Research Article

Association of *MTHFR* (C677T and A1298C) Gene Variants Polymorphisms with Migraineurs: A Case-control Study

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Abstract
In recent years, the activity of the regulatory enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), which influences migraine attacks, has been intensely discussed. In particular, this genetic risk factor, together with diagnostic and symptomatic characteristics, can predispose to Migraine. The present study assessed the functional polymorphism and its prevalence of *MTHFR* (C677T and A1298C) gene variants among 186 Migraineurs (116 (MA) and 70 (MWA)) compared with 152 healthy individuals. The incidence of (*MTHFR* 677 T and *MTHFR* 1298C) allele were significantly higher in Migraineurs (30.6%, 51.3%). Genotypes T677T and C1298C have been associated to induce migraine attacks (Odds Ratio (OR) = 3.53; 95% Confidence Interval (CI) = 1.18–27.86; *p*-value = 0.01) and (OR = 7.19; 95%, CI = 0.19–27.41; $p = 0.01$) respectively. Similarly, mutant interaction analysis was performed, and it was found that both genotypes were more closely associated with Migraine. Interactions of both variants had a higher risk for causing migraine in the genotypes CCAA ($p = 0.02$), CCCC ($p = 0.05$), CTAA ($p = 0.03$), and TTAC ($p = 0.02$). Patients having MA are more susceptible to genotypes (CCAA, CTAA, and TTAC), while MWA was more affected by (CCCC $p = 0.05$). Altogether, it could be concluded that folate metabolism plays an essential role in the onset of Migraine. Further studies involving larger populations may pave the way to clarify genotype-phenotype relationships.

Keywords: Migraine with aura, Migraine without aura, *MTHFR*, Genotypes, Homocysteine

1 Introduction

Migraine is the 3rd most common disease globally, with a predictable global occurrence of 14.7% [1]. Migraine affects women three times more often than men and most often due to hormones. It usually starts around puberty, and most affected people are between 35 to 45 [2], [3]. Migraine symptoms include blurred vision, sensitivity to light, sounds, odors, nausea, and vomiting [4]. Migraine attacks usually last 4 to 72 h, and most people have no symptoms between episodes. Migraines can have a massive impact on your work, family, and social life [5]. There are currently no biochemical tests available to confirm the diagnosis of Migraine, comparing the patient's clinical presentation with the classifications established by the International Headache Society (IHS) [6]. The two significant types of Migraine that are classified according to the IHS guidelines are Migraine with aura and Migraine without aura (MA and MWA) [7]. Although the two subtypes have significant overlap in symptoms, people with Migraine with aura (MA) experience a specific phase of neurological impairment, known as an "aura" which usually precedes the headache phase [8], [9].

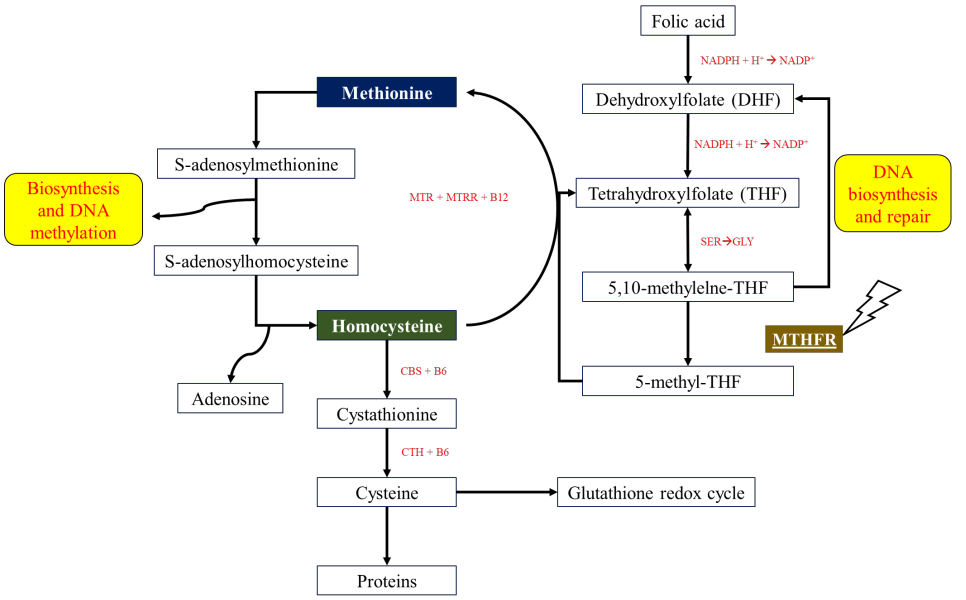

Figure 1: Schematic representation of the *MTHFR* gene, which plays a role between methionine and folate cycle.

Anxiety and depression are significantly more common in people with migraines than in healthy people [10]. In addition, it is a disease that almost certainly has a genetic basis. Among all neurological disorders, migraine research is the least funded than its economic impact [11]. Migraine is composed of an essential genetic component, irrespective of the types and number of genes [12]. It has genetic heterogeneity, a multifactorial mode of inheritance, whereby genetic factors can interact with environmental factors [13]. Genetic and ecological susceptibility are triggers that may play essential roles in the pathogenesis of Migraine [14]. The pathophysiology of Migraine is not yet fully understood [15]. It has been reported that many genes are predisposing factors for the development of migraine attacks, mainly in MA rather than MWA [16]. Many studies suggest that various genes (e.g. CACNA1A, ATP1A2, SCN1A, etc.) are involved in the cause of a migraine attack, including *MTHFR* and several other genes [17].

For protein synthesis, neurotransmitter methylation and cell regulatory functions, an essential amino acid (homocysteine) is required. In addition, the SAM precursor that produces a methyl donor for the recipient (DNA or protein) for methylation reactions results in a change in activity or function of the recipient [18]. However, the *MTHFR* gene has a unique process that regulates the methyl groups for the methylation reactions by synthesizing purines and pyrimidine, shown in Figure 1 [19]. The most common variants of this gene are C677T and A1298C [20]. The numbers of people who carry these genotypes are varied from population to population [21]. The average amount of folic acid in the blood of the *MTHFR* 677 TT genotype is only slightly lower (about 16% less) than that of people with the *MTHFR* 677 CC genotype. [22]. This mutation is characterized by the conversion of cytosine (C) > thymine (T) at position 677 of catalytic domain alanine 222 to valine (Ala222Val). It has been reported that a homozygous genotype causes about 15% of people in North America and Europe compared to the TT genotype, which is the most commonly found in the African population [23]. Another common gene variant in the *MTHFR* gene is A1298C [24]. The position 1298 in the *MTHFR* gene, "A" is expected to be having a DNA base and "C" is a variant of the gene, which substitutes glutamate to alanine amino acid (Glu429Ala) [25]. This mutant has been reported to cause *MTHFR* activity at lower levels than the C677T polymorphism [26]. However, the combinations of A1298C and C677T heterozygotes yield similar results to individuals of the 677TT genotype [27]. In addition, the genetic variation present in this gene is associated with a wide range of diseases in various organs, such as heart and

cerebrovascular, Migraine, Alzheimer, Parkinson, and ovarian cancer, and its significance is still controversial [28]–[30]. This study evaluated the role of the *MTHFR* gene using both variants (C677T and A1298C) with its migraine susceptibility. The effects caused by variants, individual or combined, were also assessed.

2 Materials and Methods

2.1 *Collection of samples*

In the present study, patients were recruited from the Department of Neurology, Government Vellore Medical College and Hospital (GVMCH), Vellore, Tamilnadu. In addition, this study was approved by the Ethical Committee of GVMCH and VIT, Vellore. A total of 186 (32.4 ± 12.1 years) migraine patients (116 (MA) and 70 (MWA)) were recruited. Blood samples were collected after informed consents. Patients were diagnosed based on IHS guidelines. Medical history was obtained from each patient, and all cases underwent a thorough physical examination. The migraine patients who had comorbidities (hypertension and other neurological disorders) were excluded from this study. In the control group, a total of 152 healthy individuals with the mean age of 33.3 ± 12.3 years, clinically diagnosed and with no signs of migraine attacks and other neurological disorders, were selected for the study.

2.2 *DNA extraction*

Venous blood samples (3–4 mL) were collected from both groups in ethylene tetra acetic acid (EDTA) vacutainers. DNA was extracted by the standardized protocol [31], and the DNA content was quantified using a Bio photometer (Eppendorf). Later, the samples were stored at –20 °C until further use.

2.3 *Genotyping*

The primers were designed for both variants (C677T and A1298C) using primer- BLAST shown in Table 1 purchased from Shrimpex Biotech, Chennai, India. Primers were reconstituted using sterile water (pyrogen-free) to make a stock solution. From the stock working solution of 10 pmol/µL was prepared. The primers were diluted by a 1:5 ratio from the stock primers by adding sterile water. All amplicons were stored at –20 °C and used for standardization.

Table 1: Primers used in the study

F/R	Primers	Size (bp)
F	TGAAGGAGAAGGTGTCTGCGGGA	198
R	AGGACGGTGCGGTGAGAGTG	
F	TTGGGGAGCTGAAGGACTAC	159
R	CTTTGTGACCATTCCGGTTT	

The polymerase chain reaction (PCR) was performed using the master gradient thermal cycler (Eppendorf). For both genotypes of *MTHFR* (C677T & A1298C) with different annealing conditions, PCR was performed. The PCR mixture was prepared in 0.2 mL vials. The mixture was vortexed thoroughly in Thermo-cycler. The components of the PCR mixture and its volumes are shown in Table 2.

Table 2: PCR master mix components per reaction for both variants (C677T and A1298C)

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

PCR thermal cycling conditions were standardized for both the variants *MTHFR* (C677T & A1298C) by gradient technique, and the optimum annealing temperature were obtained for the amplification at 60 and 55 °C, respectively. PCR conditions are tabulated in Tables 3 and 4.

After PCR, the amplified products of both variants (C677T and A1298C) were subjected to electrophoresis in 2% agarose gel to determine the conformation of the amplicon bands at 198 and 159 bp, respectively. Electrophoresis was performed using 1X TBE buffer and ethidium bromide (EtBr). A 6-fold dye was used to load the sample and 100 bp DNA marker. The procedure was carried out at a constant voltage of

100 V for 30 min. After the electrophoresis process, the gel was checked under a UV trans-illuminator for PCR amplification

Table 3: PCR thermocycler condition for *MTHFR* C677T

Reaction	Temperature (°C)	Duration (min)	Cycles
Denaturation	95	5	
Final Denaturation	95	1	
Annealing	60	1	
Extension	72	1	35
Final Extension	72	5	
On hold	4	∞	

Table 4: PCR thermocycler condition for *MTHFR* A1298C

Reaction	Temperature (°C)	Duration (min)	Cycles
Denaturation	95	5	
Final Denaturation	95	1	
Annealing	55	1	
Extension	72	1	35
Final Extension	72	5	
On hold	4	∞	

The PCR product was further processed for Restriction Fragment Length Polymorphism (RFLP). *Hinf*I restriction enzyme was used to digest *MTHFR* (C677T) by incubating the mixture at 37 °C for 2 h. followed by electrophoresis. The reaction mixture details are shown in Table 5. The wild types (CC) resulted in a single band at 198bp, heterozygous (CT) showed 198, 175, and 110 fragments. Homozygous (TT) had bands in 175, 87, and 23 bp.

Table 5: RFLP reaction mixture for *Hinf*I restriction enzyme

Reaction Mixture	Volume (µL)
Sterile Milli-Q water	1.75
PCR product	5.0
Cut Smart Buffer	0.5
Restriction Enzyme (*HinfI*)	0.25
Total volume	7.5

Similarly, for another variant, *MTHFR* (A1298C) RFLP was carried out using *Mbo*II restriction enzyme by incubating the mixture for 2 h. at 37 °C and followed by electrophoresis. The reaction mixture is shown in Table 6. The wild types (A1298A) produced five fragments of 159, 77, 56, 31, and 28 bp, heterozygous (A1298C) produced six fragments 159, 104, 77, 56, 31, and 28 bp, and the homozygous (C1298C) produced four fragments of 104, 77, 31, and 28 bp.

Table 6: RFLP reaction mixture for *Mbo*II restriction enzyme

Reaction Mixture	Volume (µL)
Sterile Milli-Q water	1.75
PCR product	5.0
Cut Smart Buffer	0.5
Restriction Enzyme (*MboII*)	0.25
Total volume	7.5

2.4 *Statistical analysis*

The statistical analysis of the data was performed with Odds ratio (OR), 95% confidence intervals and an error of 5% and ($\alpha = 0.05$) were analyzed. Interaction analysis between *MTHFR* (C677T and A1298C) was performed based on the ratio analysis. Descriptive statistics for all the data were performed, and the variables' mean, standard deviation, minimum, and maximum values 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 *Patients (Age and Gender)*

Out of 186 migraine patients, 116 (62.9%) were diagnosed under the category of MA, and 70 (37.1%) were MWA. In addition, gender-wise distribution was done for migraine patients, and it was found that males (14.5%) were less affected by Migraine than females (85.5%). It implies Migraine is predominant in females when compared to males. Similarly, migraine headaches have been reported to be in females than males [13]. Female reproductive milestones may be directly or indirectly associated with migraine [32]. Assessment of samples is shown in Figure 2. In conjunction with controls, males (26%) and females (74%) had never experienced Migraine and other neurological disorders. It has also been reported that abnormalities of the pituitary gland, neurotransmitters, hypothalamus, diamines, endocrine glands and minerals can lead to pathological complications [33].

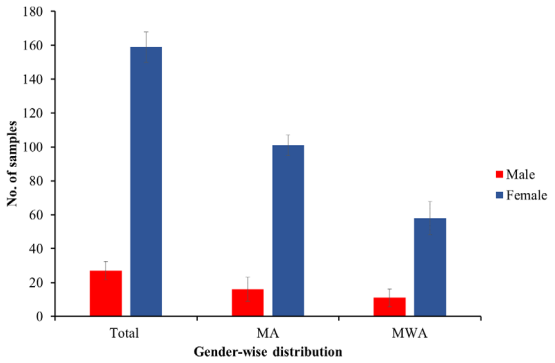

Figure 2: Assessment of samples that are categorised as MA and MWA concerning males and females.

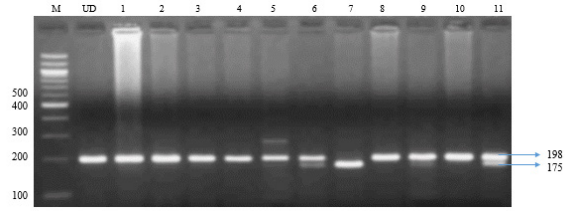

Figure 3: Polymorphism analysis of *MTHFR* C677T using ethidium bromide stained 2% agarose gel electrophoresis showing *Hinf*I digested products. (Lanes 1, 2, 3, 4, 8, 10 = CC; lanes 5, 6, 9, 11 = CT; lanes 7 = TT; UD = Undigested sample; M = 100 bp molecular marker).

3.2 *MTHFR (C677T and A1298C) Genotyping*

In the present study, RFLP technique was performed to determine the presence or absence of bands for both variants. The incidence of the polymorphism in *MTHFR* 677T had 30.6% in cases compared to 27% among healthy individuals. The frequencies of the *MTHFR* genotypes C677T, C677C, and T677T were 23.7, 57.5, and 18.8% in patients and 28.9, 58.5, and 12.6% in controls, respectively. It implies that the C677T polymorphism was positively correlated with Migraine—the Digested products obtained from the RFLP for the variant *MTHFR* C677T as shown in Figure 3. Similarly, comparisons of migraine patients (74) with healthy people (261) were reported in the Caucasian population, and it was found that the frequency of the homozygous (TT) genotype in Migraine (20.3%) was significantly higher than in the control group (9.6 %). In addition, Migraine with aura (40.9%) with a TT genotype frequency was a higher risk factor for Migraine in comparison with the control group (9.6%) [34]. However, in the Asian and non-European populations, the TT genotype was riskier for MA and MWA. It has been reported that the C677T polymorphism in cardiovascular risk patients is more vulnerable to migraine [35]. In addition, a study based on the age of Migraineurs was conducted in Iceland, and an association between *MTHFR* C677T and Migraine was reported.

The incidence of TT genotype among the healthy individuals (12.6%) was lesser than in the Migraineurs (18.8%). Association of C677T polymorphism and genetic models with the risk of Migraine compared with control are tabulated in Table 7. In addition, the TT genotype has a significant effect on lower levels of thermolability and enzyme activity by increasing plasma homocysteine levels [36].

In this study, an association was found between the *MTHFR* gene polymorphism and the predisposition to Migraine. In addition, the TT genotype has a significant effect on lower levels of thermolability and enzyme activity by increasing plasma homocysteine levels [36].

Another variant C allele present in the *MTHFR* 1298 was found to be a polymorphic allele frequency of 51.3% in cases compared to 51.7% in control. The frequencies of the *MTHFR* genotypes AA, AC, and CC were 20, 57.5, and 22.5% in migraine patients and 9.2, 78.3, and 12.5% in controls, respectively. Digested products were obtained from the RFLP for the variant *MTHFR* A1298C as shown in Figure 4. In addition, this study found an association between the *MTHFR* A1298C polymorphism and genetic patterns that cause migraines with and without aura. Likewise, among the Iranian population, it has been reported that this polymorphism is strongly associated with Migraine, and it can influence several disorders (cardiovascular, neurological disorders and inflammatory) [37]–[39]. In the present study, genetic models based on genotypes were performed. It was found that AC heterozygous with mutant CC was significantly higher with the risk of migraine attacks ($p = 0.00$). In addition, it was reported that the enzymatic activity in individuals with 1298CC was 60% compared to carriers of the 1298AA genotype [40].

It signifies a link between migraine predisposition and the *MTHFR* gene. An increase in statistical significance

Table 7: Association of *MTHFR* C677T polymorphism with the risk of migraine

Variables	Patients (%) n = 186	Controls (%) n = 152	OR (95% CI)	*p*-value
Genotype				
CC	107 (57.5)	89 (58.5)	Reference	
CT	44 (23.7)	44 (28.9)	0.83 (0.50–1.37)	0.47
TT	35 (18.8)	19 (12.6)	3.53 (1.18–27.86)	**0.01***
Genetic model				
Dominant model				
CT + TT vs CC				
CT + TT	79 (42.5)	63 (41.4)		
CC	107 (57.5)	89 (58.6)	1.04 (0.67–1.61)	0.84
Co-dominant model				
TT vs CT/ CT vs CC				
TT	35 (18.8)	19 (12.6)	Reference	
CT	44 (23.7)	44 (28.9)	1.8 (0.92–3.70)	0.08
CC	107 (57.5)	89 (58.5)	1.20 (0.73–1.98)	0.47
Recessive model				
TT vs CC + CT				
TT	35 (18.8)	19 (12.6)	Reference	
CC + CT	151 (81.2)	133 (87.5)	1.62 (0.89–2.97)	0.11
Allele				
C	258 (69.4)	222 (73)	Reference	
T	114 (30.6)	82 (27)	1.52 (1.05–2.20)	0.02*
*-significant (*p*-value = 0.05)				

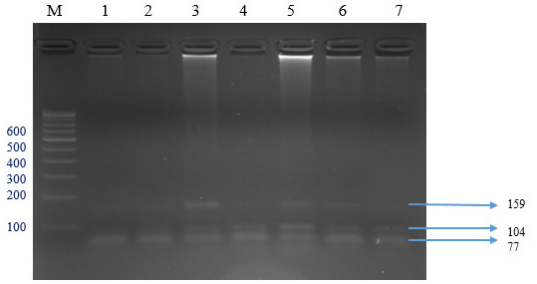

Figure 4: Polymorphism analysis of *MTHFR* A1298C using ethidium bromide stained 2% agarose gel electrophoresis showing *MboII* digested products. (Lane 1 = AA ; lanes 3, 5, and 6 = AC ; lanes 2, 4, and 7 = CC ; M = 100bp molecular marker).

was shown for those who were T677T and C1298C. In addition, these two polymorphisms have been reported to alter susceptibility in the central nervous system (CNS), especially in ischemia, stroke, Parkinson's disease, and so on. However, the role of these polymorphisms in migraine predisposition requires further confirmation [41]. It has been reported that migraine headaches do not increase blood homocysteine levels [42]. Association of A1298C polymorphism with the risk of Migraine compared with control is summarized in Table 8. On the other hand, there is controversy over whether MA and MWA are two different diseases or variations with complex genetic backgrounds [43]. However, serum homocysteine levels were higher in patients with MA than in patients with MWA. Additionally, vitamin supplements were more effective in MA patients. Russel *et al.* reported that MWA is caused by a combination of environmental and genetic factors, while MA is known to be caused only by genetic factors [44]. It implies additional evidence of the contradiction mentioned earlier. In the present study, the effect of the *MTHFR* C677T variant is less than the A1298C variant, and these results indicate the importance of personalized treatment based on genetic factors.

In a meta-analytical study reported that the *MTHFR* 1298CC polymorphism was found with an increased likelihood of migraine headaches, especially in MWA. On the other hand, there is controversy over whether MA and MWA are two different diseases or variations with complex genetic backgrounds [43]. However, serum homocysteine levels were higher in patients with MA than in patients with MWA. Additionally, vitamin supplements were more effective in MA patients. Russel *et al.* reported that MWA is

Table 8: Association of *MTHFR* A1298C polymorphism with the risk of Migraine

Variables	Patients (%) n = 186	Controls (%) n = 152	OR (95% CI)	*p*-value
Genotype				
AA	37 (20)	14 (9.2)	Reference	
AC	107 (57.5)	119 (78.3)	2.93 (1.51–5.73)	**0.00****
CC	42 (22.5)	19 (12.5)	7.19 (0.19–27.41)	**0.01***
Genetic models				
Dominant model				
AC + CC vs AA				
AC + CC	149 (80)	138 (90.8)		
AA	37 (20)	14 (9.2)	0.41 (0.21–0.88)	**0.00****
Co-dominant model				
CC vs AC/AC vs AA				
CC	42 (22.)	19 (12.5)	Reference	
AC	107 (57.5)	119 (78.3)	2.46 (1.35–4.5)	**0.00****
AA	37 (20)	14 (9.2)	0.34 (0.2–0.7)	**0.00****
Recessive model				
CC vs AA + AC				
CC	42 (22.5)	19 (12.5)	Reference	
AA + AC	144 (77.5)	133 (87.5)	2.04 (1.13–3.69)	**0.01***
Allele				
A	128 (48.7)	147 (48.3)	Reference	
C	191 (51.3)	157 (51.7)	0.71 (0.52–0.98)	**0.02***
**-significant (*p*-value = 0.00), *-significant (*p*-value = 0.05)				

caused by a combination of environmental and genetic factors, while MA is known to be caused only by genetic factors [44]. It implies additional evidence of the contradiction mentioned earlier. In the present study, the effect of the *MTHFR* C677T variant is less than the A1298C variant, and these results indicate the importance of personalized treatment based on genetic factors.

Interaction analysis between the two polymorphisms in cases and controls was performed based on the GMDR method, and the results were summarized in Table 9. It was found that in individuals with the C677C/C1298C diplotype, the risk of developing Migraine increased 5.70 times at a *p*-value of 0.05 concerning controls. In addition, CCAA, CTAA, and TTAC genotypes were also significantly higher in the cases with a *p*-value of (0.02, 0.03, 0.08, and 0.02).

Similarly, interaction analysis was performed for the patients with and without aura. It was found that CCAA, CTAA, and TTAC were highly significant interaction models with the *p*-value of 0.01, 0.02, 0.01 respectively. In addition, in MWA patients CCCC (*p*-value = 0.05) had greater susceptibility. However, the genetic makeup is not clear for healthy people who have undergone the study; the allele frequency

Table 9: Interaction analysis between two polymorphisms of cases and controls

Variables	Patient			Control		
	T	M	F	T	M	F
CCAA	144*	21	123*	103	24	79
CCAC	214	31	183	208	50	158
CCCC	149*	23	126*	108	26	82
CTAA	81*	11	70*	58	18	40
CTAC	151	21	130	163	44	119
CTCC	86*	13	73	63	20	43
TTAA	72	10	62	33	10	23
TTAC	142*	20	122*	138	36	102
TTCC	77	12	65	38	12	26
T- total no of genotypes, M- males, F- females, *-significant (*p*-value = 0.05)						

was similar to recent studies [45]. In the present study, among healthy individuals, the incidence of the variant in *MTHFR* 677T is 27% and in 1298 of C variant was 51.7%. Likewise, people with the TT/AA genotype have a 4.18-fold time increased threat of emerging Migraine. In addition, the progression of Migraine affected individuals with CT/CC genotype had equal importance in the progression of this disorder (*p*-value = 0.02). Interaction studies have recently shown a combined effect of the CT and AC heterozygous

polymorphisms and change in the folate metabolism. In addition, having these polymorphisms in people with adequate folate levels can reduce the effect of migraine attacks [46].

On the other hand, the TT/CC genotype may lead to decreased activity of the enzyme, although the 677C-T conversion occurs in the *MTHFR* catalytic domain. In contrast, the 1298A-C occurs in the regulatory domain [47].

4 Conclusions

This study examined the effect of the C677T and A1298C genotypes of the *MTHFR* gene variants on migraine susceptibility. Interestingly, TT of genotype 677 was significantly correlated with Migraine. Similarly, it was found that CC 1298 was also associated with more Migraineurs. Individuals with CC/CC genotypes have a higher risk to cause migraine susceptibility. Patients with MA are more susceptible to genotypes CCAA, CTAA, and TTAC, while MWA was found CCCC $p = 0.05$. To conclude, folate metabolism plays a vital role in the onset of Migraine. Hence, further studies involving large population may pave the way for elucidating the genotype-phenotype relationship.

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