Original Article

The Role of Methylenetetrahydrofolate Reductase Gene Polymorphisms Hypercoagulable Status of Coronavirus Disease

Ibraheem Kais Taha, Ibrahim Abdulla Mahmood, Qasim S. Al-Mayah[1]

Department of Physiology, College of Medicine, Al-Nahrain University, [1]Medical Research Unit, College of Medicine, AL-Nahrain University, Baghdad, Iraq

Abstract

Background: Hypercoagulation is a hallmark in coronavirus disease (COVID-19). The activity of the enzyme methylenetetrahydrofolate reductase (MTHFR) determines homocysteine levels, and polymorphisms in the enzyme's gene can influence the enzyme activity with a consequence of hypercoagulability in patients with COVID-19. **Objectives:** To investigate the association of two single nucleotide polymorphisms (SNPs) in the *MTHFR* gene with hypercoagulability status in COVID-19. **Materials and Methods:** This is a retrospective cross-sectional study, which included 90 patients diagnosed with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with variable severity. Patients were classified according to D-dimer level at admission into two groups: with and without hypercoagulability. Nucleic DNA was extracted from leukocytes and gene fragments corresponding to C677T and A1298C *MTHFR* gene were amplified and genotyped using allele specific polymerase chain reaction. **Results:** Hypercoagulation was reported in 42.22% of the patients. The mutant homozygous genotype (TT) was more frequent among hyper - than normocoagulable patients (13.6% vs. 1.92%) with a significant difference (odds ratio [OR] = 9.28, 95% confidence interval [CI] = 1.02–84.78, $P = 0.048$). Furthermore, T allele was more common among hyper- than normocoagulable patients (28.95% vs. 13.46%) with a significant difference (OR = 2.62, 95% CI = 1.24–5.5, $P = 0.012$). In contrast, the SNP A1298C had no significant impact. **Conclusions:** The TT genotype and T allele of C677T polymorphism but not A1298C in cMTHFE gene could be considered a risk factor for the hypercoagulable status in COVID-19.

Keywords: COVID-19, hypercoagulability, MTHFER gene, single nucleotide polymorphism

Introduction

Coagulopathy, represented by venous and arterial thromboembolism, has emerged as one of the most severe consequences of coronavirus disease (COVID-19) which associated with poor prognosis even the prophylactic and therapeutic use of anticoagulants.[1,2] Besides the effects of the virus itself and the induced cytokine storm on coagulation profile,[3,4] genetic factors may also have a crucial role.[5] Methylenetetrahydrofolate reductase (MTHFR) catalyzes the transformation of the methyl group of the 5-methyltetrahydrofolate to homocysteine during metabolism. As such, it reasonable to assume that metabolic alterations in the enzyme's activity could lead to homocysteine accumulation and the appearance of some pathologies such as thrombosis.[6]

MTHFR gene is a polymorphic gene located on chromosome 1p36.3. Two single nucleotide polymorphisms (SNPs) in this gene were intensively investigated and found to affect the encoded enzyme's activity. Practically, the *C677T* polymorphism was found to be associated with high serum level of homocysteine,[7] and is prevalent in coronary artery disease.[8] A meta-analysis revealed that the SNP *C677T* and *A1298C* were associated with elevated serum levels of homocysteine in patients with deep venous thrombosis and pulmonary embolism (PE).[9] There are few studies analyzing the frequency of *C677T* and/or

Address for correspondence: Dr. Qasim S. Al-Mayah, Medical Research Unit, College of Medicine, AL-Nahrain University, Baghdad, Iraq.
E-mail: sciencefond2015@gmail.com

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A1298C polymorphisms in hypercoalability in patients with COVID-19. The present study aimed to investigate the association of two SNPs in the *MTHFR* gene with hypercoagulability status in COVID-19.

Materials and Methods

The study population

This is a cross-sectional correlational study which was conducted at Al-Nahrain University, College of Medicine, Medical Research Unit and Baghdad Medical City, National Centre of Teaching Laboratories during the period from March 1, 2022 to October 2, 2022. The study was approved by the Institute Review Board (the local Ethical Committee of the College of Medicine, Al-Nahrain University. All participants were informed about the study and their consent was taken before any sample or data collection had taken place. Patients who were on anticoagulant were excluded from the study.

Demographic characteristics of the patients including age, gender, body mass index (BMI), and comorbidities were collected in preformed questionnaire. Furthermore, the serum D-dimer value at admission was also obtained from patients' records. Hypercoagulable status was defined as D-dimer as >1.5 times increase in D-dimer beyond the upper normal limit (220–500 ng/mL) based on a previous study.[10]

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document Number 99, Date January 31, 2022.

Gene amplification

The genomic DNA was obtained from 2 mL of blood in ethylene diamine tetra acetic acid tube stored at –20ºC from previous work by the authors. This extraction was carried out using a ready-made commercial kit (Geneaid, New Taipei, Taiwan) in accordance with the manufacturer's instructions. Using allele specific polymerase chain reaction (AS-PCR) and amplification refractory mutation system (ARMS)-PCR, two sets of primers [Table 1] were utilized to amplify MTHER gene segments corresponding to C677T and A1298C polymorphisms, respectively.

Primers were obtained from Bioneer Company, Korea provided in lyophilized form with variable concentrations. The lyophilized primers were dissolved in DNase/RNase-free water to make a stock solution with a final concentration of 100 pmol/L. The stock solution was then mixed with 90 L of distilled water to make a working solution with a concentration of 10 pmol/L. Each master mix tube was filled with template DNA (4 L) from each sample and primers (4 L from each). The mixture was then transported to the thermocycler (MyGenie 32 thermal block, Bioneer, Korea) after mixing.

Regarding PCR conditions, both SNPs were subjected to an initial denaturation at 95ºC for 4 min, followed by 40 cycles of denaturation for 1 min at 94ºC, annealing for 45 s at 57ºC (for C677T) and 62ºC (A1298C), and extension for 1 min at 72ºC. Finally, a 7-min extension at 72ºC was done. The PCR products were stained with ethidium bromide and separated on 1.5% gel electrophoresis before being seen under ultraviolet light.

Statistical analysis

Statistical package for the social sciences (SPSS) software version 25.0 was used for statistical analysis (SPSS, Chicago, Illinois). The Student's *t* test was used to evaluate continuous data, which were provided as mean and standard deviation (SD). Categorical variables were reported as numbers and percentages, and the Chi-square test was used to examine them. The binary logistic regression test was used to examine the relationship between C677T and A1298C polymorphisms and hypercoagulability. The odds ratio (OR) and 95% confidence interval (CI) were obtained based on this test. SHEsis software[11] (SHEsis online software, http://analysis.bio-x.cn/myAnalysis.php) was used to calculate the haplotype frequency and linkage disequilibrium between the two SNPs. A statistically significant difference was defined as a *P*-value less than 0.05.

Results

Demographic characteristic of the patients

The mean age of patients was 48.81 ± 17.67 years (range = 14–78 years). The majority of patients (71.1%) were men. Their mean BMI was 25.17 ± 4.18 kg/m^2

Table 1: Primers and their corresponding single nucleotide polymorphisms

SNP	Primers 3′→5′	Product size
C677T	F-TGC TGT TGG AAG GTG CAA GAT RW-GCG TGA TGA TGA AAT CGG RM-GCG TGA TGA TGA AAT CGA	226 bp
A1298C	Common F: GAAGAAGTTTGCATGCTTGTGGTTG Common R: CAGGCAAGTCACCTGGGAGAGA Primer A: GGCAAAGAACGAGACTTCAAAGACACATT Primer C: GAGGAGCTGACCAGTGATGC	A allele: 281 bp C allele: 361 bp

(range = 18–40 kg/m^2). Minority of patients had associated type 2 diabetes mellitus (T2DM) (25.6%) and hypertension (18.9%) as indicated in Table 2.

Hypercoagulability state

Out of 90 patients with COVID-19 infection, 38 patients (42.22%) have hypercoagulable state, while the other 52 patients (57.78%) have normocoagulable state.

Association of demographic characteristics with hypercoagulability

No significant difference was found between hypercoagulability state with age, gender, BMI, and T2DM. However, the vast majority of patients with hypercoagulability (89.47%) had a severe disease compared with only 11.54% of patients with normocoagulability, with a significant difference. Furthermore, hypertension was more common among patients with hyper- than those with normocoagulability (34.21% vs. 7.69%) with a significant difference [Table 3].

Table 2: Demographic data of the study population (*N* = 90)

Variable	Value
Age, years	
Mean ± SD	48.81 ± 17.67
Range	14–78
Gender	
Male	64 (71.1%)
Female	26 (28.9%)
BMI, kg/m^2	
Mean ± SD	25.17 ± 4.18
Range	18–40
Comorbidities	
Diabetes mellitus	23 (25.6%)
Hypertension	17 (18.9%)

Molecular assays

In this study, two SNPs were evaluated for their link with the development of hypercoagulation in patients with COVID-19. The distribution of these SNP genotypes in patients and controls was found to be in good agreement with Hardy Weinberg Equilibrium.

C277T

AS-PCR and RT-PCR were used for gene amplification and genotyping of this SNP. Gel electrophoresis of PCR products revealed that this SNP had three genotypes in patients and controls. These were CC, CT, and TT.

The mutant homozygous genotype (TT) was more frequent among hyper- than normocoagulable patients (13.6% vs. 1.92%) with a significant difference (OR = 9.28, 95% CI = 1.02–84.78, P = 0.048). This polymorphism appears to act in a recessive inheritance as CT+TT was far more common among hyper- than normocoagulable patients (44.74% vs. 25%) with a significant difference (OR = 2.43, 95% CI = 1.0–5.95, P = 0.050).

Analysis of allele distribution revealed a higher frequency of T allele among hyper- than normocoagulable patients (28.95% vs. 13.46%) with a significant difference (OR = 2.62, 95% CI = 1.24–5.5, P = 0.012) [Table 4].

A1298C

AS-PCR was used for gene amplification and genotyping of this SNP. Gel electrophoresis of PCR products revealed that this SNP had three genotypes patients and controls. These were AA, AC, and CC.

The frequency of different genotypes and allele of this SNP in patients and controls is shown in Table 5. The

Table 3: Association of demographic characteristics with hypercoagulability

Variable	Hypercoagulability		*P*-value
	No (*n* = 52)	Yes (*n* = 38)	
Age, years			
Mean ± SD	44.71 ± 16.99	54.42 ± 17.23	0.361
Range	14–78	24–75	
Gender			
Male	40 (76.2%)	24 (63.16%)	0.155
Female	12 (23.08%)	14 (36.84%)	
BMI, kg/m^2			
Mean ± SD	25.53 ± 4.2	24.68 ± 4.17	0.837
Range	18–40	18–35	
Disease severity			
Severe	6 (11.54%)	34 (89.47%)	<0.001
Mild-moderate	46 (88.46%)	4 (10.53%)	
Comorbidities			
Diabetes mellitus	11 (21.15%)	12 (31.58%)	0.263
Hypertension	4 (7.69%)	13(34.21%)	**0.002**

Bold face indicates $P < 0.005$

Table 4: The frequency of different genotypes and allele of C277T polymorphism in patients and controls

C277T	Hypercoagulable (n = 38)	Normal (n = 52)	P-value	OR (95% CI)
Genotypes				
CC	21 (55.26%)	39 (75%)	0.085	1.0
CT	12 (31.58%)	12 (23.08%)	0.206	1.85 (0.71–4.85)
TT	5 (13.16%)	1 (1.92%)	**0.048**	**9.28 (1.02–84.78)**
Dominant model				
CC + CT	33 (86.84%)	51 (96.15%)	0.067	1.0
TT	5 (13.16%)	1 (1.92%)		7.73 (0.86–69.13)
Recessive model				
CC	21 (55.26%)	39 (75%)	**0.050**	1.0
CT + TT	17 (44.74%)	13 (25%)		**2.43 (1.0–5.95)**
Alleles				
C	54 (71.05%)	90 (86.54%)	**0.012**	1.0
T	22 (28.95%)	14 (13.46%)		**2.62 (1.24–5.55)**

Bold face indicates $P < 0.005$

Table 5: The frequency of different genotypes and allele of A1298C polymorphism patients and controls

A1298C	Patients (n = 38)	Controls (n = 52)	P-value	OR (95% CI)
Genotypes				
AA	9 (23.68%)	15 (28.85%)	0.182	1.0
AC	15 (39.47%)	27 (51.92%)	0.885	0.93 (0.33–2.62)
CC	14 (36.84%)	10 (19.23%)	0.152	2.33 (0.73–7.43)
Dominant model				
AA + AC	24 (63.16%)	42 (80.77%)	0.066	1.0
CC	14 (36.84%)	10 (19.23%)		2.45 (0.94–6.36)
Recessive model				
AA	9 (23.68%)	15 (28.85%)	0.585	1.0
AC + CC	29 (76.32%)	37 (71.15%)		1.31 (0.5–3.41)
Alleles				
A	33 (43.42%)	57 (54.81%)	0.132	1.0
C	43 (56.58%)	47 (45.19%)		1.58 (0.87–2.87)

frequency of the mutant genotype (CC) was higher in hypercoagulable patients (36.84%) than normocoagulable patients (19.23%) with no significant difference. Neither dominant nor recessive model showed a significant association with the disease. Analysis of allele distribution revealed a close frequency of the C allele among patients with hyper and normocoagulability (56.58% vs. 45.19%) with no significant difference.

Haplotype analysis

As the two polymorphisms are located on the same gene, haplotype blocks were constructed using SHESIS software. Table 6 shows the most frequent haplotypes in patients and controls. The haplotype block CA was more frequent among normal than hypercoagulable patients (53.85% vs. 42.1%); however, the difference was not significant. In contrast, the haplotype block TC was more common in hypercoagulable patients (14.47%) compared with normal coagulation patients (3.85%) with a significant difference (OR = 4.23, 95% CI = 1.29–13.85, P = 0.017) [Table 6].

Table 6: The most frequent haplotype blocks

Haplotype blocks	Hypercoagulable (n = 76)	Normal (n = 104)	P-value	OR (95% CI)
CA	32 (42.1%)	56 (53.85%)	0.121	0.62 (0.34–1.13)
CC	30 (39.47%)	38 (36.54%)	0.688	1.13 (0.62–2.08)
TC	11 (14.47%)	4 (3.85%)	**0.017**	**4.23 (1.29–13.85)**

Bold face indicates $P < 0.005$

Linkage disequilibrium

The result of linkage disequilibrium (LD) analysis is presented in Figure 1. LD plot was constructed using combined genotype data from all patients and controls. The SNP C277T was in a moderate LD (the measure D' was 0.69) with A1298C.

Discussion

The present study aimed to investigate the association of two SNPs in the *MTHFR* gene with hypercoagulability status in COVID-19. According to the result of the study, T allele of C277T polymorphism was significantly associated with hypercoagulation. Many worldwide studies are in

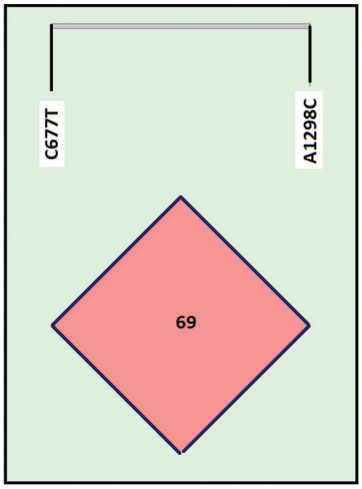

Figure 1: Linkage disequilibrium pattern in MTHFR gene between C277T and A1298C polymorphisms. The red area indicates a linkage disequilibrium. The maximum *D*' value is 1

concordance with this result. In a meta-analysis by Jang *et al*.[12] which included four studies on Asian populations (Korean, Chinese, Japanese, and Taiwanese) with a total of 655 patients with venous thromboembolism (VTE) and 1125 controls. The analysis showed that CT genotype, TT genotype, and the T allele were strongly linked to a greater risk of VTE in the populations of Korean and Chinese populations, but not in Japanese and Taiwanese populations.

In another meta-analysis by Zhang *et al*.[13] on 24 studies with a total of 2339 cases and 4048 controls, significant association was found between VTE risk and T allele and both CT and TT genotypes. Additionally, the TT homozygous genotype was linked to a greater incidence of VTE in European populations but not in North American populations, according to a meta-analysis by Den Heijer *et al.*[14]

Basol *et al.*[15] conducted a study on 118 PE patients and 126 controls, and they have shown that T allele frequency was higher in the PE patients' group than the control group with a significant difference. Ponti *et al.*[16] reported a clear trend toward the worldwide prevalence of *MTHFR* 677 T and COVID-19 incidence and mortality.

The amino acid alanine is converted to the amino acid valine in the protein as a result of the *MTHFR* polymorphism C677T, which translates into decreased enzyme activity. This subsequently increases plasma homocysteine level. This, in addition to low folate intake and low plasma folate levels, increases the risk factor for hypercoagulability.[18]

As the incidence of the *MTHFR* gene variants differs between ethnic and racial groups and the fact that folic acid intake habits varied amongst them, these factors can help to explain the non-significant correlation identified in some of the earlier research.

The haplotype block TC is more frequent in hypercoagulable patients with a significant difference.

In accordance with this study, CT haplotype was reported to be significantly higher in patients as compared with controls (OR [95% CI] = 1.71 [0.25–2.32], $P = 0.002$) in a study on 318 Iranian patients and 400 controls to assess associations between *MTHFR* SNPs and risk of ischemic stroke.[17]

A total of 13,473 healthy adult Chinese women who came to local maternal and children's hospital for pre-pregnancy examination provinces were evaluated by Fan *et al*.,[18] and the haplotype analysis showed a frequency of TA (43.6%), CA (37.9%), CC (17.6%), and TC (0.9%). Komiyama *et al*.[19] reported a frequency similar to those reported in the Japanese population from the 1000 Genome Project[20]: CA, 44.2%; TA, 38.0%; CC, 17.8%; TC, not detected in the Japanese population while only one patient was detected in Komiyama *et al.* study.[19] A similar result was reported also by Al-Motassem *et al.*[21] on lung cancer (LC) patients in comparison to controls and revealed the frequencies: CA (LC: 33.1%; controls: 36.9%) and CC (LC: 43.9%; controls: 33.4%) followed by TA (LC: 21.3%; controls: 24.7%), while the rare haplotype was TC (LC: 1.7%; controls: 5%).

Except for the TA haplotype, which was not reported in this study as a frequent block, the frequency order of the other reported blocks is in harmony with what reported in the mentioned studies.

The present study reveals a moderate linkage disequilibrium ($D' = 0.69$) in the *MTHFR* gene between C677T and A1298C polymorphisms. In the study of Fan *et al*.,[19] the LD analysis showed that the *D*' and r^2 values were 0.883 and 0.143, respectively, which indicates that the *MTHFR* C677T and A1298C polymorphisms were at high LD. Al-Motassem *et al.*[21] also reported a relatively high LD in their study on 98 Jordanian patients with lung cancer and 89 controls ($D' = 0.84$, R^2: 0.18; $D' = 0.56$, r^2: 0.08, respectively).

Trifonova *et al*.[22] conducted a study on 837 individuals from nine population groups inhabiting different regions of Northern Eurasia and belonging to seven ethnic groups, in addition to 4 population groups from the HapMap project database. They reported that the functionally significant C677T and A1298C polymorphisms were not linked in all the populations except for the southern Kyrgyz and Ket samples, as well as populations from the HapMap project.

In the work of Komiyama *et al.*[19] on 240 Japanese patients with non-syndromic cleft lip with/without cleft palate (CL/P), 103 fathers and 153 mothers of these patients, and 68 controls, they reported that the haplotype analysis showed no linkage disequilibrium between C677T and A1298C.

The incoherence of this study's result with the abovementioned studies' results can be explained by the difference in genetic and environmental background among different ethnic populations, which can lead to differences in their linkage disequilibrium patterns and haplotype–disease associations.[22]

Conclusions

COVID-19 hypercoagulability is significantly associated with the mutant homozygous genotype (TT) and the presence of the T allele of C677T polymorphism as compared with CC and CT genotypes and the C allele. COVID-19 hypercoagulability is significantly associated with TC haplotype block. Large-scale study of the *MTHFR* gene polymorphism in the Iraqi population as its both distribution and effect differs worldwide depending on the geographical region and ethnic decent (genetics).

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Conflicts of interest

There are no conflicts of interest.

Authors' declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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