



Association of haptoglobin phenotypes with susceptibility to migraine in an Iranian population: a preliminary study

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Alireza Khosravi & Alireza Nakhaee designed the study and contributed to analysis, interpretation of data, and drafting of manuscript. Jafar Poodineh collected data, reviewed and edited the manuscript for intellectual content. Masoud Sadeghi contributed to analysis and interpretation of data and revised the manuscript of this review. All authors gave final approval of the version to be published.

Article History

Received: 08 December 2017

Accepted: 27 January 2018

Published: March-April 2018

Citation

Alireza Nakhaee, Alireza Khosravi, Jafar Poodineh, Masoud Sadeghi. Association of haptoglobin phenotypes with susceptibility to migraine in an Iranian population: a preliminary study. *Medical Science*, 2018, 22(90), 169-175

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ABSTRACT

Background: Oxidative stress and nitric oxide (NO) are key factors in the pathogenesis of migraine. Haptoglobin-Hemoglobin (Hp-Hb) complex can reduce NO production and inhibit oxidative stress. Since the ability of different Hp phenotypes in the reduction of NO and oxidative stress are diverse; one influential factor in the pathogenesis of migraine may be Hp phenotypes. Therefore, the present study assessed the association of Hp phenotype with migraine. **Materials and Methods:** In this study, 243 patients with migraine and 250 healthy persons were evaluated in terms of phenotypes Hp. Hp phenotype each individual was determined by electrophoresis of serum proteins in polyacrylamid gel and specific peroxidase staining. Phenotype frequency in two groups (migraine and healthy) were compared. **Results:** A significant difference in sex and age distribution was not found between patient and control groups. Hp phenotype 2-2 was present in 74.9% of migraine patients and in 48.4% of healthy persons. Hp2-1 and Hp1-1 were present in 20.6% and 4.5% of patients, respectively, and in 40.8% and 10.8% of controls. The distribution of Hp phenotypes in two groups of healthy and patients showed a significant difference ($P < 0.001$). **Conclusion:** The results of present study showed that Hp phenotype 2-2 was significantly associated with the occurrence of migraine. It seems in people with Hp1-1, a risk of migraine attacks is less than Hp2-2 phenotype. Possibly because three types of Hp have different functional efficiencies.

Keywords: Migraine, Haptoglobin, Phenotype, Nitric oxide, Oxidative stress

Abbreviations:

NO: Nitric oxide

Hp: Haptoglobin

Hb: Hemoglobin

PG: Prostaglandins

MO: Migraine without aura

MA: Migraine with aura

OR: Odds ratio

TGVS: Trigeminovascular system

CGRP: Calcitonin gene-related-peptide CGRP

COX: Cyclooxygenases

ROS: Reactive oxygen species

CI: Confidence interval

RBC: Red blood cells

DBH: Dopamine beta-hydroxylase

DDC: DOPA decarboxylase

MAOA: Monoamine oxidase A

1. INTRODUCTION

Migraine is a neurological syndrome characterized by an attack of severe headaches associated with nausea, vomiting hypersensitivity to light, sound or smell [1]. This disorder affects about 18% of general population and is three times more common in women than in men [2]. The two main categories are migraine without aura (MO) and migraine with aura (MA). An aura is a group of symptoms including vision disturbances, which serve as the warning sign before that intense headache is outbreak. In migraine without aura, peoples do not have such alert signals [3].

The pathophysiology of migraine has been not well understood, but several hypotheses have been presented. According Wolff theory, migraine attacks are begun by vasoconstriction of cranial vascular leading to reduction in cerebral blood flow that then was followed by compensatory vasodilatation in intracranial or extracranial blood vessels [4]. Increase in cerebral blood flow due to vasodilatation was assumed to result in perivascular edema and inflammation that in turn triggered headache pain. Molecules such as nitric oxide (NO) or nitroglycerin are involved in the induction of inflammatory responses and pain generation in migraine [5]. Moreover, Yilmaz et al. [6] showed that increased oxidative and nitrosative stress in migraine attack may affect cerebral blood flow and cause the headache. Although triggering factor(s) that activates inflammatory responses and oxidative stress leading to

migraine attack is still unknown, it is possible that genetic background is involved in these processes [7]. An ideal genetic candidate linking to NO, oxidative stress and inflammatory reactions that may affect migraine attack can be haptoglobin (Hp). Hp is a plasma protein synthesized in liver which binds to free hemoglobin (Hb) and removes it from circulation [8], thereby suppresses its oxidative activity in tissues [9]. In addition, Hp is involved in other physiological processes, including inhibition of prostaglandin synthesis, inhibition of NO, angiogenesis and immunomodulation [8]. In human populations, Hp is polymorphic protein with three common phenotypes: Hp1-1, Hp2-2 and the heterozygous Hp2-1, which is determined by two alleles HP^1 and HP^2 [10]. The effects of Hp in almost all above mentioned physiological events are phenotype dependent. For example, Hp 1-1 is biologically the most effective in binding to free Hb and preventing oxidative stress whereas, Hp 2-2 has the least effective [11].

Since NO and oxidative damage are known to be involved in the pathogenesis of migraine [12,13] and Hp phenotypes affect NO levels and severity of oxidative stress [11], and we postulated that functional differences between the Hp phenotypes may have an effect on the risk of migraine. For the evaluation of this opinion, in the present study, we tried to find out whether patients with migraine have different Hp phenotype distribution compared with non-migraine peoples.

2. MATERIAL AND METHODS

Subjects

The study was approved by the Ethics Committee of Zahedan University of Medical Sciences, Zahedan, Iran. The study included 243 patients suffering from migraine (age: 29 ± 14 years). Migraine diagnosis was performed according to the criteria of Headache Classification Committee of the International Headache Society [1]. The control group consisted 250 matched healthy individuals (age 26 ± 15 years) without personal or family history of chronic headache and other neurological or psychiatric disorders. The controls were recruited from relatives of patients admitted to local clinical lab and from medical students. Blood samples of patients and controls were collected and after clotting, centrifuged for 5 minutes at 3000 rpm and serum was stored at -20°C until analysis.

Determination of Hp phenotypes

For the determination of Hp phenotype, a solution of 10% Hb was required. For this purpose, the heparinized blood sample was withdrawn from a health donor and was centrifuged at 2500 rpm for 5 min. Plasma and Buffy coat were removed and red blood cells (RBCs) were washed five times in phosphate buffer saline. Then, one ml RBC was mixed with 9 ml distilled water and was incubated for 20 min at 4°C . Hemolysate was centrifuged at 10,000 g for 30 min and the supernatant containing Hb was stored at -20°C until use.

Hp phenotypes were determined using native polyacrylamide gel electrophoresis and peroxidase staining as described by Hochberg [14] with slight modification in staining step. Briefly, 10 μL of serum was mixed with 2 μL of 10% Hb solution and in order to the formation of Hp-Hb complex, mixture incubated for 5 min at room temperature. The 12 μL loading buffer containing 125 mM Tris pH 6.8, 20% glycerol and 0.001 bromphenol blue was added to 12 μL of sample and 20 μL of the mixture was loaded on polyacrylamide gel. The electrophoresis was performed on stacking gel 4% (29.2:0.8% acrylamide:bisacrylamide) and resolving gel 6% using running buffer containing 25 mM Tris pH 8.6 and 250 mM glycine at constant voltage 150 V for 3 h. After completion of electrophoresis, gel was removed and incubated in 20 ml of 3,3',5,5'-tetemethylbenzidine substrate solution (Prepared for ELISA by Pishtazteb Company, Iran), containing 3% acid acetic and 0.3% potassium ferricyanide. The bands representing Hp-Hb complex in the gel were visualized within 10 min and were stable for over 48 hr in water. In order to document, gel was photographed during 24 hr after staining.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 software. Continuous variables were compared using T-test. A Chi-square test was used to compare the frequency of phenotypes and alleles between the patients and controls. The Hardy-Weinberg equilibrium was tested for allele frequencies in both patients and controls groups to detect any possible deviation. Multiple logistic regression analysis was used to determine odds ratio (OR) and 95% confidence interval (95%CI) as a measure of relative risk. A p-value <0.05 was considered statistically significant.

3. RESULTS

Patients

The characteristics of the patients and controls are summarized in Table 1. There were no significant differences between the migraine patients and controls in terms of age and sex. From 243 migraine patients, 23% were with aura, while 77% had migraine without aura.

Table 1 Characteristics migraine patients and controls

	Patients (N=243)	Controls (N=250)	P value
Age	29±14	26±15	0.44
Male	82	93	0.40
Female	161	157	0.40

Hp phenotyping

As shown in Figure 1, three different Hp phenotypes were distinguishable by pattern of bands representing Hp-Hb complexes. Hp1-1 phenotype showed only a fast single band, whereas Hp 2-2 phenotype showed multiple slow-migrating polymer bands. Hp 2-1 phenotype represented both 1-1 and polymer forms.

The phenotype and allele frequencies of Hp in the patients and controls are shown in Table 2. Phenotype of Hp 1-1 was more prevalent in the control group compared with that in the patients with migraine (10.8 vs. 4.5%) and was associated with reduced risk of migraine 0.271 (95% CI 0.13-0.566; $P < 0.001$). In addition, the allelic frequency was revealed statistically significant difference in Hp 1 allele between the two groups (31.2% in controls vs. 14.8% in cases). Also, the Hp 1 allele significantly decreased the risk of migraine (OR=0.385, 95%CI: 0.282–0.524; $P < 0.001$) compared to Hp 2 allele. There was no significant difference in the distribution of Hp phenotypes between MO and MA ($P > 0.05$).

The distributions of phenotype frequencies in the healthy controls were in Hardy–Weinberg's equilibrium ($\chi^2=0.616$, $P = 0.432$) as expected for Iranian population [15], but not in the migraine patients ($\chi^2= 8.3$, $P = 0.004$).

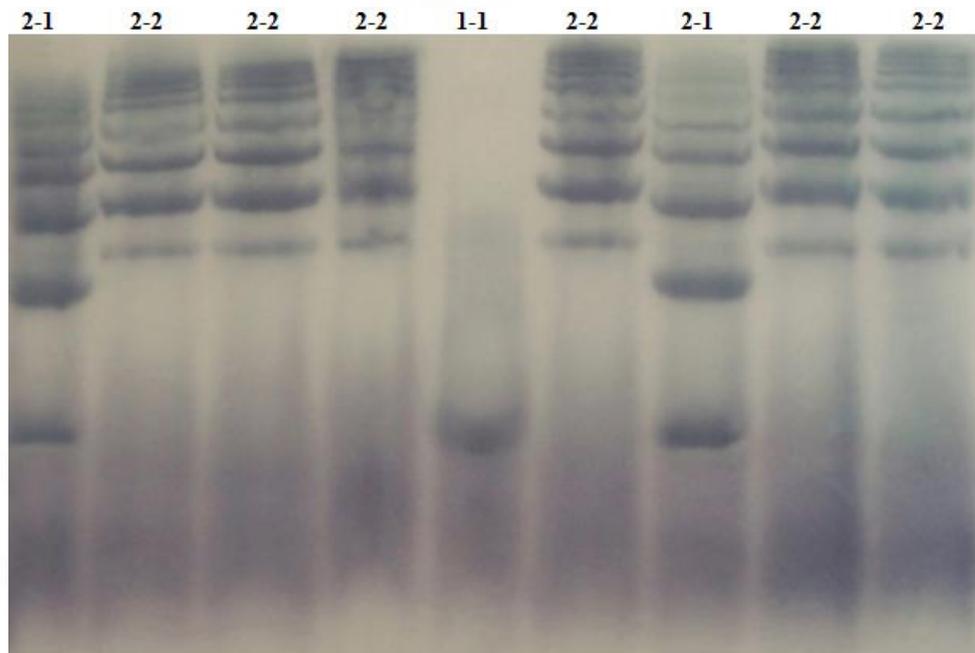


Figure 1 Electrophoresis pattern of haptoglobin phenotypes. 10 μ L of serum were mixed with 2 μ L of 10% hemoglobin solution and after incubation for 5 min at room temperature, 12 μ L loading buffer was added to 12 μ L of sample. 20 μ L of this mixture were placed in each well of the polyacrylamide gel and the run was started with a constant voltage 150 V. The separated haptoglobin phenotypes were stained by 3,3',5,5'-tetramethylbenzidine (TMB) solution containing 3% acid acetic and 0.3% potassium ferricyanide. The phenotypes shown, from left to right, are (2-1), (2-2), (2-2), (2-2), (1-1), (2-2), (2-1), (2-2) and (2-2).

Table 2 Phenotype and allele frequencies of haptoglobin case and control groups and their association with risk of migraine

	Patients N (%)	Controls N (%)	OR (95%CI)	(P value)
Hp1-1	11 (4.5)	27 (10.8)	0.271 (0.13-0.566)	0.001
Hp2-1	50 (20.6)	102 (40.8)	0.326 (0.216-0.491)	0.001
Hp2-2	182 (74.9)	121 (48.4)	1.00	
Hp1 allele	72 (14.8)	156 (31.2)	0.385 (0.282 – 0.524)	<0.001
Hp2 allele	414 (85.2)	344 (68.8)	1.00	

4. DISCUSSION

To our knowledge, this is the first study to assess the association of Hp phenotypes and predisposition to migraine attacks in an Iranian population. Interestingly, we found that individuals with Hp2-2 phenotype have an increased risk for developing migraine *headaches*. Furthermore, we showed for the first time that Hp1-1 phenotype may protect individuals versus migraine *headaches*.

Migraine is a disabling brain ailment whose disturbs the central nervous system, causing significant pain of headache [4]. Although the molecular *mechanism of migraine* headache remains vaguely understood, it has been proposed that the variability in the frequency of migraine attacks is linked to both the genetic component carried by the individual and environmental activating factors [7]. Several candidate genes polymorphism including estrogen receptor 1, potassium channel (KCNN3), dopamine beta-hydroxylase (DBH), DOPA decarboxylase (DDC), and monoamine oxidase A (MAOA) have been shown to be involved in the pathogenesis of migraine [15]. Hp polymorphism has been suggested as candidate genetic marker in a number of neurological disorders. For example, Hp 2-2 is a risk factor for schizophrenia and epilepsy [8], whereas unipolar major depression and Parkinson's disease are associated with Hp 2-1 and Hp 2-1 phenotypes [16, 17]. The results of present study showed that Hp 2-2 phenotype may predispose individuals to development of migraine, while Hp 1-1 can confer protection from migraine risk. The associations of Hp phenotypes with migraine susceptibility can be attributed to the functional difference between phenotypes. Three main Hp phenotypes are formed through Hp polymorphisms, which each has different structural and functional characteristic [16].

Hb is the effluent source of iron in the body and free iron may produce reactive oxygen species (ROS) through Fenton reaction. ROS such as hydroxyl radical can impact on the tone of vessels via direct effect on the smooth muscle cells, or via indirect effect thorough alteration of vasoactive substances (production or biological actions) and lead to migraine [18]. Hp possesses an innate antioxidant activity. This protein may inhibit oxidative stress via removing free Hb from the circulation and tissues [17]. Once Hp binds to Hb, this complex is quickly eliminated by means of monocyte/macrophage scavenger receptor CD163 from circulation and at the site of injury. Hp 1-1 is the smallest phenotype of Hp that not only has high plasma concentrations and high affinity for Hb, but also, the clearance of Hp1-1-Hb complex via CD163 receptors is processed more rapidly than other phenotypes [19]. So, with overproduction of free Hb, individuals with Hp2-2 phenotype are more susceptible to oxidative stress, because the affinity of this phenotype to Hb is lower than other phenotypes and free Hb can produces free radicals via its iron. Since, oxidative stress appears to play a crucial role in the development of migraine; individuals with Hp2-2 phenotype have an increased risk for developing migraine *headaches* [20]. Moreover, Hp1-1 with small size diffuses in the interstitial cerebral fluid more avidly than other phenotypes [8], for this reason; probably the concentration of this phenotype will be more than others in the brain.

Neuropeptides and substances that induce inflammation, release from trigeminal afferents accompanies vascular alterations and the feeling of pain by sensitizing neurons in the trigeminovascular system (TGVS). Also, new research validated that some neuropeptides such as calcitonin gene-related-peptide (CGRP), which were found to be increased during migraine attacks, may cause the feeling of pain when injected to migraineurs [21]. Recently, researches have been concentrated on vasoactive substances that released via endothelium. These substances can initiate and maintain feeling of pain during migraine attacks by mediating modifications in the vascular tone and cerebral blood flow. NO is one of the most important substances released by endothelium, playing an essential role in the regulation of cerebral and extra cerebral cranial blood flow, arterial widths, mediation of pain, and neurotransmission in the *central nervous system* [14]. NO can dilate cerebral vessels through cGMP pathway directly and initiate the

early phase of migraine attack. Also, NO can promote synthesis and release of CGRP from nociceptive afferent fibers in the TGVS and trigger the deferred phase of migraine attack [22]. Both unbound Hb and Hb-Hp complex readily bind to NO and scavenge it, with consequent changes in vasomotor constriction [23,24]. Hb binding capacity is a criterion, which measures the protective ability of Hp phenotypes against a hemolytic episode (Hb-related cell damage and release of hem. This criterion and also, binding affinity of the Hp-Hb complex to CD163 are phenotype dependent [25]. This activity, which is ranked in the order Hp1-1>Hp2-1>Hp2-2, partially explains the ability of the phenotypes to prevent Hb-related oxidative stress.

In addition, one of the fundamental processes in the onset of migraine attack is dura matter inflammation [4].

Prostaglandins (PG) are powerful mediators of inflammation and pain, which are derived from membrane phospholipids such as arachidonic acid. These substances produce in the endothelium and perivascular sites and have vasoactive and pain-producing activities. In the large cerebral arteries, PG could donate to decline in cerebrovascular tone, stimulation of alterations in local cerebral blood flow and mediation of the headache during migraine occurrences. One of the major enzymes involves the production of PG is cyclooxygenases (COX-1 and COX-2). COX-2 acts as the main source of PG synthesis in inflammation [26,27]. Inflammatory stimuli such as extravascular damages activate cytokines, with consequent COX enzymes induction. Also, Hb stimulates the production of PG and causes inflammation. Both in vitro and in vivo studies demonstrated that Hp effectively prevents PG synthesis through COX-inhibiting. Therefore, Hp has anti-inflammatory properties [26,28]. Guetta and coworkers have recently revealed that the Hp 1-1-Hb complex induces considerable greater levels of anti-inflammatory cytokines like IL-10, compared to Hp 2-2-Hb complex. [29] Thus the greater incidence of Hp 2-2, which is an inferior anti-inflammatory compared to Hp1-1, may increase the predisposition for migraine [29,30]. It has been revealed that CD163 receptors may down-regulate and up-regulate in altered clinical situations. Hence, in addition to Hp polymorphism, CD163 appearance might also have a clinical significance in mediating the rate in which Hb-Hp complexes are cleared in migraine, but this prospect remains to be examined [31].

5. CONCLUSION

In conclusion, our findings showed that the Hp2-2 phenotype associated with a noteworthy increase in migraine risk as compared to the Hp 1-1 and Hp 2-1 phenotypes. This is the first study to evaluate the relationship between Hp polymorphisms with migraine headache. Further studies would help in the clarification of the mechanisms by which Hp phenotypes affect the risk of migraine progress.

FUNDING

The authors gratefully acknowledge the Research Council of Kermanshah University of Medical Sciences (Grant Number: 95042) for the financial support.

CONFLICT OF INTEREST

The authors have declared that there was no conflict of interest.

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