi 2$ test from 3×2 or 2×2 contingency tables for genotypes and alleles, respectively.

Table 5. Comparison of migraine characteristics between patients with G allele and those without G allele.

	A (n=123)	(A/G+G/G) (n=47)	OR	95% CI	P Value
Age	34.6 ± 10.87	29.6 ± 8.87			0.005
Female	88 (71.5)	42 (89.4)			
Male	35 (28.5)	5 (10.6)	3.34	1.22-9.14	0.015
MA	42 (34.1)	19 (40.4)	0.76	0.38-1.53	0.48
MoA	81 (65.9)	28 (59.6)			
Positive family history of migraine	75 (61.0)	28 (59.6)	1.1	0.53-2.1	0.86
Age upon onset	27.35 ± 9.58	23.55 ± 8.1			0.017
Duration (median)	5	3			0.448
Migraine episodes (median)	2	2			0.203

The values are n (%), mean ± SD, or median.

To better evaluate the effect of mutant G allele on disease development, we categorized the migraineurs into two subgroups; carriers of G allele ((A/G+G/G) and carriers of wild type A allele. As shown in Table 5, there were significant differences in age, sex, and age in disease onset between the two subgroups. Indeed, variant G allele carrying patients had a lower age upon onset of the disease and were predominantly females.

DISCUSSION

This study showed an association between the TLR-4 896A/G (rs4986790) polymorphism and migraine, suggesting that TLR-4 is involved in the pathogenesis of migraine headaches. Moreover high prevalence of G allele and G allele carrier genotypes in patients with migraine were also observed. Increased frequency of variant G allele and heterozygous A/G allele has been observed in female patients. G allele carriers were more prone to migraine headaches in the younger age group.

Migraine is a complex disease wherein various genetic factors interact with environmental factors. Genetic variants in innate immunity-related inflammatory pathways may participate in several facets of brain injury and therefore contribute to the pathogenesis of migraine It is worthwhile to note that migraine is associated with stroke (22) due to the possible dysfunction of arteries during migraine attacks and the presence of indolent brain lesions in migraineurs (23). Therefore, the vascular dysfunction involved in migraine pathophysiology might have a role in the development of ischemic stroke in migraineurs (24). Indeed, a number of studies have noted that inherited variants in genes encoding for TLR pathway are associated with stroke and other neurodegenerative diseases (13,25,26).

Brain endothelial cells can be activated by microbial and non microbial ligands through TLRs with a resultant activation of NF-κB pathway and subsequently expression of inflammatory mediators (17,27,28). Increasing evidence reveals that TLR-4 signaling induced by ischemic injury which leads to increased oxidative stress, might play a role in the development of brain damage and inflammation (13,29,30,31). These reports seem to support the inflammatory theory in the phatophysiology of migraine (31). Concordant with this theory is the literature showing an association between migraine and *Helicobacter pylori, Chlamydia Pneumoniae,* and *Toxoplasma* infections (10,33,37). Indeed, TLR-4 initiates cellular signaling in response to structurally diverse microbial molecules such as bacterial LPS, respiratory syncytial virus fusion protein, and chlamydial heat shock protein 60 (38). It has been demonstrated that 896A/G mutation which is encoded within the fourth exon of the *TLR-4* gene affects its ligand-binding region and is associated with hyporesponsiveness to LPS in macrophages and epithelial cells (17). Functional polymorphism in TLR-4 modulates the levels of inflammatory cytokines (39) and also increases host susceptibility to bacterial infection (20,21). Therefore, higher prevalence of TLR-4 896G allele in migraineurs may increase vulnerability of pathogens in the patients compared to controls. In addition, it has also been demonstrated that TLR-4 activation increases the risk of atherosclerosis (40).

To the best of our knowledge, this is the first report on the association between TLR-4 896A/G polymorphism and migraine. Interestingly this association was stronger in women and remained significantly associated with migraine after controlling for age and history of migraine. This result is in line with the high prevalence rate of migraine

in females (41). Although these findings need to be confirmed in further studies in different population, it might support the theory of neurogenic inflammation in migraine pathophysiology. Higher prevalence of G allele carriers in younger patients at the time of disease onset seems to support the role of TLR-4 in the pathogenesis of migraine. On the other hand, TLR-4 would play a role not only as a risk factor for ischemic stroke, but also as a causative agent of worsening conditions in higher inflammation and brain damage. In addition, it has been demonstrated that TLRs expression is not static and could be modulated rapidly in response to pathogens, various cytokines and environmental stresses (42).

Although we did not find any significant difference in G allele frequency between MA and MOA, given that TLR-4 plays a fundamental role in the production of proinflammatory cytokines and chemokines by monocytes and macrophages, it seems that TLR-4 dysfunction due to the presence of minor A allele may alter the production of proinflammatory cytokines and consequently make changes in brain vasculature. Concordant with this speculation is upregulation of the expression of TLR-4 in glial cells during inflammation (43). Indeed, induction of an experimental subarachnoid hemorrhage in rodents induces a high expression of TLR-4 in the brain and basilar artery (44).

Concerning the hereditary pattern, (45,46) our results seem to highlight the role of TLR-4 polymorphism as a genetic risk factor for migraine. However, further studies in different populations are required to elucidate the precise role of TLR-4 896G/A mutation in susceptibility to migraine.

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