Research Article

Association of Serum Copper Levels and Amine Oxidase Copper Gene 1 (*AOC1*) with Migraineurs

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Abstract
Migraine is a common neurovascular multifactorial disease with biochemical abnormalities in the central nervous system (CNS). It is characterized by episodes of frequent headaches, affecting about 14% of the world's population. Trace elements are essential to play an important role in neurotransmission and causing oxidative stress in patients with migraine. Also, it is hypothesized that Histamine (biogenic amine), catabolized by Diamine oxidase (DAO), induces a vascular headache. DAO contains Copper as a cofactor and is coded by the Amine oxidase copper containing 1 (*AOC1*) gene. This study aims to determine the level of serum copper (Cu), an association of the *AOC1* gene and antioxidant capacity in migraine patients. In this study, a total number of 200 individuals (patients and controls) were equally distributed in each group according to demographic details obtained. The results obtained from this study were found to be significant to migraine. The frequency of T allele (rs10156191) in exon 4 *AOC1* was 7.5% in migraineurs OR of 16.13; 95% CI- 0.63 to 47.97, and the p-value was observed to be 0.074. The mean concentration of Cu was found to be 0.09 ± 0.02 mg/L and 0.22 ± 0.10 mg/L in patients and controls, respectively. Antioxidant capacity of serum was found to be lower in patients (3 ± 1.2 µM ascorbic acid equivalents) when compared to controls (7 ± 0.9 µM ascorbic acid equivalents). Decreased Cu and a nonsynonymous of rs10156191 are associated with migraine, which may decrease DAO activity. Further research, needs to be focused on the DAO activity that can determine the migraine-inducing effect.

Keywords: Antioxidant, Copper, Diamine oxidase, Histamine, Migraine

1 Introduction

Migraine is a recurrent disease, which occurs due to neurovascular pathophysiology with a strong genetic basis that affects cortical, subcortical, and brainstem areas [1]. Migraine is classified as a primary headache along with tension-type headache and trigeminal autonomic cephalalgias [2]. It was ranked as the 3rd most prevalent disorder globally, in the Global Burden of Disease (GDB) study [3]. As per the guidelines given by the International Headache Society (IHS), Migraine is classified into migraine with aura (MA) and migraine without aura (MWA) [4]. Sensitivity to light, sound, and smell, nausea, and vomiting are the significant symptoms of migraine [5]. Aura physiology includes genetic abnormalities, headache anatomy, and activation of the trigeminal cervical complex [6]. These are the crucial factors that provide important clues about the pathophysiology of migraine. In addition, it provides insights that ultimately lead to new treatments [7]. The diagnostic criteria for MWA are based on the incidence of headache over 4–72 h. It is diagnosed by any two of four characteristics, such as unilateral position, moderate to severe pain intensity, quality of pulsation, worsening due to withdrawal from routine exercise [8]. On the other hand, MA is diagnosed by one or more of the following aura symptoms: visual, sensory, speech, motor, brainstem, and retinal disorders [7]. Migraine characteristics are diverse and genetics can elucidate the pathophysiology of this disorder. Classification and treatments for migraine are based on biological causes and genes [9]. Genetic associations, along with the physiological parameters of this neurovascular disorder, can provide insights and views for therapeutic strategies [10]. It has been reported that cortical spreading depression (CSD) and inflammatory soup on the dura have produced neurogenic inflammation in animal models [11]. In addition, the pain-sensitive structures of the trigeminal nerve (sinuses, meninges, and arteries) contain nociceptors that respond to chemical and mechanical stimuli [12]. Nociceptor structures have peptidergic secretory vesicles; when they are activated, they signal to secondary neurons and release a substance P (SP), a calcitonin gene linked peptide (CGRP), a polypeptide that activates pituitary adenylate cyclase, somatostatin, and neuropeptide Y [13]. The release of neuropeptides, causes inflammation leads to plasma vasodilation, extravasation, and degranulation of mast cells, which further increases the sensitization of the trigeminal vascular system [14]. Mast cell vesicle contains histamine, prostaglandins, serotonin, etc., which is capable of causing lowering pH, local inflammation, sensitizing other neurons, and releasing more neuropeptides [15].

Histamine is a biological amine, also known as 2- (1H-imidazole-4-yl) ethane amine, which modulates the anaphylactic reaction. The biological functions of histamine are mediated by binding to four specific receptors Histamine Receptors, namely (H1R, H2R, H3R, and H4R). This biogenic amine regulates allergic reactions and inflammation via H1R and induces gastric acid secretion via H2R. It supports the release of neurotransmitters by binding to H3R in the central nervous system and releasing inflammatory mediators via H4R [16]. It is synthesized intracellularly from the amino acid histidine by vitamin B6, containing L-histidine decarboxylase [17]. Histamine is metabolized extracellularly by diamine oxidase and intracellularly by Histamine-N-methyltransferase. Diamine oxidase uses oxygen to oxidatively deaminase histamine to imidazole acetaldehyde, ammonia, and H_2O_2 in the presence of copper as a cofactor [18]. Disruption of histamine degradation and imbalances in its levels lead to histamine intolerance. This histamine intolerance leads to headaches, gastrointestinal disorders such as flatulence, nausea, abdominal pain, diarrhea, myalgia, hypotension, etc. It has been reported that the leading cause of histamine deficiency can be DAO deficiency, which results in impaired bowel deterioration and subsequently elevated plasma levels [19].

DAO deficiency a genetic mutation in gene 1 containing *AOC1* located in chromosome 7q36, which encodes an altered protein with low enzymatic activity [20]. Cu levels also play an essential role in the activity of this enzyme as it acts as a cofactor [21]. Enzymatic blockage occurs due to the use of several types of drugs, which can lead to an imbalance in histamine levels. Recently, there have been reports of the prevalence of DAO deficiency (87%) in patients with clinically diagnosed migraine [20]. DAO is strongly expressed in the intestines, kidneys, lungs, and placenta and rarely in the CNS. Still, an increase in histamine is due to the release of nitrogen monoxide upon stimulation by provoking

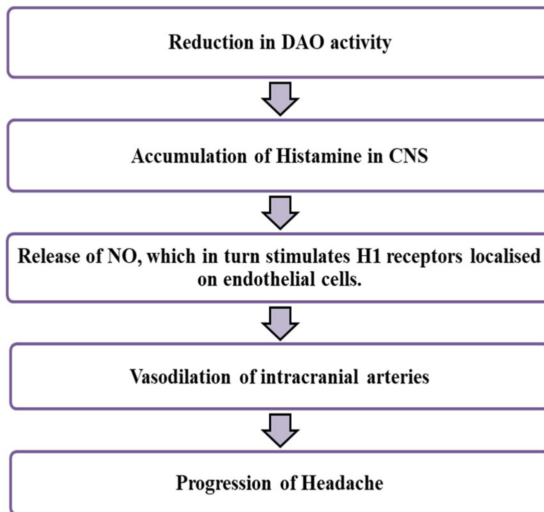

Figure 1: Schematic representation of histamine causing migraine.

hypothalamic activity of H1R in the intracranial arteries [22]. This non-synonymous single nucleotide polymorphism (SNP) which codes for altered DAO activity, was decoded on the *AOC1* gene [23]. Schematic representation of histamine causing migraine was shown in Figure 1. It has been reported that three nonsynonymous SNPs, Thr16Met, Ser332Phe, and His645Asp (rs10156191, rs1049742 and rs1049793), respectively, have been identified in the Caucasian population, which are associated with decreased DAO activity [24]. Alleles with rs10156191 have a T sequence coding for Met at position 16 instead of Thr, which in turn reduces the intrinsic activity of DAO, thereby reducing its ability to catabolize circulating histamine [19]. Cerebral hypoxia is associated with migraine attacks. Decreased blood flow causes a decrease in CSD in the cerebral cortex, causing metabolic changes, such as intracellular calcium overload and increased oxygen demand, which can lead to oxidative stress [25]. It has been reported that changes in cerebral blood flow may lead to reactive oxygen species (ROS) [26]. On the other hand, antioxidant defense system activity reduces oxidant production in migraine patients [27]. Thus, the objective of this study is to investigate the association of rs10156191, serum Cu levels, and antioxidant capacity with migraine patients and controls.

2 Materials and Methods

2.1 *Collection of samples*

The samples (n = 200) were collected from the Department of Neurology, Outpatient (OP) at Government Vellore Medical College and Hospital (GVMCH), Vellore, Tamilnadu. The study was approved by the Ethical Committee of (GVMCH) and the Institute of Ethics (VIT Vellore). Ethical standards were followed as per the guidelines throughout the study. Initially, individuals with migraine were diagnosed and sub-classified under migraine with aura (MA) and migraine without aura (MWA). The questionnaires were prepared according to the guidelines given by the International Headache Society (IHS). A total number of 200 individuals (patients and controls) were equally distributed in each group according to demographic details obtained. The control group of individuals were clinically diagnosed and does not have any evidence of migraine attacks and other neurological disorders. To avoid conflicts, informed consent was taken from all the subjects involved in this research. The whole blood samples were performed by venipuncture method and collected in Heparin and EDTA-coated vacutainers.

2.2 *Separation of serum*

Blood sample (3 mL) was taken from each individual's (patients and control) in (clot activator) CAT vacutainer tubes (Becton Dickenson [BD] # 367896) and kept upright for 30–45 min at room temperature (19–24 °C) for clot activation. The tubes were centrifuged (Thermo Scientific, SL40R) for 10 min at 3000 rpm. The supernatant was aliquoted immediately and was stored at –20 °C until further use.

2.3 *Atomic Absorption Spectroscopy (AAS)*

To determine the concentration of Cu levels in the serum of both patient and control samples, air-acetylene flame- AAS; VARIAN AA240 was used. The sample is vaporized and the element of interest atomized at high temperatures. The element concentration is determined based on the attenuation or absorption by the analyzed atoms of a characteristic wavelength emitted from a light source [28]. 0.25 mL of serum was diluted to 4.75 mL with sterile water and subjected to AAS. Different

concentrations of Copper sulfate ($CuSO_4$) were prepared and varied as 2, 4, and 6 ppm from the stock. The unknown concentration of the trace elements was determined from the standard curve.

2.4 *DPPH assay*

Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging assay was performed to determine the percentage of scavenging potential in the serum of cases and to compare with control. This method was developed by Blois in the year 1958 to determine the antioxidant activity [29]. 2,2- DPPH radical is a relatively stable compound with a peak absorbance at 517 nm (A517). The reaction of a DPPH radical along with a variety of antioxidants acting as donors of an electron and/ or a radical hydrogen leads to the formation of 1,1-diphenyl-2-picrylhydrazine and a loss of A517 [21]. Serum of 100 µL was taken in a 1.5 mL centrifuge tube and 200 µL of acetonitrile was added for deproteinization. The tubes were centrifuged at 1500 rpm for 5 min and the supernatant was collected. Then, 900 µL of 0.1 mM DPPH was added to the supernatant and incubated at room temperature for 30 min. Later, the samples were measured the absorbance at 517 nm in a UV-spectrophotometer. Ascorbic acid was used as the standard positive control of different concentrations (2–50 µM). Also, for negative control 100 µL of acetonitrile was taken. Scavenging potential was calculated for each sample (serum, positive and negative) and the % was calculated using the formula [Equation (1)].

$$SP,\% = \left[1 - \frac{S}{N_c}\right] \times 100 \quad (1)$$

Where, SP - Scavenging potential %, S - Sample absorbance, N_c - Negative control absorbance.

2.5 *Extraction of DNA*

Genomic DNA was extracted by collecting (3–4 mL) blood from the individuals in EDTA anticoagulated tubes. The standardized protocol was performed using the ammonium acetate (salting out) method [30] and stored at –20 °C until further use.

2.6 *Polymerase Chain Reaction (PCR)*

The amplification of extracted DNA samples was performed using the *AOC1* gene. For PCR analysis, the samples were amplified from the primer designed by using the UCSC Genome browser and Primer 3 software. Forward primer 5’CCCATCTCTGCCCAT AAGAC3’ and Reverse primer 5’GGAGGGCATACT CTGCTGTG3’encoding the SNP rs10156191, which causes Thr16Met substitution, and is confined to exon 4 of the *AOC1* gene. The amplicons with base pair size of 518 bp. Each PCR reaction contains a 25 µL mixture of (10x buffer, dNTP, primers, Taq DNA Polymerase, $MgCL_2$, H_2O, and DNA template). The PCR reaction setup and conditions are shown in (Tables 1 and 2)

Table 1: PCR Master mix components per reaction

PCR Mixer Components	Volume (µL)
10x Buffer	2.5
Deoxyribonucleoside triphosphate (dNTP)	0.5
Forward Primer	0.5
Reverse Primer	0.5
Taq DNA Polymerase	0.25
$MgCl_2$	0.75
H_2O	19
DNA Template	1
Total volume	25

Table 2: Condition for running PCR

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	4 min	
Denaturation	95	60 s	35 cycles
Annealing	52.5	60 s	
Extension	72	60 s	
Final extension	72	5 min	

2.7 *Gel electrophoresis*

The amplified PCR product was subjected to electrophoresis in 2% agarose gel to determine the conformation of the amplicon bands at 518 bp. Electrophoresis was performed using 100 mL of 1X TBE buffer and 8 µL of ethidium bromide (EtBr). A 6-fold dye was used to load the sample. To estimate the size, 3 µL of a 100 bp DNA marker was used. The installation was carried out at a constant voltage of 100 V for 30 min.

2.8 *Single Nucleotide Polymorphism (SSCP)*

SSCP is based on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a

non-denaturing gel is highly dependent on its size and structure [31]. After the confirmation of the amplicon bands of the PCR products were subjected to SSCP. 7 µL of PCR product was mixed with an equal loading buffer containing 95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. The mixture was denatured at 95 °C for 7 min and cooled at 40 °C for 5 min. The whole mixture was then loaded on 8% non-denaturing polyacrylamide gel and run at room temperature at 80 V and 9 A. After electrophoresis, the bands were visualized by silver staining. The samples which have shown mobility shift of bands were outsourced for Sanger sequencing and the results were analyzed.

2.9 *Headache Impact Test (HIT)*

To measure the impact of the headache considering people at society Headache Impact Test (HIT) score is measured. HIT is a series of questionnaires that were developed by an international team of headache experts from neurology, primary care medicine, and psychometricians (SF-36®) [32]. One of the diagnostic criteria used in this study for classifying MA and MWA is the HIT assessment tool. This tool is used to help patients how they feel, react during headaches and symptoms based on their response the scores have been evaluated and named as (never, rarely, sometimes, very often, and always).

2.10 *Statistical analysis*

The statistical analysis of the data were performed with a confidence interval (CI) of 95% and an error of 5% ($\alpha = 0.05$). Further, the significance of the experimental data was interpreted through a p-value < 0.05. The margin of error was used to determine the sample size. Descriptive statistics for the data were performed and mean, standard deviation, minimum, and maximum values for the variables (characteristics) were investigated. All statistical analysis of the experimental data was carried out using SPSS Statistics 13.0, a statistical tool

3 Results and Discussion

3.1 *Age and gender*

Migraine patients were clinically diagnosed and sub-classified as (MA and MWA). The migraineurs age was (31 ± 9 years) when compared to control (32 ± 8 years). Among 200 samples, female's prevalence rate was higher (82.5%) when compared to males (17.5%). Also, gender-wise classification was done for the patient MA and MWA group and it was found that female affected patients were higher when compared to males. This implies migraine has a higher prevalence in females rather than in males. Similarly, it has been reported that Migraine is commonly seen in females than males [33]. In this population group, 88% are women and 12% are men. This implies female reproductive milestones such as menarche, menstruation, pregnancy, lactation, and menopause are directly or indirectly associated with migraine headaches [34]. In addition, it has been reported that slight aberration in the precisely membranous network of the hypothalamus, endocrine glands, neurotransmitters, pituitary gland, diamines, minerals can lead to pathological complications [35]. Assessment, of samples of both migraine patients (MA and MWA) and controls concerning males and females along with controls were shown in Figure 2.

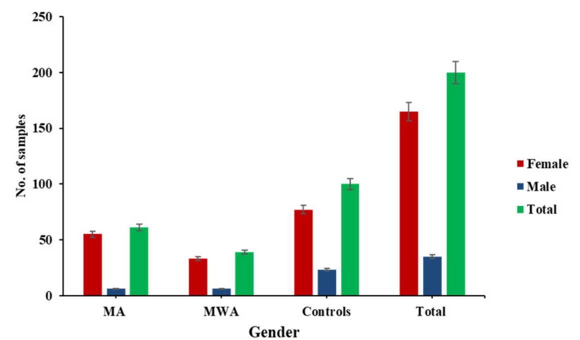

Figure 2: Assessment of samples that are categorized as (MA and MWA) concerning males and females along with controls samples.

3.2 *Cu levels in serum*

AAS results were analyzed and it was observed that the concentration of Cu levels in the blood serum of migraine patients were lower when compared to the serum of the control group shown in Figure 3. Similarly, for each parameter, the Cu levels were observed and it was found that MWA (0.111) patients had the lowest concentration of Cu compared to MA (0.151) patients. Summarization of the Cu levels in patients

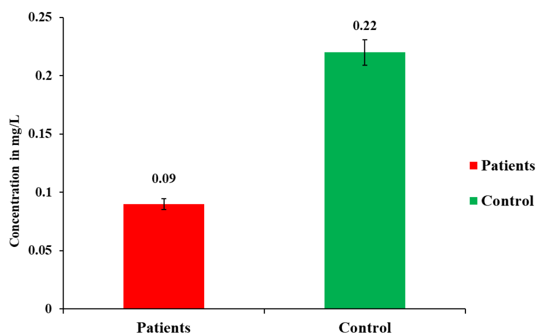

Figure 3: The mean concentration of serum Cu levels in Migraine patients and controls.

with MA and MWA and compared with control shown in Table 3. Reduced Cu levels were seen in MA (0.14 ± 0.01 mg/L) and MWA (0.08 ± 0.01 mg/L) as compared to healthy individuals (0.22 ± 0.01 mg/L). Whereas, the reference-free serum Cu levels were 0.1 to 0.15 mg/L. Dyshomeostasis minerals, especially Cu, play a significant role in the progression of migraine [36]. Low concentration of Cu levels may affect the activity of DAO since Cu acts as the cofactor. Recently, a study reported an association between the Cu concentration levels in migraine patients [37]. On the other hand, it has been reported that increased levels of Cu are observed in tumors of the gastrointestinal tract, osteosarcoma, and lung cancer [38]. In addition, histamine is an amine synthesized from L-histidine by decarboxylase of L-histidine and later catabolized by DAO extracellularly [19]. In the central nervous system, it is synthesized by histaminergic neurons of the posterior hypothalamus [39]. Worm *et al.*, reported that headaches and migraineurs are triggered and seemed to be more susceptible to vascular headaches at a minimum dosage level of histamine [40]. However, the pathophysiology of migraine with the association of Cu lacks in the literature. It has recently been reported that the concentration of Cu in levels should be associated with diamine oxidase causing the SNPs in the *AOC1* gene.

Along with the disequilibrium of Cu levels in serum, variations in the *AOC1* gene regulate DAO activity. Nonsynonymous SNPs are a major cause of decreased function of drug-metabolizing and xenobiotic-metabolizing enzymes [24]. These SNPs can cause amino-acid substitutions that are relevant to enzyme activity or kinetic properties. Alleles (rs2268999, rs2052129, rs1049742 and rs10156191) have been reported to increase the risk of decreased DAO activity [23]. In another study done in peripheral blood of migraine patients, DAO mRNA expression of homozygous minor allele carriers such as (rs2052129, rs10156191, and rs2268999) is significant (p-value = 0.002) than the major allele reported in the German population [41].

Table 3: Concentration of Cu levels in MA and MWA compared along with control

Parameters	Cu (mg/L)	Standard Deviation	*p*-value
Control- Male	0.215	0.033	0.04*
Control-Female	0.229	0.076	0.04*
MA-Male	0.130	0.028	0.018**
MA-Female	0.153	0.031	0.03*
MWA-Male	0.027	0.006	0.009*
MWA-Female	0.127	0.027	0.015**

*Denotes a significant difference ($p<0.05$)
** Denotes a highly significant difference ($p<0.02$)

3.3 *Radical scavenging activity*

Antioxidant capacity of serum was determined by DPPH assay, also the percentage of scavenging potential in the serum of cases and control was observed. This method has been widely used to estimate the activity of antioxidants in recent years [42]. When the DPPH solution is mixed with a substance (H+) it changes from violet color to residual pale yellow. The occurrence of *AOC1* allelic variant (rs10156191), serum copper levels, and antioxidant capacity were investigated in 100 cases and 100 controls. The investigated result depicts that migraine is more prevalent in females (88%) than males (12%) and migraine with aura patients (61%), and there are more in number in this population compared to without aura patients. Based on these results, it was found that the mean value of serum antioxidant capacity is reduced in the patients with Migraine (3 ± 1.2 µM ascorbic acid equivalents) when compared to controls (7 ± 0.9 µM ascorbic acid equivalents). The scavenging potential was observed as 9.53% for 3 ± 1.2 µM ascorbic acid equivalents in patients, whereas 13.21% scavenging potential was observed in 7 ± 0.9 µM ascorbic acid equivalents in controls (Figure 4.)

3.4 *PCR-SSCP analysis*

In the present study, gradient PCR was performed to

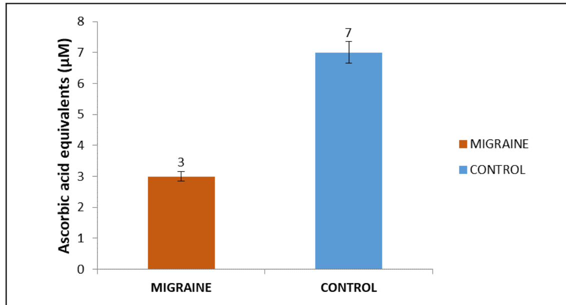

Figure 4: Serum antioxidant capacity in migraine patients and controls.

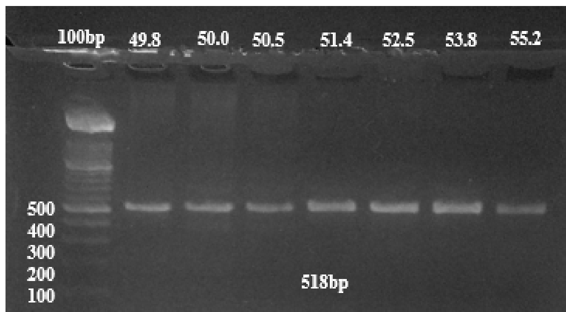

Figure 5: Gradient PCR set up for Exon 4 of *AOC1* gene using 1.8% agarose gel. Lane 1–100 bp marker, Lane 2–8 different °C.

obtain that optimum annealing temperature (Figure 5.) SSCP technique was performed to determine the mobility shift in the bands due to variations in the *AOC1* gene Exon 4 (Figure 6.) The number of bands and position of bands was varied in few cases and control samples after silver staining of native Polyacrylamide gel electrophoresis (PAGE). It was observed that rs10156191 is significantly associated with Migraine in this population. Also, it was observed that the Odds Ratio (OR) value for the genotypic distribution of heterozygous (CT) and homozygous mutant genotypes (TT) among migraineurs were 5.5 and 3.0, respectively and it is shown in OR value to be greater than 1, (Table 4.) Therefore, it implies a strong association of the rs10156191 with migraine. It has been reported in Spain that *AOC1*, which is involved in histamine metabolism is associated with the risk of developing migraine (with OR= 2.08), particularly in women. This implies rs10156191 that is related to decrease DAO enzyme activity [41]. Sequence T encodes a variant with the amino acid substitution Met in the

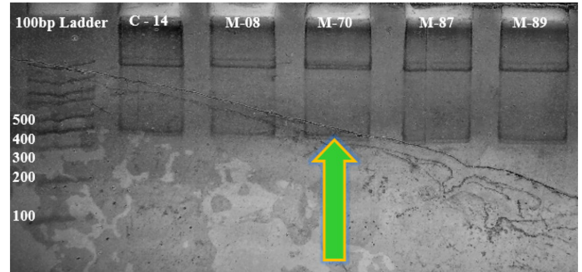

Figure 6: PCR-SSCP of *AOC1* Exon 4; L1–100bp marker, L2: control and L3–L6: migraine samples. Lane 4- band shift.

16th position, instead of Thr in the wild type protein. This substitution reduces the enzyme core activity and thus decreasing the ability to metabolize histamine.

Table 4 : Association of rs10156191 with Migraineurs (cases) and healthy individuals (controls)

Genotype	Cases (n=100), (% genotype)	Control (n=100), (% genotype)	OR, CI
Homozygous wild type (CC)	90, (90%)	99, (99%)	-
Heterozygous (CT)	5, (5%)	1, (1%)	5.5 (95% CI: 0.63 to 47.97)
Homozygous (TT)	5, (5%)	0, (0%)	3.0 (95% CI: 0.09 to 90.97)

In the present study, it was found that the presence of T allele was found to be in 7.5% in the migraineurs and 0.5% in controls. OR was found to be 16.13 with 95% CI: 2.1 to 123.36 and a *p*-value of 0.007. This implies that the rs10156191(C > T) is associated with migraine in this population shown in Table 5. This association may reduce the activity of DAO and ncrease the plasmatic and CSF levels of histamine resulting in the induction of migraine attacks. The results of the present study shed light on the oxidative stress and antioxidants in migraineurs. Antioxidant capacity of non-enzymatic antioxidants was found to be reduced in migraineurs (3 ± 1.2 µM ascorbic acid equivalents) compared to healthy individuals (7 ± 0.9 µM ascorbic acid equivalents). A study has reported that total antioxidant capacity (TAC) was significantly reduced in migraine patients compared to controls [28]. In another study, enzymatic antioxidants such as Glutathione

peroxidase, superoxide dismutase (SOD) are also reduced in migraine compared to controls [29]. Similarly, another allele of the *AOC1* gene, rs2052129, affects the expression function of the enzyme [19]. This implies an increase in the frequency of the rs2052129 T allele, decreases in the histamine metabolism. However, the rs2052129 T and rs10156191 T alleles have a positive clinical association with migraine, although they have different clinical effects [43]. It has been reported that gender is the main factor in the activity of the DAO enzyme *in vivo*, where women are more active than men [44]. This implies a link between SNPs DAO activity with gender and migraine. Nevertheless, considering the limited sample size in this study that the most significant association was observed in women, the findings obtained in this study would require replication to obtain more support to the proposed association to induce migraine [45]. Also, SNPs were reported to be related to other histamine-related disorders namely, rhinitis, hypersensitivity, and ulcerative colitis

Table 5: Allelic distribution of rs10156191 among Migraineurs and controls

Allele	Cases (n=100), allelic %	Control (n=100), allelic %	OR, CI	*p*-value
C	185, (92.5%)	199, (99.5%)	-	-
T	15, (7.5%)	1, (0.5%)	16.13 (95% CI: 2.1 to 123.36)	0.0074**

**Denotes a highly significant difference ($p<0.02$)

4 Conclusions

Current concepts of the etiology of migraine favor the neurological origin of the treatment strategy, but the exact pathophysiology of the disease has not been revealed [46]. The prevalence of this neurological disorder is almost equal in both males and females until adolescence. However, the incidence is higher in women (70%) from menarche to menopause when compared to men (30%) [47]. It could be concluded that results provide evidence of the reduced concentration Cu levels, and a nonsynonymous SNP, rs10156191 seem to be associated factors with the risk of migraine. This implies the reduced activity of DAO. In addition, DAO activity can be measured to determine the effect of decreased Cu levels and the presence of the rs10156191 (C > T) allele in migraine. To reduce oxidative stress caused by brain hypoxia, non-enzymatic antioxidants, such as vitamins may be prescribed for migraine patients. Further research needs to be focused on the DAO activity that can determine the migraine-inducing effect.

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