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Correlation between iron regulatory protein-1 (G-32373708A) and -2 (G-49520870A), gene variations and migraine susceptibility in southeast Iran: A case-control study

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ABSTRACT

Migraine is a chronic neurological disease characterized by recurrent moderate to severe headaches commonly in association with neuro-inflammation. Iron regulatory proteins (IRPs) regulate the expression of iron metabolism genes and control cytosolic iron concentrations in order to optimize cellular iron availability. The current study aimed to investigate the possible associations IRP-1 and -2 single nucleotide polymorphisms (SNPs) and susceptibility to migraine in Iranian patients. In a prospective case-control study, we studied blood samples of 190 patients with migraine and 200 healthy controls for analysis of gene variants. Genotyping for the IRP-1 SNP: G-32373708A (rs867469), and IRP-2 SNP: G-49520870A (rs17483548) were performed using PCR-ASO and PCR-RFLP respectively. Statistical analysis was performed using the SPSS version 21.0 (SPSS, Chicago) and SNPStats version 1.14.0. Among IRP SNPs, rs867469 (GG genotype, adjusted OR = 3.82, 95% CI = 1.131–12.953, $P = 0.031$) and rs17483548 (GG genotype, adjusted OR = 19.12, 95% CI = 3.69–99.05, $P = 0.001$) were significantly associated with migraine. The most frequent genotypes in the migraineurs were GA in both SNPs rs867469 (58%), and rs17483548 (68%). Moreover, there was significant statistically relationship between rs17483548 SNP (GG genotype, adjusted OR = 0.10, 95% CI = 0.013–0.745, $P = 0.025$) and different subclasses (without and with aura) of migraine. Our data indicated that G/G genotypes in both the G-32373708A-IRP-1 and G-49520870A-IRP-2 polymorphisms could be associated with increased risk for migraine. Therefore, it is suggested that in addition to other factors, IRP-1 and IRP-2 SNPs may play a pivotal role in the migraine.

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1. Introduction

Migraine is a rigorous and painful headache accompanied with sensory warning and is a public health problem of great impact on both the patient and society [1]. The two major subclasses of migraine are common migraine (without aura) and classic migraine (with aura or neurological symptoms) [2]. Since about half of migraineurs (migraine patients) do not pursue medical attention and there is no economic, social or ethnic limitation, it

is difficult to precisely determine of disease prevalence in the community [3]. It seems that about 15 to 16 percent of women and 5 to 9 percent of men are affected with migraine and its prevalence is highest among the ages of 30–49 worldwide [4]. Migraine etiology is multifactorial, involving both various genetic and environmental factors, but scientists consider three important mechanisms for its pathophysiology including: inflammatory, neurological and cardiovascular impairments [5,6]. Cell and molecular association studies may point to the novel molecules that mediate migraine disorder and enable its management. According to the theory of neuro-inflammation, in the migraine, ion channels (Na^{2+} , Ca^{2+} , K^{2+}) and inflammatory mediators activation in the meninges sensory nerves, stimulates pain receptors in these area [7,8]. It

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has been shown that headaches are a common symptom of iron deficiency and prolonged headaches may even become chronic. On the other hand iron deficiency can also lead to anemia. In addition to changes in the inflammatory cytokines, relation between iron deficiency anemia and migraine attacks have been reported but there are conflicting results on the mechanisms involved [9–14]. The accumulation of iron ions is related to the frequency of migraine attacks, being greatest in chronic migraine where the attacks may occur daily [15]. It is known that diminution of iron ions deteriorate the surface of mitochondria and decrease the efficiency of the respiratory chain and the neuronal energy substrates. Widely, iron and other trace elements are present in sensitization processes in central nervous system (CNS) and likely to be involved in pain threshold modulation [16]. Iron regulatory proteins (IRPs), also known as iron-responsive element-binding proteins (IRE-BP) regulate iron metabolism via binding to iron responsive elements in mRNA. IRP-1 or aconitase-1 (ACO-1) has aconitase activity, and catalyze conversion of citrate to isocitrate and they are iron-sulfur proteins that they require a 4Fe-4S cluster for their enzymatic activity [17]. IRP-2 is more expressed in intestine and brain, less abundant than IRP-1 in other regions and has no ACO activity [18–20]. The human IRP-1 gene is located on chromosome 9p21.1 and IRP-2 gene is located on chromosome 15q25.1. So far, several allelic variant polymorphisms have been found in IRPs gene (<http://www.ncbi.nlm.nih.gov/snp>), and g.32373708 G > A-IRP1 (rs867469) plus g.49520870G > A-IRP-2 (rs17483548) are more important. To understand the probable role of IRPs, as important iron controllers, we analyzed its imperative polymorphisms in migraineurs with two different subclass of disease and compared them to healthy controls. As we know, it is the first epidemiological studies exploring IRP-1 and IRP-2 SNPs in migraine population all over the world.

2. Materials and methods

2.1. Patients and samples

The study was approved ethically in medical research committee at Zahedan University of Medical Sciences, and was conducted with clinical samples from migraine patients (N = 190, age: 13 to 66 years) who were treated at the Department of Neurology, Ali-ebn Abitaleb Hospital, Zahedan, Iran, from August 2013 to February 2014. Healthy controls (HCs) without any inflammatory, neurological diseases, migraine headache and specific systemic disease (N = 200, age: 15–75 years) from volunteer blood donors were selected during same time. A diagnosis of migraine was made according to standardized criteria of international headache classification [21]. Patients were excluded if they had anemia related diseases or received iron supplement. Patients adjusted in two definite groups without aura (N = 112, 76 female and 36 male) and with aura (N = 78, 56 female and 22 male) subtypes of migraine. All patients were informed of the study and participated voluntarily and written consents were taken. The work is carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

2.2. Blood collection and DNA extraction

Whole blood (10 mL) samples were taken from all subjects and collected in separator tubes (contain EDTA, 0.5 M) and centrifuged for 15 min at 150g (gravity) at 20 °C and then serum was stored at –20 °C in sterile plastic tubes for DNA extraction. Genomic DNA was extracted from the serum of 190 subjects with migraine headaches and 200 HCs using the DNA extraction kit (DIAtom DNA Prep., GORDIZ, Moscow, Russia) according to the manufacturer's

instruction. DNA quality extracts were analyzed by electrophoresis. By NanoDrop DNA concentrations about 60 ng/μl was obtained and ratio of 260/280 nm around 1.7 to 1.9 was accepted [22].

2.3. IRP-1 Genotyping

Selection of SNPs was made on the basis of their location in the 5' regulatory region, which commonly contains the promoter [23]. The g.32373708 G > A-IRP1 (rs867469) single nucleotide polymorphisms (SNPs) were analyzed through allele-specific oligonucleotide-PCR method (ASO-PCR) method [24,25]. PCR amplifications were performed in a final volume of 20 μl containing, 10 μl master mix (TAKARA, Tokyo, Japan), 0.7 μl (10 pmol) of each primer, 2 μl template DNA, and 6.6 μl DNase-free water was used. The amplification was performed in Bio-Rad Thermal Cycler with an initial denaturation step at 95 °C for 5 min; followed by 35 cycles at 95 °C for 30 s, 56 °C for 35 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min. The primers designed to detect the rs867469 SNP were as follows: allele specific sense oligonucleotides 5'-TGCACACCTGCAAAGAAG-3' for G variant and 5'-TGCACACCTGCAAAG AAA-3' for A variant and antisense oligonucleotide 5'-CTAGATGAAAGGTGGTGAGG-3'. The PCR product (237 bp) was checked for size and purity by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide and viewed under UV light.

2.4. IRP-2 Genotyping

Selection of SNPs was made on the basis of their location in the 5' regulatory region, which commonly contains the promoter [23]. The g.49520870G > A-IRP-2 (rs17483548) single nucleotide polymorphisms (SNPs) were analyzed through restriction fragment length polymorphism-PCR method (RFLP-PCR) method [24,25]. PCR amplifications were performed in a final volume of 20 μl containing, 10 μl master mix (TAKARA, Tokyo, Japan), 0.7 μl (10 pmol) of each primer, 2 μl template DNA, and 6.6 μl DNase-free water was used. The amplification was performed with an initial denaturation step at 95 °C for 5 min; followed by 35 cycles at 95 °C for 30 s, 60 °C for 35 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min, using the following primers: sense 5'-CCCCCACTT GAAAACACG-3' and antisense 5'-AGATCGTCGGACAGGAAAAC-3'. The PCR product (360 bp) was checked for size and purity by 1.5% agarose gel electrophoresis. Final volume of 20 μl including 2 μl of 10 × Buffer, 0.5 μl of enzyme, 7 μl of PCR product, 10.5 μl of double distilled water was used for all amplification products overnight at 37 °C, and 10 μl sample loaded for electrophoresis. AflII (Thermo Scientific) endonuclease digested pattern for rs2243250 amplification product were 183 bp and 177 bp for the GG, 183 bp, 177 bp and 360 bp for the GA and 360 bp for the AA genotypes.

2.5. Statistical analysis

SPSS version 21.0 (SPSS, Chicago) and SNPStats version 1.14.0 were used for all the statistical analyses. The association between case-control status and each polymorphism, measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI), was estimated using an unconditional multiple logistic regression model, both with and without adjustment for sex and age. The Hardy-Weinberg equilibrium (HWE) was tested with the X2 test for any of the SNPs under consideration. Smoking habit was categorized in terms of never smokers and smokers (including current and former). Stratified analysis according to age, sex, race, and migration was also conducted. To study a possible gene-environment interaction, the patients and controls were divided into subgroups depending on sex, race, age, migration, and

Table 1

Demographic distribution of migraine patients and control subjects enrolled in the study.

Characteristics	Control (n = 200) N (%)	Case (n = 190) N (%)
Gender (male/female)	68(34)/132(66)	42(22)/148(78)
Age (year) < 50	174(87)	180(95)
Age (year) ≥ 50	26(13)	10(5)
Mean ± SD	35.1 ± 12.2	31.7 ± 10.17
Range	15–75	13–66
Smoking (yes/no)	80(40)/120(60)	88(46)/102(54)
Race (parse/other)	144(72)/56(28)	146(77)/44(23)
Migrate (yes/no)	96(48)/104(52)	72(38)/118(62)

smoking. Unconditional logistic regression analyzes were also performed to assess the association between genotypes and risk for migraine after stratification of the individuals according to sex, race, age, migration, and smoking. The significance level was set at $P \leq 0.05$ for all the tests.

3. Results

3.1. Demographic analysis and the risk of migraine independent of genotype

The characteristics of the patients with migraine and migraine free controls involved in this study are presented in Table 1. The mean ± SD age was 31.7 ± 10.17 years for the patients (range 13–66) and 35.1 ± 12.2 years for the controls (range 15–75) as well

as 22% of the patients and 34% of the controls were men, whereas 78% of the patients and 66% of the controls were women. Moreover, we explored the relationships between gender, age, smoking, race and migration and the risk of migraine independently of genotype (Table 2). Our results suggested that there were no significant different between gender, age, smoking, race and migration among the controls and patients (Table 2). Therefore, age and gender variables (with lower P -value) were further adjusted in the multivariate logistic regression model to control for possible confounding factors of the main effects of the polymorphisms.

3.2. Association of IRP-1 SNP (rs867469 G/A) and migraine

The G/G, G/A and A/A genotypes of IRP-1 were found in 9%, 81% and 10% in HCs, in comparison with 31.5%, 58% and 10.5% in migraineurs, respectively. The allele frequency of IRP-1 rs867469 (G/A) were 49.5% (G), 50.5% (A) in HCs and 60.5% (G), 39.5% (A) in migraineurs, respectively (Table 3). Regarding the genotypes frequencies for the IRP-1 rs867469 (G/A), both case and control group are not consistent with HWE. Distributions of IRP-1 allele frequency in rs867469 (G) were significantly different between patients and controls (crude OR = 1.56, $P = 0.029$). Moreover distributions of IRP-1 GG polymorphism were significantly different between patients and controls (crude OR = 3.33, $P = 0.040$). On the other hand, the GG genotype increased the risk of migraine (adjusted OR = 3.82, $P = 0.031$). In conclusion, in the migraine patients, the G allele of the rs867469 G/A-IRP1 SNP increased the risk of the disease (adjusted OR = 1.61, $P = 0.023$) and the A allele decreased such risk (Table 3). AA and AG genotypes could be

Table 2

Risk of migraine in association with gender, age, smoking, race and migration.

Characteristics	Control (n = 200) N (freq.)	Case (n = 190) N (freq.)	OR (95%CI)	P-value
<i>Gender</i>				
Males	68(0.34)	42(0.22)	0.55(0.291–1.042)	0.067
Females	132(0.66)	148(0.78)	1.81(0.960–3.433)	0.067
<i>Age (year)</i>				
Age < 50	174(0.87)	180(0.95)	2.69(0.92–7.862)	0.071
Age ≥ 50	26(0.13)	10(0.05)	0.37 (0.127–1.08)	0.071
<i>Smoking</i>				
Ever (current, former)	80(0.40)	88(0.46)	1.29(0.73–2.28)	0.37
Never	120(0.60)	102(0.54)	0.77(0.438–1.364)	0.37
<i>Race</i>				
Persian	142(0.71)	146(0.77)	1.35(0.712–2.579)	0.35
Other	58(0.29)	44(0.23)	0.73(0.388–1.404)	0.35
<i>Migrate</i>				
Yes	96(0.48)	72(0.38)	0.66(0.374–1.170)	0.15
No	104(0.52)	118(0.62)	1.51(0.855–2.677)	0.15

Comparisons were performed by independent samples t -test χ^2 of test results for categorical data, freq. = frequency, OR = odds ratio, CI = confidence interval, N = number.

Table 3

Distribution of genotypes and frequency of alleles of the g.32373708 G/A-IRP-1 polymorphisms in patients with migraine and individuals without disease (controls).

IRP-1 (rs867469)	Control (n = 200)	Case (n = 190)	Crude OR (95%CI)	Crude P-value	Adjusted. OR ^a (95% CI)	Adjusted. P-value ^a
AA, No (%)	20(10)	20(10.5)	1	–	1	–
AG, No (%)	162(81)	110(58)	0.67(0.265–1.740)	0.421	0.63(0.242–1.642)	0.345
GG, No (%)	18(9)	60(31.5)	3.33(1.055–10.530)	0.040	3.82(1.131–12.953)	0.031
	P = <0.001					
A allele	101(50.5)	75(39.5)	1	–	1	–
G allele	99(49.5)	115(60.5)	1.56(1.047–2.338)	0.029	1.61(1.068–2.428)	0.023
	P = 0.029					

CI: confidence interval, OR: odds ratio.
Significant P -values are in bold.

^a Adjusted for age and sex.

Table 4

Distribution of genotypes and frequency of alleles of the g.49520870 G/A-IRP-2 polymorphisms in patients with migraine and individuals without disease (controls).

IRP-2 (rs17483548)	Control (n = 200)	Case (n = 190)	Crude OR (95%CI)	Crude P-value	Adjusted. OR ^a (95% CI)	Adjusted. P-value ^a
AA, No (%)	16(8.0)	12(6.3)	1	–	1	–
AG, No (%)	154(77.0)	130(68.4)	2.11(0.77–5.752)	0.144	0.63(0.242–1.642)	0.133
GG, No (%)	30(15.0)	48(25.3)	7.5(2.171–25.906)	0.001	19.12(3.69–99.05)	0.001
	P = 0.002					
A allele	92(46.0)	71(37.4)	1	–	1	–
G allele	108(54.0)	119(62.6)	1.42(0.953–2.140)	0.085	1.51(0.998–2.288)	0.051
	P = 0.08					

CI: confidence interval, OR: odds ratio.

Significant P-values are in bold.

^a Adjusted for age and sex.**Table 5**

Distribution of genotypes and frequency of alleles of the g.32373708 G/A-IRP-1 and g.49520870 G/A-IRP-2 polymorphisms in patients with different migraine subtypes.

IRP-1 (rs867469)	With aura, No (%) (n = 78)	Without aura, No (%) (n = 112)	OR(95%CI)	P-value
AA, No (%)	8(10.3)	12(10.7)	1	–
AG, No (%)	34(43.6)	76(67.9)	1.49(0.372–5.973)	0.573
GG, No (%)	36(46.2)	24(21.4)	0.44(0.103–1.915)	0.277
	P = 0.033			
IRP-2 (rs17483548)				
AA, No (%)AG, No (%)GG, No (%)	8(7.1) 96(85.7) 8(7.1)	4(5.1) 34(43.6) 40(51.3)	1 1.41(0.237–8.416) 0.10(0.013–0.745)	– 0.705 0.025
	P < 0.001			

CI: confidence interval, OR: odds ratio. Significant P-values are in bold.

considered as protective and GG genotype could be considered as risk factor in migraine headaches. Otherwise, there were no significant association with migraine subtypes and IRP-1 rs2070874 (G/A) SNP in this population (Table 5).

3.3. Association of IRP-2 SNP (rs17483548 G/A) and migraine

The G/G, G/A and A/A genotypes of IRP-2 were found in 15%, 77% and 8% in HCs, in comparison with 25.3%, 68.4% and 6.3% in migraineurs, respectively. The allele frequency of IRP-2 rs17483548 (G/A) were 54% (G), 64% (A) in HCs and 62.6% (G), 37.4% (A) in migraineurs, respectively (Table 4). Regarding the genotypes frequencies for the IRP-2 rs17483548 (G/A), both case and control group are not consistent with HWE. Distributions of IRP-2 allele frequency in rs867469 were not significantly different between patients and controls (Table 4). Distributions of IRP-2 AG polymorphism were not different between patients and controls before and after adjustment for age and sex (crude OR = 2.11, $P = 0.144$ /adjusted OR = 0.63, $P = 0.133$). Distributions of IRP-2 GG polymorphism were significantly different between patients and controls before and after adjustment for age and sex (crude OR = 7.5, $P = 0.001$ /adjusted OR = 19.12, $P = 0.001$). On the other hand, the GG genotype increased the risk of migraine. AA and AG genotypes could be considered as protective and GG genotype could be considered as risk factor in migraine headaches. Otherwise, there were significant association with migraine subtypes and GG genotype of IRP-2 rs17483548 (G/A) SNP in this population (Table 5).

4. Discussion

Migraine is a severe neurological disorder that causes a strong throbbing or pulsating pain in one area of the head and can be accompanied by nausea, vomiting and extreme photophobia [2]. Several studies used a candidate gene approach to elucidate genetic contribution to neuropathic pain phenotypes; however,

the data is limited and inconsistent [26,27]. The genetic background of migraine consists of common or overlapped pathways and the responsible genes may provide insight regarding the pathophysiological mechanisms that can explain their comorbidity with migraine [28,29]. Data from the large cohort of Caucasian women (n = 25,713) in 77 different SNPs suggested that there is an association between variants in some inflammatory mediators including TNF- α rs673 (OR = 0.52, 95% CI = 0.30–0.89, $P = 0.017$), CCR2 rs1799864 (OR = 1.12, 95% CI = 1.03–1.21, $P = 0.007$), TGF β 1 rs1800469 (OR = 0.93, 95% CI = 0.89–0.89, $P = 0.009$), NOS3 rs3918226 (OR = 1.13, 95% CI = 1.01–1.27, $P = 0.04$), and IL-9 rs2069885 (OR = 1.12, 95% CI = 1.02–1.24, $P = 0.02$) with migraine [30]. Although the pathogenesis of migraine is not completely detected, a growing viewpoint of assays offers that oxidative stress and free radical damage may worsen or aggravate migraine susceptibility [31,32]. In a clinical study, migraine patients that take antioxidant supplements had shown a progressive effects in migraine therapy, offering that oxidative stress is somehow contributed in its pathogenesis [33]. Iron is assumed as a possible origin of oxidative radicals related to destructive activities affecting the CNS [34]. Although iron can cause oxidative tissue injury through the Haber–Weiss and Fenton response, it is also a necessary for many metabolic pathways. Several studies have detected that iron unbalance load may have a vital role in the pathogenesis of migraine [12,35]. In mammals, two homologous IRPs have been recognized, IRP-1 and IRP-2. IRP-1, is a multi-functional protein that acts as an iron-responsive element (IRE)-binding protein contributed in the control of iron metabolism by linkage of mRNA to suppress translation [36]. It acts also as the cytoplasmic isoform of aconitase. Aconitases are iron-sulfur proteins and requirement a 4Fe-4S cluster for their enzymatic function, in which they catalyze transform of citrate to isocitrate [37]. IRP-2 also called IREB-2 (iron-responsive element binding protein 2) have 79% similarity to IRP-1 but not have aconitase activity [38]. Under the circumstances of iron-deficiency, IRPs bind to IREs exist in the 5' UTR of mRNAs, such as in ferritin heavy and light chains and suppress

translation and at the same time IRPs bind to mRNAs including IREs in the 3' UTR, such as TfR-1 and DMT-1, that lead to increase RNA stability. And conversely, under iron overload circumstance, IRPs bind to IREs is decreased. So, under iron-deficiency circumstances, IRPs up-regulate genes related to enhance iron uptake and under iron overload circumstances, IREs up-regulates genes related to storage of iron level [39]. The significance of balance iron homeostasis is emphasized by the reality that neurological disorders has been seen in some pathological circumstance due to overload of iron. Targeted deletions of IRP-1 and IRP-2 in animals have demonstrated that they are the chief physiologic iron sensors [40]. Some data confirmed that multiple genes are contributed with extension of migraine in different populations [41,42]. In related with other neurological disorders, Deplazes et al., indicated that mutations (non-synonymous polymorphism, 1888V) in exon 21 and a -88C>T polymorphism in the promoter region) in the IRP-2 gene were not a common cause of Parkinson's disease associated with substantia nigra iron accumulation [43]. In this study, for the first time we focused on IRPs in patients with migraine headaches to examine the hypothesis that say migraine headaches could related to iron unbalance. Therefore, our results for the first time provided evidence that enhance our understanding of how migraine may relate to these IRPs gene variation. We evaluated whether the g.32373708 G>A polymorphism in the IRP-1 gene and the g.49520870 G>A polymorphism in the IRP-2 gene effect on the risk of migraine. As far as we know, the g.32373708 G>A-IRP1 polymorphism and the g.49520870 G>A-IRP2 polymorphism have not been studied in migraine patients so far. We observed that the incidence of migraine was positively associated with the attendance of the G/G genotype of the IRP1 and IRP-2 SNPs. Our study demonstrated that none of age, sex, race, and smoking factors have not association with migraine. In this study the genotypes frequencies in both case and control groups are not consistent with HWE, so we regarded this issue as an important limitation of our study. At the moment we have logical reason of this data, bating that environmental factors may have effect in migraine pathogenesis at least in this population. However, to achieve better results and determine the role of different environmental factors in migraine susceptibility should be done experiments in the broader population. Similar studies enrolling greater sample sizes and composed of other ethnic groups from different countries may contribute to confirming our findings and therefore these results should be taken as preliminary. In conclusion, our work shows that genetic polymorphisms of the IRPs genes may be associated with development of migraine. These findings may be useful in augment of the cause of migraine.

Conflict of interest

All the authors declare that they do not have financial disclosure or conflicts of interest.

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