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Association of two variants C677T and A1298C MTHFR gene polymorphisms with ischemic heart, ischemic stroke and venous thromboembolism diseases in a sample of Arabic Iraqi patients

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ABSTRACT

Background: There are genetic polymorphisms mostly single amino acid substitution occurred in the MTHFR gene, mutant type of this gene inhibit the production of MTHFR enzyme, it may result in excess homocysteine level in plasma which is thought to be the susceptibility of occlusive vascular diseases.

About 109 mutations in MTHFR have been described as causing severe MTHFR deficiency. The most common MTHFR gene polymorphism can be detected as two variants; C677T and A1298C single nucleotide polymorphism (SNP) that are relatively common in many populations worldwide.

Objective: For investigation of the association of two variants C677T and A1298C MTHFR gene polymorphisms with Ischemic heart, ischemic stroke and venous thromboembolism diseases.

Subjects and method: This study was a prospective case-control trial included 58 Arabic Iraqi patients (43 males and 15 females) aged between 20-64 years suffered from different cardiovascular and thromboembolic disorders. Whereas, control group included 52 subjects composed of 41 males and 11 females without any thrombotic event history.

The detection of MTHFR genetic polymorphism (wild, heterozygous, homozygous) by using real time polymerase chain reaction technique (quantitative real time PCR) for two specific variants of MTHFR (C677T and A1298C) as single nucleotide polymorphism to assess allele frequency or genotype distribution (CC,TT,CT and AA,CC, AC respectively).

Result: The wild type of A1298C MTHFR gene (AA) in patients group was 37.93% versus 59.62% in control group with no significant difference (p=0.054). The frequency of the heterozygous genotype (AC) was higher in patients group (39.66%) than control group (30.77%) with a signficant difference (OR= 3.66, 95%CI= 1.14-11.77, p =0.027). This association seems to be in recessive model as the frequency of AC+CC in patients was 62.07% compared with 40.38% in controls (OR= 2.42, 95% CI= 1.12-5.2, p= 0.024).

The mutant homozygous genotype (CC) was slightly more frequent among patients group than control group (22.41% vs. 9.61%) with no significant difference.

At allelic level, the frequency of C allele in patients group was higher than those with control group (42.24% vs. 25%) with a highly significant difference (OR= 2.19, 95%CI= 1.23-3.91, p= 0.008).

Conclusion: Interestingly, patients those had carried heterozygous MTHFR A1298C genotype AC were 3.5 fold risk of exposure to ischemic heart disease, ischemic stroke and venous thromboembolism when compare with those carrying wild AA genotyping of the same SNP.

Multivariate analysis between patients group and control group in the presence of A1298C MTHFR gene polymorphism appears that heterozygous MTHFR A1298C genotype AC is considered as independent risk factor for association of ischemic heart disease, ischemic stroke and venous thromboembolism.

Keywords: *MTHFR gene polymorphism, two variants (C677T and A1298C), independent risk factor*

INTRODUCTION

The human MTHFR gene is located on chromosome 1 at position 36.3, currently, it describes as containing 12exon whereas the first exon is noncoding (1). There are genetic polymorphisms mostly single amino acid substitution occurred in the MTHFR gene, mutant type of this gene inhibit the production of MTHFR enzyme, it may result in excess homocysteine level in plasma which is thought to be the susceptibility of health problems such as occlusive vascular diseases, neural tube defect and colon cancer (1).

About 109 mutations in MTHFR have been described as causing severe MTHFR deficiency. The most common MTHFR gene polymorphism can be detected as two variants; C677T and A1298C single nucleotide polymorphism (SNP) that are relatively common in many populations worldwide. In the C677T variant, nucleotide replaces cytosine with thymine at position 677 with exon 4 of the MTHFR gene whereas A1298C variant nucleotide replaces adenine with cytosine at position 1298 within exon 7 of the MTHFR gene (2).

These polymorphisms alter MTHFR enzyme structure lead to the formation of unstable and heat sensitive enzyme and reduce its activity at high temperature 37C° resulting in elevated levels of homocysteine in blood (2).

It has been demonstrated that the heterozygous polymorphism of C677T and A1298C, along with folate deficiency, lead to increased levels of homocysteine. Whereas, patients carrying C677T polymorphism of MTHFR in homozygous form TT leading to thermolabile MTHFR variant in addition of exhibition decreased formation of mrthyltetrahydrofolate under folate deficiency condition. All of these factors lead to lower production of S-adenosyl methionine and subsequently lower availability of methyl groups that is required for methylation events of DNA (3).

From the other side, decrease availability of methyl tetrahydrofolate results in reduced remethylation of homocysteine, followed by an increased in plasmatic homocysteine.

Conversely, wild type individuals for the C677T genotype (CC) are not affected by folate deficiency, and thus the conversion of homocysteine to methionine is preserved due to availability of methyltetrahydrofolate under adequate supply of folate that is required for the methylation reaction (4).

Many studies have associated MTHFR C677T and A1298C polymorphisms with an increased susceptibility to acute coronary syndrome (5-8). Another recent Comprehensive meta-analysis study includes 47 studies that concluded possible impact of C677T and A1298C polymorphisms on myocardial infarction risk (9).

Stroke is considered as the second common cause of mortality worldwide and is a major public health problem. The genetic factors can contribute to stroke pathogenesis that is supported by the association of specific gene variants and environmental factors. Several studies show indicated MTHFR C677T polymorphism as a potential risk factor for stroke in different ethnicities (10-12). Other study evaluated the effect of polymorphism MTHFR C677T on the risk for attack of ischemic stroke in Indian population and found a significant of high frequency of MTHFR T allele in the patients with ischemic stroke compared to the healthy subjects (10).

Mutations in genes encoding enzymes such as methylene tetrahydrofolate reductase for two variants C677T, A1298C and methionine synthase reductase A66G may lead to the imbalance between vascular relaxing and vascular contraction factor, and then contribute to the risk of developing hyperhomocysteinemia resulting in venous thromboembolism (VTE) (13). Venous thromboembolism can be treated by oral anticoagulants with follow up from expert pharmacist (14).

SUBJECTS AND METHOD

Study design

This study was a prospective case-control trial included 58 Iraqi patients (43 males and 15 females) aged between 20-64 years suffered from different cardiovascular and thromboembolic disorders at different wards within multi-centers in Ibn Al- Bitar Specialized center for cardiac surgery, Al-Imamein Al-Kadhimaein medical city and Dr. Saad Al-Watri hospital for neurosciences; this study was performed from September 2020 to September 2021.

Baseline demographic characteristic features of the enrolled patients (gender, age, weight, smoking and alcohol drinking status, diseases history as well as recurrent thrombosis, medications history, diagnosis for admission) were recorded. Moreover, genetic polymorphism of MTHFR for two specific variants C677T and A1298C were accomplished in the pioneer molecular pathology laboratory. This study was started after taking approval from scientific committee in the college of pharmacy/ university of Mustansiriyah. After that, approval from scientific and administrative committee in the ministry of health was taken. All participants in this study would sign a written consent. The ethical approval was given by each patient participated in the current clinical study.

The participants were divided into two groups as follow:

Patients group; included 58 patients and subdivided into three subgroups as follow below:

A- Ischemic heart disease group which include 27 patients (23 males and 4 females) have been diagnosed for ischemic artery disease after cardio angiography and with admission to cardio care unit as myocardial infarction.

B-Ischemic stroke group which include 17 patients (11 males and 6 females) have been diagnosed for ischemic stroke by computed tomography of brain for different types such as transient, acute or multiple ischemic stroke.

C-Venous thromboembolism group which include 14 patients (9 males and 5 females) suffered from deep vein thrombosis of lower limbs (right, left or bilateral), pulmonary embolism or both that was diagnosed by Doppler ultrasound.

2- Control group which included 52 subjects composed of 41 males and 11 females without any thrombotic event history.

Inclusion criteria

Patients with ischemic heart disease as well as myocardial infarction. Those patients were diagnosed under supervision of cardiologist subspecialty.

Transient ischemic attack (TIA) or ischemic stroke patients under supervision of neurologist subspecialty.

Patients with venous thromboembolism (deep vein thrombosis and pulmonary embolism) under supervision of cardiologist subspecialty.

Exclusion criteria

Any condition that may interfere with the study protocol or aim will be excluded in

Patients with venous thromboembolisms disease with history of risk factors of previous surgical procedure, bone fracture and malignancy.

Patients with chronic renal failure.

Patients of Alzheimer or Parkinson disease.

Materials

Bosphore thrombophilia detection kit composed of Real-Time polymerase chain reaction (PCR) reagents for Anatolia Genework manufacturer of Turkish origin as well as Real time PCR (Agilent manufacturer) were used in this study.

Method

EDTA anti-coagulated blood (2ml sample) samples were obtained from participants, the genetic analysis for MTHFR polymorphism was detected by using whole blood. Detection of MTHFR genetic polymorphism (wild, heterozygous, homozygous) by using real time polymerase chain reaction technique (quantitative real time PCR) for two specific variants of MTHFR (C677T and A1298C) as single nucleotide polymorphism to assess allele frequency or genotype distribution (CC,TT,CT and AA,CC, AC respectively).

Polymerase chain reaction PCR Master Mix MTHFR for both C677T and A1298C contain a highly specific and accurate Taq DNA polymerase (with hot-start property), PCR buffer and the deoxyribonucleotide triphosphate (dNTP) mix. They are also contain forward and reverse primers and dual-labeled probes specific for wild type and mutant alleles MTHFR C677T and MTHFR A1298C respectively.

A) Detection of single nucleotide polymorphism (SNPs)

It was demonstrated the successful detection of SNPs in untreated whole blood and serum by using of real-time PCR assay as applied to the MTHFR C677T, MTHFR A1298C polymorphism (15-16). The primers and probes for genotyping of SNPs to MTHFR are show in table 1 (16).

TABLE 1: primers and probe for genotyping of SNPs to MTHFR

Gene and genotype	Primer/probe	Reporter	Sequences
MTHFR	primer forward primer reverse		TGACCTGAAGCACTTGAAGGAGAA GGAAGAATGTGTCAGCCTCAAAGA
C677T	probe C probe T	FAM TET	ATGAAATCGGCTCCCG ATGAAATCGACTCCCG
MTHFR	primer forward primer reverse		GGAGGAGCTGCTGAAGATGTG TCTCCCGAGAGGTAAAGAACAAA
A1298C	probe A	FAM	AAAGACACTTTCTTCACTG
	probe c	TET	AGACACTTGCTTCACTG

FAM is 6-carboxyfluorescein whereas TET is 6-carboxy-4,7,29,79 tetra-chloro- fluorescein. The variant base in underlined. Sequence is 5' to 3'

B- Principle assay of Bosphore thrombophilia detection kit

This test utilizes the 5' exonuclease activity of Taq polymerase to cleave a dual-labeled fluorescent hydrolysis probe during the extension phase. The probe is labeled at the 5' end with a fluorescent reporter, and at the 3' end with another fluorescent molecule that acts as a quencher for the reporter. When the two fluorophores are in close proximity, even if the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step, Taq polymerase encounters and cleaves the probe bound to template. As the reporter is relieved from the suppressing effect of the quencher, fluorescence signal can be detected.

As the PCR product accumulates, the fluorescence generated by the reporter increases, the point at which the signal rises above background level and becomes detectable, is called the threshold cycle (CT).

In the tube MTHFR DNA, whether wild type or polymorphic, is amplified in a single reaction, using sequence-specific primers against mutant and wild type alleles. The fluorescent signal generated by the MTHFR polymorphism is detected by a probe labeled at the 3' end with FAM, through the FAM channel.

In contrast, the fluorescent signal generated by the wild type allele amplification, is detected by a second probe (labeled at the 5' end with a different reporter molecules, HEX) through the HEX channel.

C- Preparing the samples

The whole blood samples are incubated with EX-Tract DNA solution found in the kit in order to be prepared for PCR set-up. The thermal block/mixer is adjusted to 80°C. After that, 10 microliter of whole blood sample is added on 65 microliter of EX-Tract DNA solution and mixed. Then, the mixtures are incubated at 80°C for 20 minutes. After incubation, 20µ liter of sample is mixed with 30 microliter of deionized water.

D- Preparing the PCR

15 µ liter of the master mix into the PCR tubes or strips was added to 5 µ liter of DNA sample (positive or negative control). Then, the tube cap was closed and make sure that the solution in each tube is at the bottom of the tube. The prepared sample was centrifuged if necessary.

E- Programming the Real-Time PCR instrument

Before starting a real-Time PCR reaction using the bosphore kit, the following steps taken into consideration and should be completed: all filters (FAM and HEX) were chosen with identification of unknown samples. The correct thermal protocol was selected

The thermal protocol for Bosphore thrombophilia detection kit is composed of an initial denaturation for activation of Taq DNA polymerase (with hot start property); a second step is amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle. Timing of thermal protocol can be demonstrated as below table 2.

TABLE 2: Steps and timing of thermal protocol of PCR

Steps	Temperature	Time
Initial denaturation	95°C	14.5 minutes
Denaturation	95°C	30 seconds for 45 cycles
Annealing and synthesis	64°C	1 minute for 45 cycles
Hold	32°C	1 minute

F- Analysis and interpretation

After the end of the thermal protocol, the Real-Time PCR instrument software automatically calculated the baseline cycles and the threshold.

Samples that cross the threshold in all channels are displayed as positive, for mutant and wild type alleles respectively. Following table 3 the possible results and their interpretation.

TABLE 3: result of MTHFR gene polymorphism and their interpretation

PCR Master Mix MTHFR C677T	MTHFR C677T mutant (FAM)	MTHFR C677T wild type (HEX)	Interpretation
	+	+	Sample is heterozygous mutant for MTHFR C677T
	+	-	Sample is homozygous mutant for MTHFR C677T
	-	+	Sample is wild type for MTHFR C677T
	-	-	Inconclusive, test should be repeated
PCR Master Mix MTHFR A1298C	MTHFR A1298C mutant (FAM)	MTHFR A1298C wild type (HEX)	Interpretation
	+	+	Sample is heterozygous mutant for MTHFR A1298C
	+	-	Sample is homozygous mutant for MTHFR A1298C
	-	+	Sample is wild type for MTHFR A1298C
	-	-	Inconclusive, test should be repeated

Statistical analysis

Statistical analyses were performed by using SPSS software version 25.0 (SPSS, Chicago). Continuous data were subjected to normality test (Shapiro Wilk test). Data with normally distribution were presented as mean and standard deviation, and analyzed with Student t-test as well as ANOVA test.

Data with non-normal distribution were presented as median and range which analyzed with Mann Whitney U test for two groups' comparison. Categorical variables were expressed as number and percentage, and analyzed with Chi-square test.

Hardy-Weinberg Equilibrium (HWE) is a mathematical equation that can be used for calculation the genetic variation of population at equilibrium. The deviation of different genotypes from Hardy-Weinberg Equilibrium was calculated online using https://www.easycalculation.com/health/hardy-weinberg-equilibrium-calculator.php websites. If there is no deviation between observed genotype frequencies in a population with the frequencies predicted by the equation, the genotype frequencies in a population are in a good accordance with HWE (17).

Multivariate analysis was done to find the mode and correlation between several variables at once by using binary logistic regression which based on a set of independent variable (17).

The association of different genotypes and alleles frequency with the diseases was evaluated through the calculation of odd ratio (OR) and their corresponding 95% confidence intervals (CI) using binary logistic regression. Odd ratio illustrates and demonstrates the risks given by genetic mutations. A p-value less than 0.05 were considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Patients' demographics and disease characteristics

One hundred-ten 110 Subjects enrolled to represent the sample size of this study including 84 male (76.4%), and 26 female (23.6%). Those subjects were then allocated into two groups: the first group was 58 patients group (including 43 male patients (74.14%) and 15 female patients (25.86%)), while the second group was 52 control group that including 41 male (78.85%) and 11 female (21.15%).

The mean age of the patients was (45.17±11.62 years) which didn't differ significantly from that of control group (44.31±17.81 years). The mean body mass index (BMI) of all patients was (28.86±5.55) Kg/m² whereas the mean BMI of control group was (28.34±4.28) Kg/m². After comparing of matching process for the variables mainly (gender, age and body mass index) between patients group and control group showed no significant differences ($p > 0.05$).

However, about three-fourths of the patients were ex/current smokers compared with 34.62% of control group with a significant difference. Furthermore, the median cigarette/day in patients group and control group were 40 and 20, respectively with a highly significant difference. Alcohol consumption was more common among patients group than control group (6.9% vs. 0%); however, the difference was not significant ($p > 0.05$).

Interestingly, the comorbidity/risk factors, in general, were far more frequent in patients group than control group (75.86% vs. 26.91%) with a highly significant difference.

Hypertensions, diabetes, as well as other risk factors were more common among patients (58.62%, 36.21% and 15.52% respectively) than control group (11.54%, 13.46% and 1.92%, respectively) with significant differences ($p < 0.05$). Table 4 presented the baseline demographic disease characteristics data of all study groups.

TABLE 4: Demographic and disease characteristics of the patients group and control group

Demographic and disease characteristics	Patients group (n=58)	Control group (n=52)	p-value
Gender			
Male	43(74.14%)	41(78.85%)	0.642
Female	15(25.86%)	11(21.15%)	
Age, years			
Mean ±SD	45.17±11.62	44.31±17.81	0.562
Range	20-64	24-64	
BMI, kg/m2			
Mean ±SD	28.86±5.55	28.34±4.28	0.587
Range	19.0-45.7	20.95-39.16	
Smoking			
Never	14(24.14%)	34(65.38%)	0.012*
Ex/current smoker	44(75.86%)	18(34.62%)	
Smoking severity, cig/day			
Mean ±SD	50.4±23.04	24.44±8.56	<0.001*
Median	40	20	
Range	15-100	20-40	
Alcohol consumption			
No	54(93.1%)	52(100%)	0.120
Yes	4(6.9%)	0(0%)	
Comorbidities/ risks			
None	14(24.14%)	38(73.08%)	<0.001*
Diabetes	21(36.21%)	7(13.46%)	<0.001*
Hypertension	34(58.62%)	6(11.54%)	<0.001*
Others#	9(15.52%)	1(1.92%)	0.018*

Other comorbidities/risk factors included three cases of oral contraceptive and dyslipidemia, two cases of pulmonary hypertension and one case of atrial fibrillation among patient group, whereas one case of dyslipidemia in control group

Data presented as number of patients (n) and percentage (%) for categorical variables, or as (mean ±SD) and median for numerical variables.

Independent sample t test for numerical and normally distributed variables, Mann Whitney U test for numerical and non-normally distributed variables, chi- square test for categorical variables.

P value ≥ 0.05 is considered non-significant, P* value < 0.05 is considered significant.

From the results of the current study, Smoking and smoking severity can be considered as important modifiable risk factor which progresses the incidence of ischemic heart disease and ischemic stroke. A very recent large study was included 897975 current smokers aged ≥ 40 years that concluded and approved that smoking cessation, but not reduction, was associated with risk reduction of cardiovascular diseases (18).

Whereas, the other study was demonstrated the effect of smoking in ischemic stroke incidence by using multivariate analysis, which concluded that smoking, was the only significant associated factor in patients with atrial fibrillation and a low-risk CHA2DS2-VASc score, this score is used for stroke risk assessment in atrial fibrillation (19).

Molecular Assays for MTHFR gene

Two single nucleotide polymorphism (SNPs) were investigated for MTHFR gene (C677T and A1298C) of Iraqi participants for their association with the development of ischemic heart disease, ischemic stroke and venous thromboembolism (deep vein thrombosis and pulmonary embolism). The distribution of different genotypes of these SNPs in patients group and control group was found to be in a good accordance with Hardy Weinberg Equilibrium (HWE).

The Hardy-Weinberg Equilibrium (HWE) is an important essential principal of population genetics, which states that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. This equilibrium can be affected by many of factors such as mutations, natural selection, non-random mating, immigration, genetic drift and gene flow. All of these factors lead to introduce of new alleles into populations and then disturb equilibrium of allele frequencies (20).

MTHFR C677T

Allele specific PCR and real time PCR (quantitative PCR) were used for gene amplification and genotyping of this SNP. This SNP had three genotypes in patients group and control group. These were wild type CC, heterozygous CT and homozygous TT.

The wild type of C677T MTHFR gene (CC) in patients group was 58.62% versus 55.77% in control group with no significant difference. In addition the presence of mutant heterozygous genotype (CT) in patient group was 32.76% vs. 40.38% in control group with non-significant differences between them. Interestingly, the mutant homozygous genotype (TT) was slightly more frequent among patients group than control group (8.62% vs. 3.85%) with no significant difference. Neither dominant nor recessive model showed a significant association with the disease.

Analysis of allele distribution revealed very close frequency of T allele among patients group and control group (25% versus 24.04%) with no significant difference (table 5)

TABLE 5: The frequency of different genotypes and allele of C677T polymorphism in patients group and control group

C677T	Patients group (n=58)	Control group (n=52)	P-value	OR(95%CI)
Genotypes				
CC	34(58.62%)	29(55.77%)	0.495	1.0
CT	19(32.76%)	21(40.38%)	0.386	2.13(0.38-11.82)
TT	5(8.62%)	2(3.85%)	0.256	2.76(0.48-15.95)
HWE	0.335	0.445		
Dominant model				
CC+CT	53(91.38%)	50(96.15%)	0.318	1.0
TT	5(8.62%)	2(3.85%)		2.36(0.44-12.71)
Recessive model				
CC	34(58.62%)	29(55.77%)	0.763	1.0
CT+TT	24(41.38%)	23(44.23%)		0.89(0.42-1.9)
Alleles				
C	87(75%)	79(75.96%)	0.869	1.0
T	29(25%)	25(24.04%)		1.05(0.57-1.95)

Data presented as number of patients (n) and percentage (%) for categorical variables.

Binary logistic regression for categorical variables.

P value ≥ 0.05 is considered non-significant, P* value < 0.05 is considered significant.

This current finding is accordance with another study by Prasanna (21) that concluded there was no significant difference between control group and patients group with ischemic heart disease in the distribution of MTHFR gene C677T genotyping and allele frequency.

Previous systemic review and update meta-analysis discussed the association of MTHFR C677T gene polymorphism with ischemic heart disease; this review included 123 studies with 87020 subjects of different ethnicity (Asian, African, Caucasian and other ethnic populations). This review concluded that the variant T allele of the C677T polymorphism was associated with increased risk of ischemic heart disease.

Furthermore, subgroups analyses of the subjects' ethnicity were performed and the results showed that the MTHFR C677T gene polymorphism was associated with ischemic heart disease in Asian population, but not in Africans whereas, the association between MTHFR C677T gene polymorphism and ischemic heart disease was obscure (22).

Another previous meta-analysis study in 2016 was demonstrated that the MTHFR C677T gene polymorphism and coronary heart disease risk in the Chinese population which included 33 studies and involved 6130 case of ischemic heart disease compared with 6163 control cases. Overall, this study revealed significant association was found between C677T gene polymorphism and ischemic heart disease with odd ratio (OR) and ranged (1.41-1.88) (23).

Meta-analysis of observational studies discussed that association of MTHFR variant genotyping with increased risk and morbidity of ischemic stroke in the elderly population; this study showed that C677T MTHFR genotyping polymorphism may contribute for elevation risk of ischemic stroke in elderly population as well as the association of T-allele to increase risk of ischemic stroke. Other finding concluded that the Chinese populations were appearing more susceptible for C677T MTHFR genotyping mutation (24).

However, the role of C677T MTHFR in ischemic stroke remains unclear. Meta-analysis of existing published studies that included 65 studies involving 12390 patients group compared with 16275 control group for evaluation the overall risk of mutation in the etiology of ischemic stroke. This meta-analysis study showed that odd ratio was 1.306 and 30% higher risk of Ischemic stroke in the presence of C667T MTHFR (25).

According to deep venous thrombosis, recent Turkish study 2019 by Sefa Senal that showed and concluded the MTHFR C677T gene polymorphism and alleles frequency was not associated between patients with deep vein thrombosis and control group in Turkish populations with no significant difference. This study involved 64 patients group and 64 control group; the prevalence of C677T MTHFR genotyping polymorphism in patients group was distributed as wild CC (62.5%), heterozygous CT mutant (23.4%) as well as homozygous TT mutant (14%) (26), all of these results is accordance with results of C677T MTHFR genotyping polymorphism in the present study of Iraqi participants.

Other Turkish study demonstrated that there was no significant impact on pulmonary embolism by single mutation of MTHFR (27). Another recent study in 2020 revealed that there was no significant difference in C677T MTHFR gene with venous thromboembolism (% of mutational in patients group was 57% versus 48.2% in control group) (28).

Netherland study by Irene D. concluded that C677T MTHFR genotyping polymorphism was not associated with risk of venous thrombosis in the single large study which involved 4375 of patient cases and 4856 of control cases (29). Recent meta-analysis study in 2020 discussed the relationship between C677T MTHFR with venous thromboembolism in the Caucasian and Asian populations; this study explored that C677T MTHFR genotyping mutation could increase the risk of venous thromboembolism in the Asian populations but not in the Caucasians (30).

MTHFR A1298C

Allele specific PCR as well as real time PCR (quantitative PCR) were used for gene amplification and genotyping of this SNP. This SNP had three genotypes patients group and control group. These were wild type AA, heterozygous AC and homozygous CC. The frequency of different genotypes and allele of this SNP in patients group and control group is shown in table 6.

TABLE 6: The frequency of different genotypes and allele of A1298C polymorphism patients group and control group

A1298C	Patients group (n=58)	Control group (n=52)	P-value	OR(95%CI)
Genotypes				
AA	22(37.93%)	31(59.62%)	0.054	1.0
AC	23(39.66%)	16(30.77%)	0.029	3.66(1.14-11.77)
CC	13(22.41%)	5(9.61%)	0.338	1.81(0.54-6.08)
HWE	0.154	0.195		
Dominant model				
AA+AC	45(77.59%)	47(90.38%)	0.078	1.0
CC	13(22.41%)	5(9.61%)		2.72(0.89-8.23)
Recessive model				
AA	22(37.93%)	31(59.62%)	0.024*	2.42(1.12-5.2)
AC+CC	36(62.07%)	21(40.38%)		
Alleles				
A	67(57.76%)	78(75%)	0.008*	1.0
C	49(42.24%)	26(25%)		2.19(1.23-3.91)

Data presented as number of patients (n) and percentage (%) for categorical variables.

Binary logistic regression for categorical variables.

P value ≥ 0.05 is considered non-significant, P* value < 0.05 is considered significant.

The wild type of A1298C MTHFR gene (AA) in patients group was 37.93% versus 59.62% in control group with no significant difference (p=0.054). The frequency of the heterozygous genotype (AC) was higher in patients group (39.66%) than control group (30.77%) with a signficant difference (OR= 3.66, 95%CI= 1.14-11.77, p =0.027). This association seems to be in recessive model as the frequency of AC+CC in patients was 62.07% compared with 40.38% in controls (OR= 2.42, 95% CI= 1.12-5.2, p= 0.024).

The mutant homozygous genotype (CC) was slightly more frequent among patients group than control group (22.41% vs. 9.61%) with no significant difference.

At allelic level, the frequency of C allele in patients group was higher than those with control group (42.24% vs. 25%) with a highly significant difference (OR= 2.19, 95%CI= 1.23-3.91, p= 0.008).

Very recent Iranian study demonstrated the relationship of MTHFR A1298C gene polymorphism, homocysteine level, vitamin B12 and folate levels with coronary artery disease in the Iranian populations; this study concluded that MTHFR A1298C gene polymorphism was associated with coronary artery disease (31). Other study discussed impact of genetic defect on coronary heart failure among Turkish Cypriot patients; which concluded that those patients with atherosclerosis can be more affected by modifiable risk factors such as hypertension, diabetes, obesity, and smoking as well as genetic factors that can be responsible for coronary heart diseases (32).

Interestingly, very recent Iraqi study in 2020 was done in northern populations; this study focused on genetic polymorphism in early onset myocardial infarction in a sample of Iraqi patients without history of ischemic heart disease and ischemic stroke. Nine genetic polymorphisms and allele frequency were screened among patients group and control group, one of those polymorphisms was MTHFR gene polymorphism for two variant C677T and A1298C.

The distribution of genetic polymorphism of MTHFR C677T in patients group was (54.9%, 32.35% and 12.75%) for wild, heterozygous and homozygous respectively. While, the distribution of MTHFR A1298C genetic polymorphism in patients group was (33.34%, 47.06% and 19.6%) for wild, heterozygous and homozygous respectively (33)

Meta-analysis systemic review demonstrated the effect of MTHFR A1298C gene polymorphism on homocysteine levels in the Chinese populations; this analysis included 13 studies and reported that MTHFR A1298C did not effect on homocysteine level (34).

For ischemic stroke, very recent meta-analysis estimated the potential relationship between MTHFR A1298 gene polymorphism and ischemic stroke susceptibility; this review involved 5725 cases versus 8655 controls in 40 studies, these results indicated the polymorphism in MTHFR A1298C gene has significant association with increase susceptibility of ischemic stroke under the C allelic genetic model. Additionally, in ethnic subgroup analysis MTHFR A1298C gene polymorphism was correlated with stroke risk of Asian populations, whereas there was no significant correlation among MTHFR A1298C gene polymorphism and increase risk of ischemic stroke in Caucasian and African populations (35).

Oppositely, other Italian study showed that the allele's frequencies of MTHFR C677T were significantly higher in ischemic stroke patients than normal subjects, whereas there were no significant differences in MTHFR A1298C between patients of ischemic stroke group and control group (36). Other Chinese study concluded that the MTHFR A1298C gene polymorphism is negatively associated with ischemic stroke (37). Furthermore, another study in southern Iran 2019, this study showed that MTHFR C677T genotype was more frequent in patients with ischemic stroke and MTHFR A1298C was less frequent in younger ischemic stroke patients' (38).

The two variants of MTHFR gene have been associated with range of diseases and the prevalence of MTHFR C677T and A1298C gene polymorphism was studied previously in United States primary care populations as ethno-geographic status that concluded the mutation for both variants of MTHFR gene was rare as well as homozygous mutation for double variants of gene TT/CC was absent (39).

Multivariate analysis between patients group and control group in presence of A1298C MTHFR gene polymorphism

Multivariate analysis is complex technique that allows more than two variables to be analyzed at once. There are two types of multivariate analysis techniques: analysis of dependence and analysis of interdependence. The selection of technique is depending on type of data and reason for the analysis (40).

To find out if the different genotypes of A1298C are independent risk factor for patients with hyper-coagulation disorders (Ischemic heart disease and myocardial infarction, Ischemic stroke and venous thromboembolism), multivariate analysis was performed. In this analysis, factors which a significant association with the disease in univariate analysis was entered the model. The results are shown in table 7.

TABLE 7: Multivariate analysis between patients group and control group in presence of A1298C MTHFR gene polymorphism

Variables	Patients group (n=58)	Control group (n=52)	P-value	OR(95%CI)
Comorbidities				1.0
No	14(24.14%)	38(73.08%)	<0.001*	5.0(1.31-19.62)
Yes	44(75.86%)	14(26.92%)		
HTN				1.0
No	24(41.40%)	46(88.46%)	0.019*	8.34(3.48-20.09)
Yes	34(58.62%)	6(11.54%)		
DM				1.0
No	37(63.79%)	45(86.54%)	0.006*	4.04(1.48-11.05)
Yes	21(36.21%)	7(13.46%)		
Others				
No	49(84.48%)	51(98.08%)	0.013*	1.0
Yes	9(15.52%)	1(1.92%)		15.29(1.76-132.44)
Smoking				
No	14(24.14%)	34(65.38%)	0.017*	1.0
Yes	44(75.86%)	18(34.62%)		2.77(1.19-6.39)
A1298C				
AA	22(37.93%)	31(59.62%)	0.091	1.0
AC	23(39.66%)	16(30.77%)	0.038*	3.54(1.07-11.71)
CC	13(22.41%)	5(9.61%)	0.277	2.0(0.57-6.96)

Data presented as number of patients (n) and percentage (%) for categorical variables.

Binary logistic regression for categorical variables.

P value ≥ 0.05 is considered non-significant, P* value < 0.05 is considered significant.

There are modifiable risk factors for ischemic heart disease and ischemic stroke. These include: smoking, obesity, dyslipidemia, hypertension and diabetes mellitus (40). All of these factors are took into consideration and demonstrated in this study.

The heterozygous genotype of the SNP (AC) remained significantly associated with the ischemic heart disease, ischemic stroke and venous thromboembolism disease (OR=3.54, 95%CI=1.07-11.71, p=0.038). Interestingly, comorbidity, in general (OR=5.0, 95%CI=1.31-19.62, p<0.001), hypertension (OR=8.34, 95%CI=3.48-20.09, p=0.019), diabetes (OR=4.04, 95%CI=1.48-11.05, p=0.006), other risk factors (OR=15.29, 95%CI=1.76-132-44, p=0.013) and

smoking (OR=2.77, 95%CI=1.19-6.39, p=0.017) are independent risk factors or consequences in patients with ischemic heart disease, ischemic stroke and venous thromboembolism.

Interestingly, patients those had carried heterozygous MTHFR A1298C genotype AC were 3.5 fold risk of exposure to ischemic heart disease, ischemic stroke and venous thromboembolism when compare with those carrying wild AA genotyping of the same SNP, with statistically significant difference (p=0.038). From the result, Multivariate analysis between patients group and control group in the presence of A1298C MTHFR gene polymorphism appears that heterozygous MTHFR A1298C genotype AC is considered as independent risk factor for association of ischemic heart disease, ischemic stroke and venous thromboembolism.

Amelia K study in 2017 demonstrated that there was combination of modifiable risk factors (hypertension, diabetes mellitus, obesity and smoking) with genetic factors for increasing ischemic stroke and cardiovascular disease incidence (41). Other previous study showed that modifiable risk factors (high blood pressure, high blood glucose level, high cholesterol and triglyceride level, smoking, diet and physical inactivity) in adults were highlighted in general population with prior coronary heart disease and ischemic stroke (42).

CONCLUSION

Interestingly, patients those had carried heterozygous MTHFR A1298C genotype AC were 3.5 fold risk of exposure to ischemic heart disease, ischemic stroke and venous thromboembolism when compare with those carrying wild AA genotyping of the same SNP.

Multivariate analysis between patients group and control group in the presence of A1298C MTHFR gene polymorphism appears that heterozygous MTHFR A1298C genotype AC is considered as independent risk factor for association of ischemic heart disease, ischemic stroke and venous thromboembolism.

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REFERENCES

1. Kavrakova B, Krstevska M, et al. Hyperhomocysteinemia and of methylenetetrahydrofolate reductase (C677T) genetic polymorphism in patients with deep vein thrombosis. Mater Sociomed. 2013; 25(3): 170-174.
2. Sibani S, Christensen B, Characterization of six novel mutations in the methylenetetrahydrofolate reductase (MTHFR) gene in patients with homocystinuria. Hum mutat. 2000; 15(3): 280-7.
3. Dell'edera D, Tinelli A, et al. Effect of multivitamins on plasma homocysteine in patients with the 5, 10 methylenetetrahydrofolate reductase C677T homozygous state. Mol Med Rep. 2013; (8): 609-612.
4. Martinez-Serra J, Robles J, et al. Fluorescence resonance energy transfer-based real-time polymerase chain reaction method without DNA extraction for the genotyping of F5, F2, F12, MTHFR, and HFE. J Blood Med. 2014; (5): 99-106.
5. Kozieradzka A, Pepinski W, et al.The rs1801133 polymorphism of methylenetetrahydrofolate reductase gene- the association with 5-year survival in patients with ST-elevation myocardial infarction. Adv Med Sci. 2012; 57:106-111.
6. Angeline T, Jeyaraj N, Tsongalis GJ. MTHFR gene polymorphisms, B-vitamins and hyperhomocystinemia in young and middle-aged acute myocardial infarction patients. Exp Mol Pathol. 2007; 82:227-233.
7. Zee RY, Mora S, Cheng S, et al. Homocysteine, 5,10-methylenetetrahydrofolate reductase 677C>T polymorphism, nutrient intake, and incident cardiovascular disease in 24,968 initially healthy women. Clin Chem. 2007; 53:845-851.
8. Kaul S, Zadeh AA, Shah PK. Homocysteine hypothesis for atherothrombotic cardiovascular disease: not validated. J Am Coll Cardiol. 2006; 48:914-923.
9. Alizadeh S, Djafarian K, et al. C667T and A1298C polymorphisms of methylene tetrahydrofolate reductase gene and susceptibility to myocardial infarction: a systematic review and meta-analysis. Int J Cardiol. 2016; 217:99-108.
10. Vijayan M, Chinniah R, Ravi PM, et al. MTHFR (C677T) CT genotype and CT apoE3/3 genotypic combination predisposes the risk of ischemic stroke. Gene, 2016; 591:465-470.
11. Biswas A, Ranjan R, et al. Homocystine levels, polymorphisms and the risk of ischemic stroke in young Asian Indians. J Stroke Cerebrovasc Dis., 2009; 18:103-110.

12. Trabetti E. Homocysteine, MTHFR gene polymorphisms, and cardio-cerebrovascular risk. J Appl Genet. 2008; 49:267-282.
13. Patra, et al. Acute Pulmonary Embolism in Young: A Prospective Observational Study on Clinical Implication of Age on the Presentation and Management of Patients with Acute Pulmonary Embolism Int J Cardiovasc Res 2014, 3:4
14. Al-Mahroos M I, Abdulridha M K, Alhaleem M R. Evaluation of A discharge community- based post-discharge warfarin management protocol on Iraqi patients. IJPSR, 2017; Vol. 8(5): 1000-10.
15. Livak KJ. Allelic discrimination using fluorogenic probes and the 59 nuclease assay. Genet Anal 1999; 14:143-9.
16. Ulvik A, Ueland P. Single Nucleotide Polymorphism (SNP) Genotyping in Unprocessed Whole Blood and Serum by Real-Time PCR Application to SNPs Affecting Homocysteine and Folate Metabolism. Clinical Chemistry.2001; 47 (11): 2050-53.
17. Cathryn M. Lewis. Genetic association studies: Design, analysis and interpretation. Briefings in bioinformatics, 2002; 3(2): 146-153.
18- Su-Min Jeong, Keun Hye Jeon, et al. Smoking cessation, but not reduction, reduces cardiovascular disease incidence. European Heart Journal, 2021; 42(40): 4141-4153.
19- Soonil Kwon, Tae Jung Kim, et al. Predictors of ischemic stroke for low-risk patients with atrial fibrillation: A matched case-control study. Heart Rhythm Society.2021; 18(5):702-708.
20. Nikita Abramovs, Andrew Brass, et al. Hardy-Weinberg Equilibrium in the Large Scale Genomic Sequencing Era. Frontiers in Genetics, 2020; 11:210.
21. Prasanna, P. V. V. S.L, et al. Association of MTHFR gene polymorphisms C677T and A1298C with coronary artery disease. International Journal of Current Research. 2016; 8 (3): 27309-15.
22. Zhi Luo, Zhan Lu, et al. Associations of the MTHFR rs1801133 polymorphism with coronary artery disease and lipid levels: a systematic review and updated meta-analysis. Lipids in Health and Disease. 2018, 17:191.
23. Jiang-Gui Shan, Song Xue. MTHFR C677T polymorphism and coronary artery disease risk in the Chinese population: a meta-analysis based on 33 studies. Int J Clin Exp Med. 2016; 9(2):2822-2830.
24. Guilin Chang, Zheng Kuai, et al. The association of MTHFR C677T variant with increased risk of ischemic stroke in the elderly population: a meta-analysis of observational studies. BMC Geriatrics 2019; 19:331.
25. P A Abhinand, M Manikandan, et al. Meta-analysis study to evaluate the association of MTHFR C677T polymorphism with risk of ischemic stroke. Bioinformation. 2017; 13(6):214-219.
26. Sefa Şenol, Kürşat Kargün. Methylenetetrahydrofolate reductase polymorphisms in acute deep vein thrombosis. Turkish Journal of Vascular Surgery. 2019; 28(2):69-72.
27. Nursah Basol, Nevin Karakus, et al. The importance of MTHFR C677T/A1298C combined polymorphisms in pulmonary embolism in Turkish population. Medicina, 2016; 1-7.
28. Sabahattin Destek, Vahit Onur Gül. Hereditary Thrombophilia Risk Factors In Patients with Venous Thromboembolism. East J Med. 2020; 25(1): 26-32.
29. Irene D. Bezemer, Carine J. M. Doggen, No Association between the Common MTHFR677C→T Polymorphism and Venous Thrombosis. Arch Intern Med. 2007; 167:497-501.
30. Miao Gao, Na Feng, et al. Meta-analysis of the relationship between methylenetetrahydrofolate reductase C677T and A1298C polymorphism and venous thromboembolism in the Caucasian and Asian. Bioscience Reports, 2020; 40: 1-12.
31. Saeideh Amani, Ebrahim Mirzajani, et al. The association of methylene tetrahydrofolate reductase (MTHFR) A1298C gene polymorphism, homocysteine, vitamin B12, and folate with coronary artery disease (CAD) in the north of Iran. Turk J Biochem 2020; 45(6): 851–857.

32. Cenk Conkbayir, Rezan Fahrioglu Yamaci, et al. Impact of Genetic Defects on Coronary Atherosclerosis among Turkish Cypriots. The heart surgery forum, 2017 (5): 20
33. Ameen M. Mohammad, Galawezh O. Othman, et al. Genetic polymorphisms in early-onset myocardial infarction in a sample of Iraqi patients: a pilot study. BMC Res Notes, 2020; 13:541.
34. Jiancheng Wang , Nengtai Ouyang , et al. Effect of MTHFR A1298C and MTRR A66G Genetic Mutations on Homocysteine Levels in the Chinese Population: A Systematic Review and Meta-analysis. J Transl Int Med, 2017; 5(4):220-229.
35. Xiaobo Dong, Jun Wang, et al. MTHFR A1298C gene polymorphism on stroke risk: an updated meta-analysis. Genes and Environment, 2021; 43:40.
36. Gustavo Cernera, Marika Comegna, et al. Molecular Analysis of Prothrombotic Gene Variants in Patients with Acute Ischemic Stroke and with Transient Ischemic Attack. Medicina 2021; 57: 723.
37. Shuo Li, Edward W Randell, et al. MTHFR677T and 1298C Play Independent but Opposite Effects in Stroke - Additive Co-Effect of two Intragenic Alleles. Biomed J Sci & Tech Res. 2019; 17(1): 12563-69.
38. Seyed Mehdi Hashemi, Nourollah Ramroodi, et al. Common Variations in Prothrombotic Genes and Susceptibility to Ischemic Stroke in Young Patients: A Case-Control Study in Southeast Iran. Medicina, 2019; 55: 47.
39. James S Graydon, Karla Claudio, et al. Ethnogeographic prevalence and implications of the 677C>T and 1298A>C MTHFR polymorphisms in US primary care populations. Biomarkars in Medicine. 2019; 13(8): 649–661.
40. Sandeep Kumar, Siddharth Kumar Singh. Multivariate Analysis: An Overview. Journal of Dentofacial science. 2013; 2(3): 19-26.
41. Amelia K. Boehme, Charles Esenwa, et al. Stroke Risk Factors, Genetics and Prevention. Circulation Research, 2017; 120:472-495.
42. Julia Truthmann, Markus A. Busch, et al. Modifiable cardiovascular risk factors in adults aged 40-79 years in Germany with and without prior coronary heart disease or stroke. BMC Public Health, 2015; 15:701.