ORIGINAL RESEARCH ARTICLE
pISSN09763325 | eISSN22296816
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www.njcmindia.com
DOI: 10.55489/njcm.131020222420

Association Of the Methylene Tetrahydrofolate Reductase (MTHFR) Gene Polymorphism with Susceptibility to Recurrence of Cardiovascular Outcomes Among Ischemic Stroke- A Randomized Controlled Trial

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ABSTRACT

Background: Hyperhomocystenemia and genetic variants are factors for causing young age stroke globally. This study aims to identify homocysteine related-MTHFR gene polymorphism that associated with recurrent cardiovascular outcomes.

Methodology: A randomized controlled trial conducted upon 90 hyperhomocysteinemic ischemic stroke patients were taken from the neurology wards of a tertiary care hospital were randomly selected into vitamin B therapy group and control groups (n=45 in each group). Baseline subject details were collected venous blood sample for MTHFR genetic testing via PCR-RFLP technique along with blood homocysteine levels, vitamin B12, folic acid levels.

Results: The results showed that the frequency of CT genotype polymorphism was 15.5% vs 13.3% for the MTHFR C677T gene without any significant difference between vitamin group and control group respectively (*p*-value >0.05). The reduction in mean homocysteine up to -6.77±4.50 versus -2.08±0.71 µmol/L in the vitamin group as compared to control group respectively, *p* value 0.001.

Conclusion: Considerable amount of MTHFR gene polymorphism found among hyperhomocysteinemic ischemic stroke of sub-Himalayan region. Nutritional deficiencies including vitamin B 12 & folic acid, and some hidden reasons found, which could lead to the primary cause of hyperhomocysteinemia. Vitamin B therapy is an effective for reducing homocysteine.

Key-words: Gene polymorphism, Ischemic stroke, homocysteine, recurrence, cardiovascular disorders

INTRODUCTION

Globally, young age stroke had several causative factors, here we are focusing on hyperhomocysteinemia is a disorder caused by a disruption in homocysteine-metabolism cycle due to nutritional deficits of B-vitamins and three genes polymorphisms which leads to a greater risk of hyperhomocysteinemia and vascular disease among recent epidemiological studies.[1-9]

The sub-Himalayan region is more susceptible to hyperhomocysteinemia due to high altitude, asymptomatic thrombophilia, polycythemia, or changes in platelet, counts, and dehydration, which causes thrombosis. [10] There are only a few previous studies

How to cite this article: Kataria N, Kalyani VC, Mirza AA, Vivekanandhan S, Kumar M, Bharupi Y, Ranjan S, Kumar N, Kumar N. Association of the Methylene Tetrahydrofolate Reductase (MTHFR) Gene Polymorphism with Susceptibility to Recurrence of Cardiovascular Outcomes Among Ischemic Stroke- A Randomized Controlled Trial. Natl J Community Med 2022;13(10):692-697. DOI: 10.55489/njcm.131020222420

Financial Support: None declared | **Conflict of Interest:** None declared | **Date of Submission**: 07-09-2022 | **Date of Acceptance**: 11-10-2022 | **Date of Publication**: 31-10-2022

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published within Asia on the MTHFR C677T,[8; 4] gene polymorphisms involving in well-known homocysteine metabolism pathway.

Their association with higher homocysteine levels among cardiovascular disease population without any matching hyperhomocysteinemic ischemic stroke with normal renal functions status. Previous literature on Caucasians and Indo-European population found a relationship between homocysteine [11] upon cardiovascular patients, however, contradictory results for the possibility of this association reported in the above-mentioned comes especially among cardiovascular disorder population. The results of the present study help an enhanced consideration of mentioned genetic polymorphisms involved in homocysteine metabolism enzymes with their effects on homocysteine levels, and risk of cardiovascular outcomes which finally provide suggestions for planning & designing clinical trials and their subgroup analysis. This study intended to determine 1) magnitude of the MTHFR C677T, D919G & I278T gene polymorphisms, and 2) their associations with the risk of recurrent major cardiovascular outcomes (cardiovascular events and vascular death) among hyperhomocysteinemic ischemic stroke patients at a tertiary care hospital from sub-Himalayan regions.

METHODOLOGY

Study population: A randomized controlled trial design was adopted upon a sample size of 90 ischemic brain stroke human participants (calculated by using G power 3.1 version software with power=80, alpha=0.05) [12] who were randomly allocated with 1:1 ratio between vitamin B therapy group (n=45, daily a single tablet of vitamin B6-5mg, B9-5mg & B12-500mcg) and control group (n=45, standard hospital treatment for stroke) for four months duration. Inclusion criteria for participants were confirmed cases of clinically stable (NIHSS score <21) ischemic stroke within 72 hours of attack having age within adult limit of 18-70 years, having hyperhomocysteinemia, and normal renal function test. Participants with disorders, which may enhance homocysteine levels such as migraine, Alzheimer's disease, Parkinson's disease, were excluded from the trial. Emergency room, neurology- medicine ward from a tertiary care hospital from Rishikesh, Sub-Himalayan region were study settings for data collection from 2020-2021. Randomization sequence generated by an independent person is done by simple random sampling technique of computer-generated random number table method (block size-4). Open list of random number tables was used to allocated participants in two each group. Primary investigator enrolled patients and assigned the interventions. Physicians and statisticians were blinded in this trial. The institutional ethical committee given approval to this study. Written informed consent was obtained from each participant. Research team members throughout the study duration-maintained confidentiality and anonymity of the records of the participants. Ethical guidelines mentioned in good clinical practices, the Declaration of Helsinki, and the Indian council of medical research (ICMR) were followed in the present study.

Data collection and laboratories techniques for blood sample: This study intended to determine these gene polymorphisms associated with the recurrence of major cardiovascular outcomes [(CVDs) or in-hospital vascular death)] among ischemic stroke. Primary outcome was to identify (MTHFR-methylene tetrahydrofolate reductase) gene polymorphism and recurrence of cardiovascular outcomes (CVD) and in-hospital vascular death. Secondary outcomes were to assess change in homocysteine levels after vitamin B therapy at follow-up of four months among its association with genes polymorphism. Initially, a subject datasheet including socio-demographic, and clinical variables related to stroke had obtained from both groups of the participants. Only homocysteine blood test repeated after four months of the trial. At baseline, a venous blood sample was withdrawn for investigating levels of homocysteine (µmol/L), folic acid (ng/mL), vitamin B12 (pmol/L), levels (via *chemiluminescent Immunoassay from Advia-Centaur XP immunoassay system)* and MTHFR-C677T (*Gene ID:4524; NLM catalog; GTExPortal.org; 2020;* MTHFR methylenetetrahydrofolate reductase [Homo sapiens (human)] - Gene - NCBI (nih.gov)_genes polymorphism via PCR-RFLP [13] technique for all 90 participants. EDTA vial is used to collect blood and freeze at -20^0 C temperature then DNA was extracted from blood cells by using *Qiagen blood Mini* kit (a method used as manufacturer protocol). The regions containing all genotypes polymorphisms were amplified separately with primers (from *Integrated DNA Technologies, USA*) a cycling program consisting of pre-polymerase chain reaction (PCR) on *the Eppendorf machine.* Primers for MTHFR C677T are forward-(MT1 - 5' –TGAAGGAGAAGGTGTCTGCGGGA-3' and reverse – (MT2 -5' – AGGACGGTGCGGTGAGAGTG-3'). Restriction fragment length polymorphism (RFLP) was done with *Hinf-1* restriction enzymes (*Imperial life sciences, India*) used for overnight incubation at 37^0 C temperature to determine MTHFR C677T genotypes respectively. Two laboratory personnel confirmed the quality of DNA analyses independently. The mutation status for all three SNPs was detected in the amplified products and captures on final images for each genotype were captured from *the Chemiluminescence analysis system (UV gel doc machine). All details of PCR-RFLP protocol given in supplementary files.*

Statistics: The data was analyzed in SPSS software 23.0 version (*IBM, Chicago)* by using appropriate descriptive and inferential statistics with the two-sided, *p*-value considered as significant ≤0.05 and 95% confidence interval. All socio-demographic and clinical variables had tabulated in form of frequency (percentage) and blood parameters in the mean ± SD form. Gene polymorphisms, recurrence of cardiovas-

cular outcomes as an event, and vascular death were described as frequency (percentage) and analyzed by using the *Chi-Square test* or *Fisher's exact test* accordingly. *Student 't'-test* or *Mann-Whitney U test* (accordingly) had used to determine the difference in blood parameters between both the groups. However, we performed ITT analysis for these participants, hence, 45 in each group were analyzed in the study.

Approval of Institutional Ethical Committee was obtained**.** (AIIMS, Rishikesh-IEC-352/IEC/Ph. D/2019).

RESULTS

Table 1**,** the mean age of the vitamin group (n=45) was 49.14 ± 14.22 years and the majority of participants were male, belonged to the Hindu religion, hypertensive, resided in rural areas, and affected with left-sided ischemic stroke. The majority of the participants were smokers, and non-alcoholics. However, the mean age of the control group (n=45) was 51.18 ± 14.78 years and the majority of participants were male, belonged to the Hindu religion, hypertensive, resided in rural areas, and affected with left-sided ischemic stroke. The majority of the participants were non-smokers, non-alcoholic. Results from this table proved that the vitamin B therapy found to be an effective, safe and cost-effective remedy and no-harm reported for ischemic stroke patients for reducing homocysteine levels significantly.

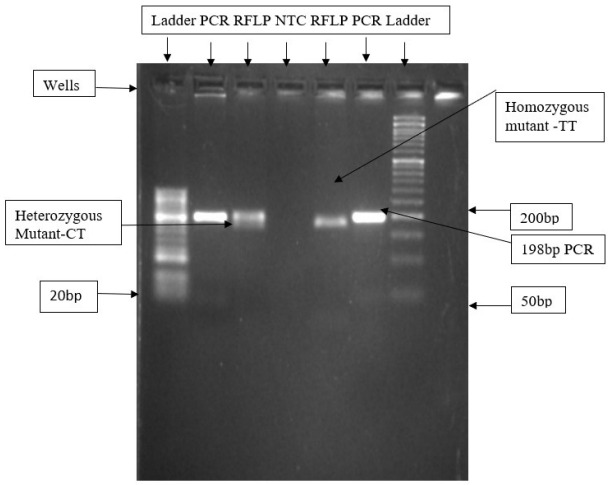
Table 1: Baseline distribution of socio-demographic & clinical characteristics between both groups.

Variable	Frequency (%)		p value
	Vitamin group (n=45)	Control group (n=45)	
Age (Mean±SD)	49.14±14.22	51.18±14.78	0.50@
Gender - Male	34 (75.55%)	29 (64.4%)	0.25@
Religion - Hindu	39 (86.6%)	37 (82.2%)	0.56$
Hypertensive	30 (66.6%)	25 (55.5%)	0.19$
Habitat - Rural	33 (73.3%)	30 (66.6%)	0.49$
Type of smoking abuse			
Non-user	16 (35.5%)	20 (44.4%)	0.69$
Tobacco	5 (11.1%)	6 (13.3%)	
Smoking	20 (44.4%)	17 (37.7%)	
No Alcohol-abuseNo	29 (64.4%)	30 (66.6%)	0.82$
Site of stroke - Left-sided	24 (53.3%)	30 (66.6%)	0.19$,
Green leafy vegetables (number of serving per week) (Mean±SD)	3.22±1.25	3.69±1.4 2	0.10@
Baseline mean Vitamin B12 levels (Mean±SD)	586.8±459.47	676.6±522.7	0.58&
Baseline mean Folic acid levels (Mean±SD)	7.82±4.30	6.42±2.87	0.15&
Mean reduction in homocysteine level post four months (µmol/L) (Mean±SD)	-6.77±4.50	-2.08±0.71	0.0001*&
Recurrence of CVD	3 (6.6%)	5 (11.1%)	0.71
In-hospital vascular death events	1 (2.2%)	5 (11.1%)	0.20

Chi-square test$, Fisher's exact test and Student 't' test @, Mann Whitney U test&, p value considered as significant <0.05*

Polymorphism in homocysteine metabolizing enzymes

Fig 1: **Gel image of MTHFR C677T genotypes (via PCR-RFLP technique)**



In sequence, ladder of 20 base pair PCR product, RFLP product of MTHFR C677T gene, heterozygous mutant-CT genotype, NTC (No template control), RFLP of homozygous mutant-TT genotype, PCR product, and 50 base pair ladder respectively.

In sequence, ladder of 20 base pair PCR product, RFLP product of MTHFR C677T gene, heterozygous mutant-CT genotype, NTC (No template control), RFLP of homozygous mutant-TT genotype, PCR product, and 50 base pair ladder respectively.

Table 2 Baseline genotypic distribution of all types of SNPs between both groups (n=90)

Gene Polymorphisms	Vitamin group (n=45) (%)	Control group (n=45) (%)
MTHFR-C677T (Homozygous wild-CC)	39 (86.6%)	37 (82.2%)
Heterozygous mutant – CT	6 (13.3%)	7 (15.5%)
Homozygous mutant-TT	0	1 (2.2%)

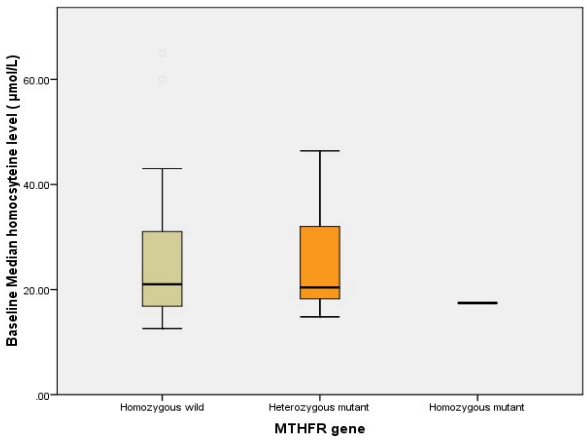
p value considered as significant <0.05*, P value NA-not assessable

Fig.1 & Table 2 showed the magnitude of MTHFR C677T polymorphism as CT & TT genotypes were 6 (13.3%), & 0 % respectively in vitamin group and 7 (15.5%) & 1 (2.2%) respectively, in control group.

We merged TT genotype to CT as only one participant had this genotype, hence, analysis was done on only CC & CT genotypes in subsequent tables 3.

Association of polymorphism with baseline and at four months homocysteine (tHcy) levels

Fig 2 showed the baseline median homocysteine levels with each respective genotypic group of MTHFR C677T, MS D919G, and CBS I278T genes. **Table 3** demonstrates no significant association of MTHFR C677T, with baseline homocysteine levels and at four months, the interaction was found non-significant at *p-value* for interaction=0.26 without adjustment to covariates (with adjustment data mentioned in above regression analysis interpretation). In addition to this, the SNPs affects the tHcy lowering effect of vitamin B therapy as mentioned in the present trial.



MTHFR C677T-CC=homozygous wild, CT=heterozygous mutant.

Fig 2 Box-Whisker graph - Association of homocysteine level with all MTHFR gene (n=90)

Table 3 Effect of MTHFR gene polymorphism on tHcy levels at baseline and at 4 months (n=90)

SNPs	Baseline homocysteine (µmol/L) Mean ± SD	Four months (µmol/L)			
		Vitamin group (n=45) Mean ± SD	Mean difference	Control group (n=45) Mean ± SD	Mean difference
MTHFR C677T					
CC (n=76)	24.70±10.86	15.85±5.66	-6.60±4.68	22.39±8.98	-2.14±0.71
CT (n=14)	25.02±9.73	19.10±10.17	-7.67±3.58	23.11±8.43	-1.86±0.72
p value	0.91	0.26*	0.38$	0.85	0.65$

Student 't' test used, $ p value considered as significant <0.05; * *p* value for interaction

Table.4 Odds ratio for recurrence of CVD events and in-hospital vascular death of MTHFR genotypes (n=90)

SNPs of gene	Yes (%)	No (%)	OR (*p* value)	95%CI
Odds ratio for recurrence of CVD events				
MTHFR C677Tgenotype				
CT (Heterozygous mutant)	0	14 (100)	0.29 (0.40)	0.01 - 5.38
CC (Homozygous wild)	8 (10.5)	72 (94.7)	1	
Odds ratio for in-hospital vascular death				
MTHFR C677Tgenotype				
CT (Heterozygous mutant)	0	14 (100)	0.37 (0.51)	0.01- 7.01
CC (Homozygous wild)	6 (7.8)	70 (92.1)	1	

Note:-p value considered as significant <0.05*

Recurrence of cardiovascular disorder (CVD) events and in-hospital vascular death

Total recurrence of cardiovascular disorders events was eight and six for in-hospital vascular death events out of 90 ischemic stroke participants.

For recurrent cardiovascular disorders (CVD) events, in MTHFR C677T gene, did not differ between CT (mutant) & CC (wild) genotype (OR=0.25, 95% CI-0.01 to 5.38; *p*= 0.40). Hence, the number of cardiovascular events were not affected by the variants of this gene polymorphism.

For in-hospital vascular death, in MTHFR C677Tgene, did not differ between CT (mutant) and CC (wild) genotype (OR=37, 95% C.I. 0.01 to 7.01; *p* = 0.51). Hence, the number of in-hospital vascular deaths were not affected by the variants of this gene polymorphism.

DISCUSSION

The sub-Himalayan region's habitants and strict vegetarians from India had a higher risk of getting hyperhomocysteinemia as reported in previous literature.[10; 14; 4] The homocysteine levels were found to be an independent factor in causing stroke as mentioned in existing recent literature. [11; 15]

The present study findings showed that the combined frequency of CT genotype was 14.4% from MTHFR C677T polymorphism. A slightly higher frequency found in previous studies among hyperhomocysteinemia with cardiovascular patients of CT

genotype frequency as 23% in Pakistan, [16] 30.4% in South India,[8] 52% in West India, 31% in China, [17] and 41% in Italy.[18] Although these studies did not have the same comparator group as in the present study, which includes hyperhomocysteinemia among ischemic stroke between both vitamin and control, groups, which leads to variation in the frequency of MTHFR polymorphism. Variation in ethnic origin of the present study North Indian population can also lead to a change in frequency of MTHFR gene polymorphism. Although, MTHFR gene polymorphism not found to be associated with homocysteine levels.

In the present study, hyperhomocysteinemic patients responded to combined vitamin B therapy by reducing mean homocysteine up to -6.77±4.50 versus -2.08±0.71 µmol/L in the vitamin group and control group respectively. VISP trial had a 2 µmol/L reduction [19] and VITATOPS trial [20] had -3.18 µmol/L which was reported low in the vitamin group as compared to the present study findings. Hence, gene polymorphism patients also reported a significant reduction in homocysteine levels effectively with the help of oral vitamin B therapy.

For the MTHFR C677T gene: The study results showed no relationship between baseline as well as four months homocysteine and all genotypes (wild & mutant) of the MTHFR gene. Three Indian studies, [11] supported these findings. Contradictory, a significant association between homocysteine level, and MTHFR gene polymorphism among other country studies of the hyperhomocysteinemic population. [16; 11] There was a small amount of polymorphism found in the study; hence, the reason for no association of MTHFR polymorphism with homocysteine may have been affected by the diversity in ethnic origin of the studied population from the sub-Himalayan region.

Second objective to find out association of the gene polymorphisms with susceptibility to getting recurrent cardiovascular events revealed that the total numbers as 6 events occurred without any significant association with all genotypes of these three SNPs. For MTHFR, single previous literature supported the findings [11] but was contradicted by few meta-analyses [9] that showed TT vs CC genotype had more coronary heart disease risk. Similarly, a 4-5-fold higher risk of getting a stroke attack was found in the TT genotype in previous literature. [8; 4] Lastly, vascular death was revealed as six events occurred without any significant difference among all genotypes of these three SNPs and previous literature found regarding this data.

Strength of the study: The methodology was quite strong which included randomization, control group, blinding of physician and participants done and generated a level-1 evidence out of it. The present study reported no harm (adverse reactions) to ischemic stroke participants during the trial among polymorphic participants too.

LIMITATIONS OF THE STUDY

The multivariable analysis was not done, as it only possible upon large data size. The reason could be the sample size, due to time constraints in COVID pandemic, only desired sample was collected. Reduction in homocysteine levels can be affected by improvement in vitamin B12 and folic acid deficiency from the nutritive diet taken by the patient. Any other laboratory techniques along with PCR-RFLP can be used for better weightage.

CONCLUSION

Our study concluded that the sub-Himalayan region which is ethnically diverse region, the amount of polymorphism was found considerable for MTHFR C677T gene. The MTHFR C677T genetic determinants had a lesser whereas nutritional deficiency (vitamin B12 and folic acid) was found most predominant for causing hyperhomocysteinemia condition among ischemic stroke population from Sub-Himalayan region. There must be some hidden reasons such as geographical location or ethnic diversity, which could lead to hyperhomocysteinemia. Vitamin B therapy found to be effective in reducing homocysteine levels among genetically polymorphic populations too which leads to had significant clinical impact. It ultimately prevents the number of recurrent of cardiovascular events, by using vitamin B therapy among genetically polymorphic participants. The researcher recommended there is a need for a longitudinal study upon homocysteine related genes with a larger sample size conducted among different ethnic groups for getting clarity in association with cardiovascular outcomes occurrence.

ACKNOWLEDGMENT

We would like to express sincere thanks to the participants for cooperating throughout our data collection period. Next, I would like to express enormous gratefulness for Prof. Madhulika Kabra, HOD & Dr. Madhumita Roy Choudhary, Senior scientist, Division of Pediatrics, Molecular biology laboratory, AIIMS, New Delhi, as well as Mr. Raja Solomon, Senior demonstrator, Neurochemistry laboratory, Dept. of neurological sciences, CMC Vellore, India for helping us in initiation of laboratory work so dedicatedly. Lastly, I would like to acknowledge Dr. Divya MR, Assistant Professor, Dept. of Neurology, AIIMS, New Delhi and Dr. Rajesh Kumar, Associate Professor, Dept. of Nursing, AIIMS, Rishikesh for their technical help in initial phase of the study.

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