Journal of Medical Sciences

ISSN 1682-4474

science alert

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OPEN ACCESS Journal of Medical Sciences

ISSN 1682-4474
DOI: 10.3923/jms.2018.76.86

Research Article
Influence of TNF-α and ESR1 Polymorphisms on Vascular, Hormonal and Inflammatory Biomarkers in Migraine

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Abstract

Background and Objective: Migraine is a highly prevalent multifactorial and polygenic neurological disorder associated with genetic, environmental and hormonal changes. Methylenetetrahydrofolatereductase (MTHFR), tumor necrosis factor (TNF-α), estrogen and genetics have been reported to play significant roles in migraine. The purpose of this study was to determine the impact of TNF-α and ESR genetic polymorphisms on migraine development. **Materials and Methods:** Female patients diagnosed with migraine (n = 129) and healthy controls (n = 100) were recruited for the study. Plasma concentrations of vitamin B6 and TNF-α were measured using enzyme linked immunosorbent assay kits. Vitamins B9 and B12 were measured using a competitive immunoassay based on a direct chemi-luminescent technology while genotyping was performed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. **Results:** The G-allele of ESR1 325C>G (OR = 2.61, p = 0.001), A allele of ESR1 594G>A (OR = 2.99, p<0.001) and A-allele of TNF-α-308G>A (OR = 9.47, p<0.001) were significantly associated with migraine among female Malays. However, the T-allele of MTHFR 677C>T was not associated with migraine. Vitamin B6 (10.9 vs 20.3 ng mL^{-1}) was significantly (p<0.001) lower and TNF-α (16.8 vs 2.9 pg mL^{-1}) was significantly (p<0.001) higher among migraine patients compared to healthy subjects. In addition, vitamin B6 level was significantly lower among migraine patients having the MTHFR 677C>T (8.6 vs 16.7 ng mL^{-1}, p = 0.008) and TNF-α-308G>A (9.6 vs 21.9 pg mL^{-1}, p<0.001) compared to the healthy subjects. Similarly, the TNF-α levels were significantly higher (44.7 vs 15.7 pg mL^{-1}, p = 0.013) among migraine patients with TNF-α-308G>A polymorphisms compared to the healthy subjects. The variant allele of TNF-α-308G>A, ESR1 325C>G and ESR1 594 G>A significantly predisposed patients to higher risk for migraine, but not the MTHFR 677C>T variant allele. **Conclusion:** Serum levels of TNF-α and vitamin B6 were associated with TNF-α-308G>A polymorphism among Malay or female subjects.

Key words: MTHFR, ESR1, TNF-α, migraine, vitamin B, homocysteine, estrogen

Citation: Munvar Miya Shaik, Murtala Bello Abubakar, Huay Lin Tan and Siew Hua Gan, 2018. Influence of TNF-α and ESR1 polymorphisms on vascular, hormonal and inflammatory biomarkers in migraine. J. Med. Sci., 18: 76-86.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Migraine is highly prevalent globally and is a leading cause of disability worldwide[1,2]. The cerebral cortex of patients with migraine is hyper-excitable and is excessively responsive to external stimuli[3]. Although genetic variations contribute to hyper-excitability, the exact mechanism linking genes to phenotypes remain unknown and are likely to be multifactorial. Overall, migraine has a strong (up to 50%) genetic component with multifactorial, polygenic inheritance[4] that may predispose migraineurs to increased susceptibility to cortical hyper-excitability.

The 5,10-Methylenetetrahydrofolatereductase (MTHFR) enzyme (encoded by the *MTHFR* gene) and homocysteine play pivotal roles in migraine pathogenesis. Homocysteine, a key intermediate in one-carbon metabolism further clarifies the involvement of B vitamins in transferring one-carbon units[5,6]. Hyperhomocysteinemia is caused by abnormal methionine biosynthesis due to deficiencies in vitamins B6, B9 and B12[7] and also by MTHFR 677C>T allele resulting in reduction of MTHFR activity[8]. Individuals homozygous for this variant have been reported to express only approximately 30% of the mean MTHFR activity when compared with individuals without the substituted allele[9,10]. Even though the 677C>T allele is commonly found in Caucasians with migraine (frequency of approximately 23-41%)[11,12], however, a Finnish study could not establish any association of the gene with migraine[13], indicating that the association of the gene and migraine may differ from one population to another.

Neurogenic inflammation may lower the nociceptive threshold required to stimulate meningeal sensory fibers and also act on vascular tissues to cause vasodilatation, plasma protein extravasation in the surrounding area, endothelial changes and platelet aggregation leading to (1) Subsequent release of serotonin (5-Hydroxytryptamine, 5-HT) and other mediators, (2) White cell adhesion and (3) Inflammation. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) play significant roles in the sensitization of trigeminal nerve fibers and also in migraine[14,15]. Polymorphism in the TNF-α gene (-308G>A) increases the production of TNF-α and is associated with migraine[16,17]. In addition, estrogen has been reported to play a vital role on the activity of homocysteine, trigeminal vascular system and on the onset of migraine[18]. A recent meta-analysis further indicated the association of polymorphisms in 325C>G and 594G>A of estrogen receptor 1 (*ESR1*) gene with migraine[18].

Despite the high prevalence and disabling nature of migraine, there is paucity of data on susceptibility genes related to the disorder, particularly among Malaysians and among other Asians. Detection of genetic polymorphisms in the TNF-α, ESR1 325C>G and ESR1594G>A may therefore, help in predicting Malaysians who may be at high risk of developing migraine. In this study, authors aimed to determine the frequency of the MTHFR 677C>T, ESR1 325C>G, ESR1 594G>A and TNF-α-308G>A gene polymorphisms in a Malaysian population and to determine the association between the levels of homocysteine, vitamin B6, B9 and B12 polymorphisms between those with and without migraine. The reason of this study was to find out the impact of TNF-α and ESR genetic polymorphisms on migraine development.

MATERIALS AND METHODS

Sample collection: This study was carried out in Hospital Universiti Sains Malaysia (HUSM), a multi-specialty teaching hospital located in KubangKerian, Kelantan, Malaysia, between August, 2012 and December, 2013. The ethical consent for this study was obtained [ethical number: USMKK/PPP/JEPeM (231.3.(08))] from the Ethical Committee of University Sains Malaysia which complies with the declaration of Helsinki.

Migraine patients (n = 1576) were identified from the computerized patient database over the previous 3 years based upon the inclusion/exclusion criteria. Inclusion criteria include patients between 15-60 years old diagnosed with migraine for more than one year prior to the study. Pregnant women and migraine patients having neurological and cardiovascular diseases or history of trauma were excluded. After the initial screening process against the inclusion and exclusion criteria, 129 patients were recruited and verbally informed about the purpose of the study. Consented patients were requested to complete written informed consent forms. All participants were then subsequently examined by a neurologist to confirm the migraine diagnoses. Socio-demographic information was also collected by the researchers. Only female Malay subjects were included in the study because neither male patients nor patients from other races (Chinese, Indian and Siamese) were registered at the clinic during the study period. A total of 100 healthy subjects were used as control for the study.

Biomarkers assay: Vitamin B6 and TNF-α levels were measured using a Cusabio Enzyme-Linked Immunosorbent Assay (ELISA) kit. Vitamins B9 and B12 levels were measured using a competitive immunoassay based on a direct chemi-luminescent technology (CMIA).

Genotyping: DNA was extracted from whole blood (2 mL) collected from the patients using a GeneAll kit (South Korea) followed by quantification using an automated DNA quantification machine (NanoQuant). DNA samples were investigated for the presence of the MTHFR 677C>T[9,19], TNF-α -308G>A[20], ESR1 325C>G[21] and ESR1 594G>A[22]mutations according to a previously published method with some slight modifications. Restriction Fragment Length Polymorphism (RFLP) was performed by using specific digestion enzymes (for MTHFR 677 C/T and ESR1 325C>G variants HinfI, for TNF-α-308 G/A variant NcoI and for ESR1 594G>A variant Btg-I).

Digestion of each PCR product was conducted by adding doubly distilled water (12.8 µL), PCR product (5 µL), restriction enzyme (2.0 U) and NEB buffer (5 µL) (New England Biolabs, Boston, USA) followed by incubation at 37°C for 2 h. The PCR products were analyzed by ethidium bromide stained agarose gel electrophoresis (2.0%) using 0.5×tris borate ethylenediaminetetraacetic acid (TBE). The fragments were then visualized using an Alpha Innotech® Ultraviolet Trans-illuminator (Alpha Innotech® USA) before the image was captured.

Confirmatory DNA sequencing was performed using Applied Biosystems 3730 XL Genetic Analyzer (Applied Biosystems, Foster City). Then, the SNPs were analyzed by aligning the sequence with that of the reference gene sequence using the Bio Edit Sequence Alignment Editor (version 7.0.5.3, North Carolina, USA). The expected genotypic frequencies were calculated by using the Hardy Weinberg Eq.:

$$p^2+2pq+q^2 = 1$$

Where:

p^2 = Frequency of the homozygous genotype AA
q^2 = Frequency of the homozygous genotype aa
2pq = Frequency of the heterozygous genotype Aa

Statistical analysis: Data analysis was performed using IBM SPSS Statistics (Version 20.0. Armonk, NY: IBM Corp). Independent t-test was used to compare vitamin B6, B9 and B12, homocysteine, estrogen and TNF-α levels between migraine patients and healthy controls. Pearson chi-square test was used to determine the association between migraine and MTHFR 677C>T, TNF-α-308G>A, ESR1 325C>G as well as ESR1 594G>A gene polymorphisms. Independent t-test was used to compare the vitamin B6, B9 and B12, homocysteine, estrogen and TNF-α levels between migraine patients with the genetic variation and healthy subjects with genetic variations. Pearson chi-square test was used to determine the odds ratio of genetic polymorphism and migraine. A p-value of <0.05 was considered as statistically significant.

RESULTS

Demographic data: The subjects characteristics are described in Table 1.

Allelic frequency: The genotypic and allelic frequencies among study subjects and controls are depicted in Table 2.

Biomarkers level: The mean serum level of biomarkers in migraine and healthy controls are shown in Table 3.

Association of MTHFR with homocysteine and vitamins B6, B9 and B12 levels: Serum vitamin B6 levels were significantly lower among migraine patients harboring the mutant MTHFR 677C>T (8.6 vs 16.7 ng mL^{-1}; p = 0.008) when compared with healthy patients without the genetic polymorphism as depicted in Fig. 1.

Association of ESR1 and estradiol levels: The serum estradiol levels among the study subjects and controls are depicted in Fig. 2.

Association of TNF-α and vitamin B6 levels with TNF-α-308G>A: The serum TNF-α levels and vitamin B6 levels for the study subjects and health controls are shown in Fig. 3a and b, respectively.

Table 1: Patient characteristics

Characteristics	No migraine (n = 129) (%)	Migraine (n = 129) (%)	p-value
Age	25.67 (6.87)	26.71 (9.10)	0.302
Duration of education (years)	14.84 (2.90)	13.50 (2.68)	<0.001
Age of menarche	12.71 (1.20)	12.47 (0.96)	0.086
Headache pain score	1.99 (2.04)	6.67 (2.05)	<0.001
Qualification			
Standard six	0 (0.0)	1 (0.8)	0.003

Table 1: Continued

Characteristics	No migraine (n = 129) (%)	Migraine (n = 129) (%)	p-value
SPM	35 (27.1)	52 (40.3)	
STP	17 (13.2)	27 (20.9)	
Diploma	15 (11.6)	20 (15.5)	
Degree	55 (42.6)	26 (20.2)	
MSc	5 (3.9)	3 (2.3)	
PhD	2 (1.6)	0	
Relation			
Married	27 (20.9)	31 (24.0)	0.482
Single	101 (78.3)	95 (73.6)	
Widow/divorcee	1 (0.8)	3 (2.3)	
Income (RM)			
Below 450	0	5 (3.9)	0.001
450-800	4 (3.1)	9 (7.0)	
801-1500	16 (12.4)	27 (20.9)	
1501-3000	77 (59.7)	48 (37.2)	
3001-6000	26 (20.2)	24 (18.6)	
Above 6000	6 (4.7)	16 (12.4)	
Profession			
Student	100 (77.5)	73 (56.6)	<0.001
Housewife	0 (0.0)	14 (10.9)	
Government job	28 (21.7)	29 (22.5)	
Private job	1 (0.8)	6 (4.7)	
Self employed	0 (0.0)	5 (3.9)	
None	0 (0.0)	2 (1.6)	
Menstruation stage			
Follicular phase (1-13 days)	64 (49.6)	61 (47.3)	0.150
Mid-cycle phase (14-16 days)	15 (11.6)	24 (18.6)	
Luteal phase (17-28 days)	48 (37.2)	38 (29.5)	
Irregular menstrual cycle	2 (1.6)	6 (4.7)	

Table 2: Genotype and allele frequencies among migraine patients and healthy subjects

	Migraine (n = 129)				Healthy subjects (n = 129)				
Polymorphisms	Genotype	Frequencies (%)	Allele	Frequencies (%)	Genotype	Frequencies (%)	Allele	Frequencies (%)	p-value
MTHFR 677 C/T	CC	89.92	C	94.19	CC	94.57	C	97.29	0.219
	CT	8.53	T	5.81	CT	5.43	T	2.71	
	TT	1.55			TT	0.00			
ESR1 325 C/G	CC	65.89	C	74.03	CC	85.27	C	92.64	0.001
	CG	16.28	G	25.97	CG	14.73	G	7.36	
	GG	17.83			GG	0.00			
ESR1 594 G/A	GG	65.12	G	82.56	GG	82.95	G	91.47	<0.001
	GA	34.88	A	17.44	GA	17.05	A	8.53	
	AA	0.00			AA	0.00			
TNF-α-308 G/A	GG	76.74	G	88.37	GG	96.90	G	98.45	<0.001
	GA	23.26	A	11.63	GA	3.10	A	1.55	
	AA	0.00			AA	0.00			

Table 3: Mean serum biomarker levels in migraine and healthy subjects

	Mean (SD)		
Parameters	Migraine	No migraine	Significance
Vitamin B6 (ng mL^{-1})	10.90 (9.6)	20.24 (17.2)	<0.001*
Vitamin B9 (nmol L^{-1})	11.15 (7.0)	12.91 (17.8)	0.296
Vitamin B12 (pg mL^{-1})	441.71 (190.9)	461.40 (175.0)	0.389
Homocysteine (μmol L^{-1})	9.32 (3.3)	9.62 (3.3)	0.474
Estradiol (ng mL^{-1})	384.47 (373.7)	334.52 (317.3)	0.248
TNF-α (pg mL^{-1})	16.79 (20.5)	2.93 (3.9)	<0.001*

*p-value is significant

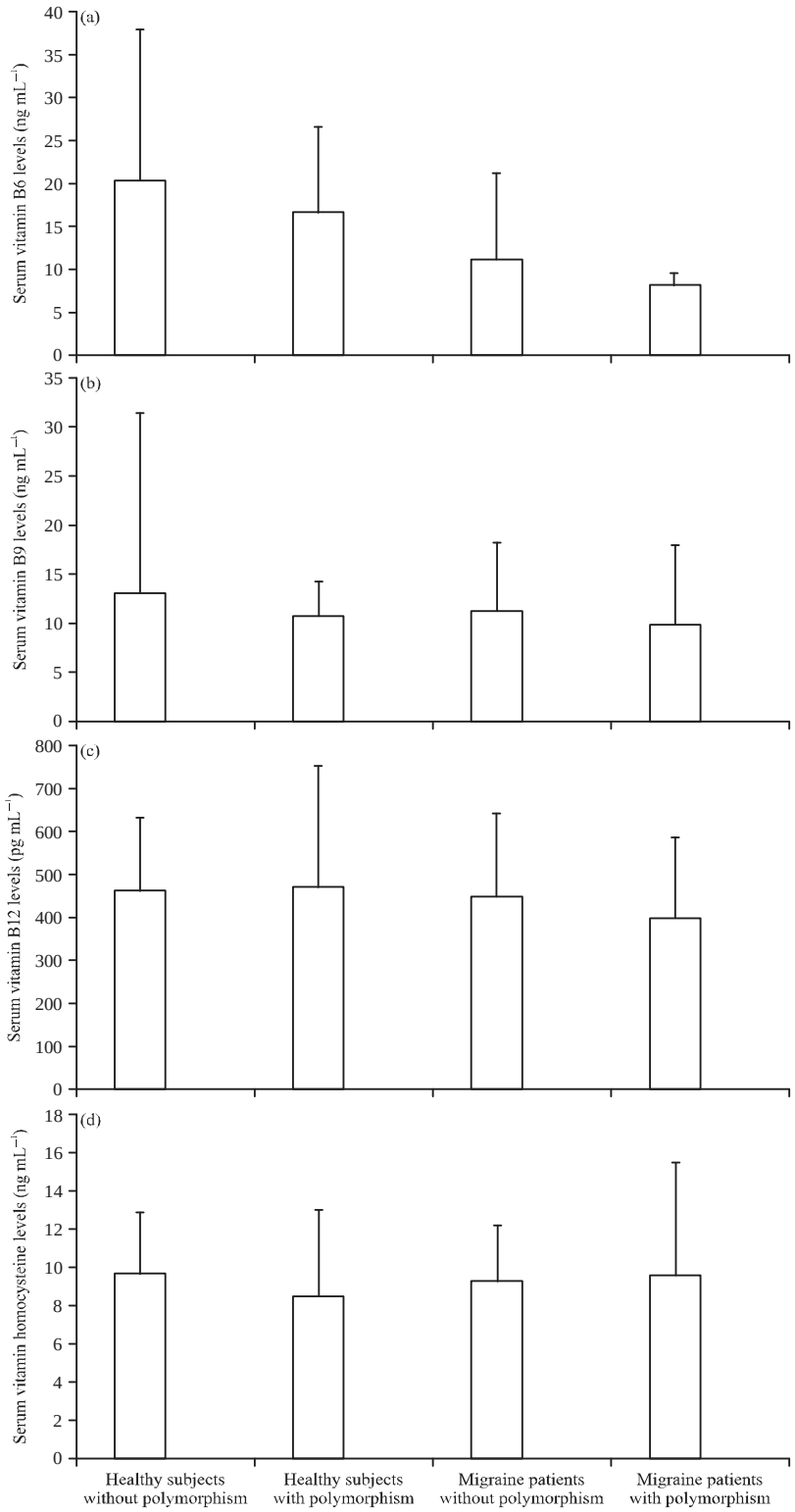

Fig. 1(a-d): (a) Serum vitamin B6, (b) Serum vitamin B9, (c) Serum vitamin B12 and (d) Serum homocysteine levels among migraine and healthy subjects having the MTHFR 677C>T genetic polymorphisms

The bars represent standard deviation

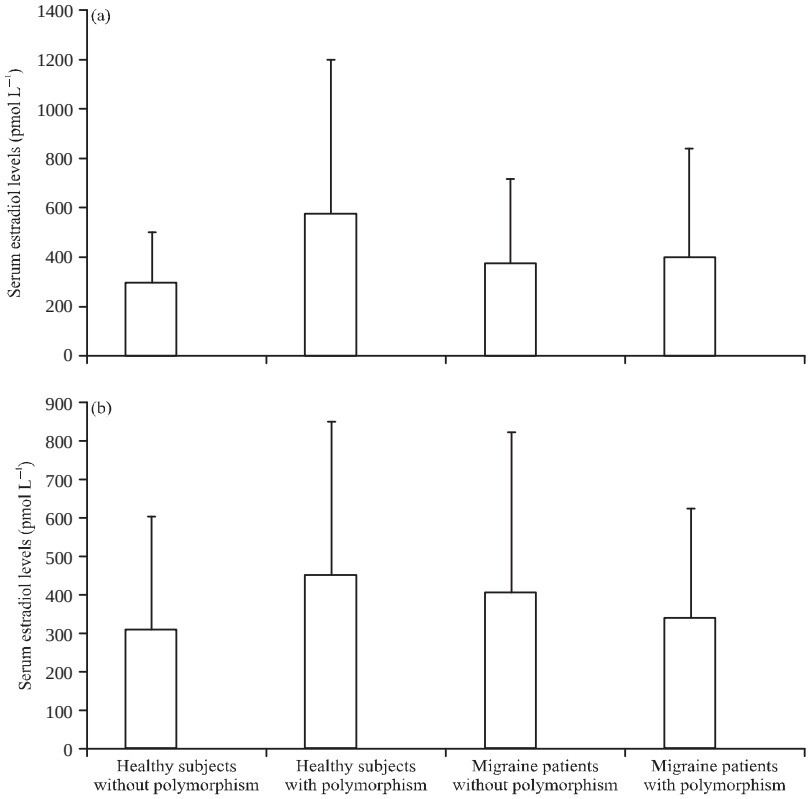

Fig. 2(a-b): Serum oestradiol levels associated with (a) ESR1 325C>G and (b) ESR1 594G>A genetic polymorphisms among healthy subjects and migraine patients
The bars represent standard deviation

DISCUSSION

This was the first study to investigate the biomarkers for migraine and their association with genetic polymorphism among Malaysian migraine patients. The mean age of migraine patients and healthy subjects was below 30 years indicating that the majority of the sample consisted of young adults as migraine prevalence is higher among females and peeking in the late teens and 20s[23]. Additionally, the prevalence ratio for females versus males was highest during the female reproductive/child-bearing years, consistent with a relationship between menstruation and migraine.

Allelic frequency: In the study frequency of C allele MTHFR 677C>Twas higher than the T allele among Malay migraine patients. To authors knowledge, there is no previously reported study on the allelic frequency of MTHFR 677C>T specifically among Malay migraine patients. The T-allele frequency in Malay migraine patients (5.8%) in this study is lower compared to that reported by other studies from Asia in which the T-allele frequency among Chinese (33.8%)[24], Indians (38%)[25] and Japanese (38.5 and 48%)[26,27] migraine patients were higher. A few more studies conducted among Caucasian migraine patients[11,13,28] also reported higher T-allele frequency (40%). The lower T-allele frequency may be attributed to the unique study population in present study which consisted of all females. In comparison, the T-allele frequency among Malaysian Chinese (14%)[19] was much higher than that for the Kelantanese Malays. The Kelantanese Malays who reside in the remote North-Eastern regions of the Malaysia are believed to have a unique genetic signature[29]. Therefore, besides ethnicity, it is also important to investigate the genetic basis of migraine based on races.

A meta-analysis on 16 studies from 2000 to 2013 reported a lack of association of MTHFR 677C>T genetic polymorphism with migraine[30] and reported that the odds of association of T-allele with migraine was 1.18, 0.99 and 1.00 among Asians, Caucasians and all populations in general, respectively which did not indicate a strong association of MTHFR 677C>T with migraine as in present findings. The frequency of G-allele of

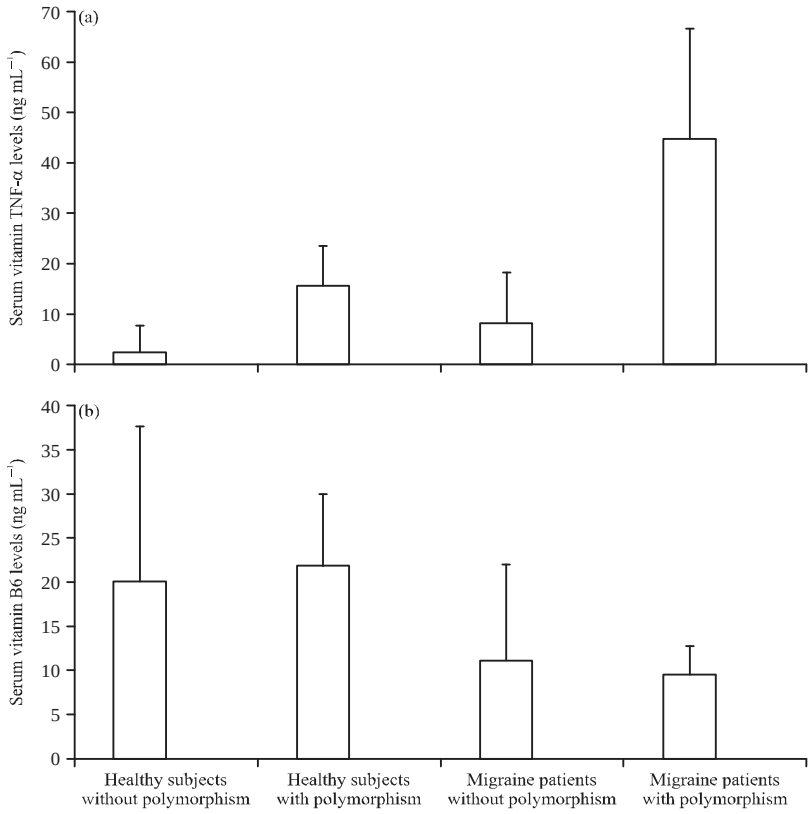

Fig. 3(a-b): (a) Serum TNF-α and (b) Serum vitamin B6 levels among migraine and healthy subjects with TNF-α-308G>A genetic polymorphisms
The bars represent standard deviation

ESR1 325C>G among Malay female migraine patients was significantly higher (26%) compared to that of healthy Malay female subjects (7.4%). To date, no study was conducted among migraine patients to determine the allele frequency for ESR1 325C>G. The study among female Australian population[21] reported higher G-allele frequency among migraine patients (24%) when compared with healthy subjects (20%). Similarly, the two studies conducted among female Italian populations[31,32] reported higher G-allele frequencies among migraine patients (24.5 and 23.6%) when compared with healthy subjects (16.5 and 16.7% indicating that genetic predisposition may play a big role in migraine.

The mutant A-allele frequency of TNF-α-308G>A among female Malay migraine patients is comparable with the studies conducted among Turkish[33], Korean[34] and Indian[35] among migraine patients. However, the mutant A-allele frequency of TNF-α-308G>A among Iranian[17] and another Turkish[36,37] studies conducted among migraine patients were higher than that for the Malay migraine patients. The different reported frequencies can be attributed to the genetic variation among population and to author knowledge; present study is the first to be conducted in the South Asian region. Study results indicated that there is association of TNF-α-308G>A with migraine risk among female Malay which is similar with that reported in another meta-analysis[38].

Association of MTHFR and homocysteine, vitamins B6, B9 and B12 levels: In this study, vitamin B6 levels among migraine patients were significantly lower compared to healthy subjects in which <10% from the total sample had vitamin B6 deficiency. Present study is the first to report on vitamin B6 levels among Malaysian migraine patients. However, few studies reported serum vitamin B6 levels among Malaysian healthy subjects. Higher vitamin B6 levels (6 ng mL^{-1}) were also reported[19,39] among migraine patients compared to this study. This may be attributed to the smaller sample size which comprised of all races and gender among those studies as compared to this study which was restricted only to female Malay subjects. More healthy subjects and migraine patients tend to have vitamin B9 deficiencies but not vitamin B12 deficiency. Serum vitamin B9 and B12 levels in this study were similar with those reported in other studies

suggesting that vitamin B9 deficiency occurs among healthy Malaysians[19,39,40]. This could be attributed to various eating habits among Malaysians.

Serum levels of vitamin B9 and B12 among healthy Malaysian population with MTHFR 677C>T genetic polymorphism in this study are similar with those reported by Chew *et al.*[39] but were lower when compared to another study by Liew *et al.*[41]. This can be attributed to the lower sample size (n = 41) and recruitment of both genders in the study conducted by Liew *et al.*[41] compared with the present study.

Serum vitamins B6, B9 and B12 levels in the present study are similar with those reported[42] among Australian migraine patients (n = 52) having the MTHFR 677C>T genetic polymorphism but were lower when compared with another similar type of study (n = 206) conducted in Australia[43]. Higher levels of vitamin B6 seen among Australian migraine patients can be attributed to the higher sample size (n = 206) and possibly also their different dietary habits which generally consists of high proteins diet like meat which tend to contain higher levels of B vitamins.

Association of ESR1 and estradiol levels: This study excluded menstruating migraine patients in order to specifically investigate the role of estrogen and its association with genetic polymorphism among migraine patients. The findings from present study clearly indicated that serum estradiol levels were slightly higher among migraine patients when compared to healthy subjects although this difference was not statistically significant. Moreover, serum estradiol levels were higher among migraine patients having the ESR1 325C>G genetic polymorphism compared to migraine patients not harboring the polymorphism. On the other hand, estradiol levels were lower among migraine patients having the ESR1 594G>A genetic polymorphism when compared to migraine patients without the said polymorphism.

To date, no study reported on estrogen levels either among Malaysian healthy subjects or migraine patients. To author knowledge, our study is the first to report on estrogen level and to establish its association with genetic polymorphism of *ESR1* gene among migraine patients. The studies conducted among Caucasian menstruating migraine patients reported that percutaneous estradiol reduced migraine attacks by 66% in comparison to a placebo group[44-47]. It is also reported that withdrawal of estradiol tend to trigger migraine attack indicating that estrogen has significant role in menstruating migraine patients but not among non-menstruating migraine patients as similarly reported in this study.

Association of TNF-α gene and TNF-α levels: In the present study, serum TNF-α level was significantly higher among migraine patients compared to healthy subjects. In addition, serum TNF-α level among migraine patients was much higher than the healthy cut-off range indicating the presence of higher concentrations of inflammatory mediators. To date, no study reported on serum TNF-α level among Malaysian healthy subjects and migraine patients.

Similarly, the findings from studies conducted among Italian migraine patients (n = 22) and Turkish migraine patients (n=64) also reported elevation of TNF-α[48,49]. To date, no study has reported on the association between TNF-α levels and genetic polymorphism of TNF-α-308G>A. In the present study, migraine patients with TNF-α-308G>A genetic polymorphism showed significantly higher TNF-α levels compared to migraine patients without genetic polymorphism and also to that of the healthy subjects with mutations. This indicates the important role played by TNF-α-308G>A which contributed to the higher levels of TNF-α and thus causing inflammation among migraine patients. It was already reported that genetic polymorphism in TNF-α (-308G>A) have been confirmed to increase the production of TNF-α *in vitro*.

Association of TNF-α gene and vitamin B6: To date, no study has established the association of vitamin B6 with inflammation among migraine patients. Markers of immune activation and inflammation have previously been associated with hyperhomocysteinemia (homocysteine levels >15 μmol L^{-1})[50]. However, lower vitamin B6 levels increase the risk for cardiovascular diseases through inflammation which is independent of homocysteine lowering mechanisms[51-53]. It is plausible that the significantly inverse correlation between vitamin B6 levels and inflammatory markers may explain the relationship between vitamin B6 levels with inflammation. In this study, vitamin B6 and TNF-α levels were significantly correlated with each other among migraine patients indicating the potential role of lower vitamin B6 levels and inflammation in the pathogenesis of migraine.

Vitamin B6 levels were lower in migraine patients having TNF-α-308G>A genetic polymorphism compared to those without the polymorphism although this difference was not statistically significant. Present study findings indicate that generally, vitamin B6 levels were significantly lower among migraine patients and the low TNF-α levels were significantly correlated with TNF-α-308G>A genetic polymorphism. The

TNF-α levels were significantly higher among migraine patients when compared with healthy subjects indicating the potential role of vitamin B6 and TNF-α in migraine pathogenesis among Malay female subjects.

However, there were some limitations in this study. The sample was limited only to female Malay subjects because neither male patients nor patients from other races (Chinese, Indian and Siamese) were registered at the clinic during the study period. The higher number of female migraineurs seen was consistent with the already published report[54], which reported a higher prevalence of female than male migraine sufferers (i.e. three times higher)[55]. The sample was restricted to Malay patients because the majority of Kelantanese are Malay with other races such as the Chinese and Indians constituting a lower proportion of the population in Kelantan. Migraine is a genetically associated disease[56] and there is a possibility that genetic differences may modify the outcome among migraine patients. Measurement of inflammatory biomarkers in cerebrospinal fluid which was not conducted in this study may be more accurate to associate inflammation with migraine. In addition, the expression and function of genes can alter when there is epigenetic modification. Further studies on epigenetics in association with biomarker levels can give better understanding of migraine among migraine patients.

SIGNIFICANCE STATEMENT

The present study is the first study to investigate the biomarkers of migraine and their association with genetic polymorphisms in a Malaysian population and found that genetic polymorphism in TNF-α, ESR1 325C>G and ESR1 594G>A is a potential predictive biomarker of migraine in a Malaysian population. The study also found an association of vitamin B6 with inflammation among migraine patients. This study would help the researchers in determining the detail mechanism of genes and vitamin B6 association with migraine. Thus, best theory on it may be arrived at.

CONCLUSION

It is concluded that the mutant alleles of TNF-α-308G>A, ESR1 325C>G and ESR1 594 G>A but not the MTHFR 677C>T allele significantly predispose patients to higher risk of migraine, In addition, serum levels of TNF-α and vitamin B6 are associated with TNF-α-308G>A polymorphism among Malay female subjects.

ACKNOWLEDGMENTS

We acknowledge the financial support from a Research Universiti grant (1001/PPSP/815073). The authors acknowledge the USM Global Fellowship and Tertiary Education Trust Fund (TETFUND) awards.

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