Original Article

TT Genotype of the Methylenetetrahydrofolate Reductase C677T Polymorphism is an Important Determinant for Homocysteine Levels in Multi-Ethnic Malaysian Ischaemic Stroke Patients

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Abstract

Introduction: The functional point mutation C677T in the methylenetetrahydrofolate reductase (MTHFR) gene, has been reported to contribute to hyperhomocysteinaemia which is a risk factor for atherothrombotic ischaemic strokes. This study evaluated the prevalence of the C677T polymorphism of the gene in Malaysian ischaemic stroke subjects of Malay, Chinese and Indian ethnicities, and its association with homocysteine levels (tHcy). Materials and Methods: A total of 292 subjects were recruited, comprising 150 ischaemic stroke patients and 142 control subjects who were age and sex matched. Plasma homocysteine, serum folate and vitamin B_{12} were measured in all subjects. Genotyping was carried out using PCR-RFLP. Results: The homocysteine levels were significantly higher ($P = 0.001$) in the stroke group (11.35 ± 2.75 µmol/L) compared to the control group (10.38 ± 2.79 µmol/L). The MTHFR C677T genotype distribution for the stroke group was 46%, 40% and 14%, respectively for CC, CT and TT genotypes and 59.9%, 33.8% and 6.3%, respectively for the control group. The genotype and allelic frequencies were significantly different between the 2 groups, with $P = 0.02$ and $P = 0.004$ respectively. No significant difference was seen in the genotype distribution inter-ethnically. An increasing tHcy was seen with every additional T allele, and the differences in the tHcy for the different genotypes were significant in both the control ($P < 0.001$) and stroke groups ($P < 0.001$). Conclusion: This study shows that TT genotype of the methylenetetrahydrofolate reductase C677T polymorphic gene is an important determinant for homocysteine levels in Malaysian ischaemic stroke patients.

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Key words: Homocysteine, Ischaemic stroke, Malaysia, MTHFR polymorphism

Introduction

Stroke is the major cause of adult disability in developing countries.[1] Traditional risk factors contribute up to 69% of the total risk of ischaemic stroke.[2] There is a substantial body of knowledge to suggest that there are novel risk factors which may confer additional risks.[3] Hyperhomocysteinaemia (hyperHcy) has been identified as a potentially modifiable risk factor for stroke. Homocysteine (Hcy) has also been shown to be atherogenic and thrombogenic in experimental models.[4-6] The 5,10-methelenetetrahydrofolate reductase (MTHFR) is an important enzyme which regulates plasma homocysteine levels. A C677T point mutation (p.A222V) has been linked to increased risk for ischaemic stroke in various population studies,[7-9] but no systematic study has been done in the Malaysian population. The MTHFR gene is located at 1p36.3, consisting of 11 exons with the polymorphism occuring at exon 4.[10] The aim of this study is to investigate if there is an association between Hcy and MTHFR C677T polymorphism in the multi-ethnic Malaysian ischaemic stroke patients.

Materials and Methods

Study Design and Subjects

This was a case-control study. The study protocol was approved by the University of Malaya Medical Centre (UMMC) Ethics Committee. Written informed consents were obtained from all subjects prior to the study. A total

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of 292 subjects were recruited, comprising 150 stroke patients from UMMC and 142 control subjects who were age and sex matched to the patients. Patients recruited were all at least 6 months post-stroke. The subjects were interviewed and only individuals with the same ethnicity for 3 generations were selected.

The inclusion criteria for patients were those with a history of ischaemic stroke (single or recurrent) or those who presented with neurological deficits and confirmed by neuroimaging with computerised tomography (CT) or magnetic resonant imaging (MRI) scan. Echocardiography, vascular evaluation with transcranial Doppler ultrasound, CT angiography and MR angiography and other ancillary investigations were performed to evaluate stroke mechanisms. Patients were excluded if they have proven intra-cerebral bleed, cardioembolic stroke or other unusual aetiologies such as arterial dissection, Moyamoya disease or autoimmune diseases. Inclusion criteria for controls were those with no history of ischaemic stroke. Subjects were excluded if they were on medications or supplements which are known to interfere with homocysteine levels.

Conventional risk factors were also assessed. The height and weight of all subjects were taken. Subsequently, Body Mass Index (BMI) was calculated using the formula weight (kilograms) / height^2 (metres^2). Diabetes mellitus (DM) was defined as being present when, (i) the subjects were previously diagnosed by a clinician or, (ii) on treatment with hypoglycaemic agent(s) or, (iii) as outlined by the National Institutes of Health (revised criteria, 1980).[11] Hypertension (HPT) was defined as being present when, (i) the subjects were previously diagnosed by a clinician or, (ii) on treatment with anti-hypertensive(s) drugs or, (iii) having systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on at least 2 different occasions.[12] Ischaemic heart disease (IHD) was defined as being present when the subjects were previously diagnosed by a clinician. Hypercholesterolaemia was defined as being present when, (i) having total cholesterol levels of >5.2 mmol/L[13] or, (ii) use of cholesterol lowering treatment. Subjects were defined as consuming a significant amount of alcohol if they consumed 30 g per day of alcoholic beverage.[14] Smoking status was defined as currently smoking ≥ 1 cigarette per day. Positive family history (FH) was defined as the occurrence of stroke, myocardial infarction in first degree relatives.

Biochemical Parameters

The sample collection method for homocysteine (tHcy) assay was based on previously verified methods.[15] Homocysteine assay was done using AxSYM Hcy assay kit by Abbott Laboratories on automated Abbott AxSYM Analyser. Serum vitamin B_{12} and folate analysis was carried out by the Clinical Diagnostic Laboratory (CDL), University Malaya Medical Centre. Serum creatinine was also measured in all subjects, as Hcy clearance is influenced by renal function. Hence, only subjects with serum creatinine levels less than 132 μmol/L were included in this study.

DNA Extraction and Genotyping

DNA was extracted from whole blood using PUREGENE DNA extraction kit by Gentra Systems according to manufacturer's instructions. The MTHFR C677T genotyping was carried out using previously validated methods[16] with some modifications in the cycling conditions as follows: Initial denaturation was carried out at 94°C for 30 seconds. The samples were then amplified for 39 cycles consisting of denaturation at 94°C for 15 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 40 seconds, followed by a final extension step at 72°C for 5 minutes. The amplicon size for the MTHFR C677T genotyping was 246 bp. The PCR products (5 μL/1μg), was then digested with 2U of the restriction enzyme HinfI (NEB) for 2 hours at 37°C. The products were then electrophoresed on a 9% PAGE and genotyping results were documented. The homozygous 677CC gave only 1 band of 246 bp, heterozygous 677CT gave 3 bands of 246 bp, 177 bp and 69 bp; homozygous 677TT gave 2 bands of 177 bp and 69 bp, as seen in Figure 1.

Statistical Analysis

Non-parametric analyses were used for plasma Hcy, serum folate and vitamin B_{12} calculations, as the distributions were skewed. Conventional risk factors between groups were compared using Mann-Whitney U test for continuous variables and chi-square tests for discrete variables.

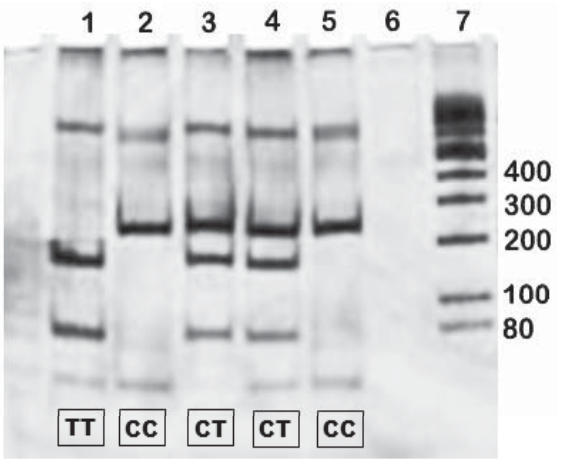

Fig. 1. Silver-stained PAGE gel for MTHFR C677T genotyping. Lanes 1-5: PCR-RFLP pattern for representative patients' samples with the corresponding genotypes indicated. Lane 6: negative PCR control. Lane 7: 100bp DNA ladder.

Kruskal-Wallis test were applied for comparison between 3 non-parametric variables, with post-hoc comparisons using Mann-Whitney U test for significant findings. Correlation studies were done using Spearman's rho. All analyses were carried out using SPSS version 11.5. Allele and genotype frequencies among the patients and control subjects were also compared by the χ^2 test with Hardy-Weinberg predictions.

Results

A summary of the subjects' demography for this study is as listed in Table 1. Overall, the tHcy mean & median for the control group was 10.39 μmol/L and (10.31 ± 0.23) μmol/L while for the stroke group, the mean and median was 11.35 μmol/L and (10.83 ± 0.22) μmol/L, respectively. Hyperhomocysteinaemia (hyperHcy) in this study was defined according to the highest quintile,[17] or ≥ 11.89 μmol/L. In the control group, 19.7% had hyperHcy, while in the ischaemic stroke group, 35.3% had hyperHcy. The difference between the highest quintile and lower 4 quintiles in the control and ischaemic stroke groups was significant (χ^2 = 7.88, *P* = 0.005). Odds ratio was also calculated between the top fifth and lower fourth groups, and it was found that the hyperHcy was 2.23 (95% CI, 1.31-3.79) times more likely in the ischaemic stroke group compared to the

Table 1. Demographic Data and Conventional Stroke Risk Factors

	Control (n = 142)	Stroke (n = 150)	*P* value
Mean age, years (Mean ± SD)	(60.6 ± 7.1)	(61.0 ± 10.1)	0.48
Gender (n,%)			0.052
Female	71 (50.0)	58 (38.7)	
Male	71 (50.0)	92 (61.3)	
Ethnic group (n,%):			
Malay	32 (22.5)	32 (21.3)	0.75
Chinese	62 (43.7)	72 (48.0)	
Indian	44 (31.0)	42 (28.0)	
BMI (Mean ± SD)	25.4 ± 4.8	25.0 ± 2.6	1.00
HPT (n, %)	61 (43.0)	123 (82.0)	<0.0001
IHD (n, %)	7 (4.9)		0.17
DM (n, %)	24 (16.9)	82 (54.7)	0.0001
Total cholesterol, mmol/L (Mean ± SD)	5.47 ± 0.91	5.22 ± 1.20	0.032
Hypercholesterolaemia (%)	28 (21.9)	100 (78.1)	<0.0001
Alcohol consumption (%)	13 (9.2)	36 (24.0)	0.001
Smoking (%)	16 (11.3)	66 (44.0)	<0.0001
Positive family history (%)	61 (43.0)	92 (61.3)	0.002

BMI: Body mass index; HPT: hypertension; IHD: ischaemic heart disease; DM: diabetes mellitus

Table 2. Overall MTHFR C677T Genotype and Allelic Frequencies, Mean tHcy, Vitamin B_{12} (Vit. B_{12}) and Folate Levels and by Different Ethnicities

	Overall (n = 292)		Malay (n = 72)		Chinese (n = 134)		Indian (n = 86)	
	Control	Stroke	Control	Stroke	Control	Stroke	Control	Stroke
MHFR C677T (genotype)								
CC, n (%)	85 (59.9)	69 (46.0)	23 (63.9)	13 (36.1)	37 (59.7)	39 (54.2)	25 (56.8)	17 (40.5)
CT, n (%)	48 (33.8)	60 (40.0)	10 (27.8)	18 (50.0)	22 (35.5)	22 (30.6)	16 (36.4)	20 (47.6)
TT, n (%)	9 (6.3)	21 (14.0)	3 (8.3)	5 (13.9)	3 (4.8)	11 (15.3)	3 (6.8)	5 (11.9)
P value	0.02		0.06		0.14		0.3	
MTHFR C677T (allele)								
C, n (%)	218 (76.7)	198 (66.0)	56 (77.8)	44 (61.1)	96 (77.4)	100 (69.4)	66 (75.0)	54 (64.3)
T, n (%)	66 (23.2)	102 (34.0)	16 (22.2)	28 (38.9)	28 (22.6)	44 (30.6)	22 (25.0)	30 (35.7)
P value	0.004		0.03		0.14		0.14	
tHcy (μmol/L ± SE)	10.38 ± 0.23	11.35 ± 0.22	10.27 ± 1.83	11.23 ± 1.60	10.45 ± 0.39	11.44 ± 0.37	10.40 ± 0.39	11.30 ± 0.36
P value	0.001		0.10		0.03		0.08	
Folate (nmol/L ± SE)	28.09 ± 0.96	23.90 ± 0.74	24.76 ± 1.83	23.74 ± 1.60	30.27 ± 1.41	24.62 ± 1.12	27.73 ± 1.79	22.83 ± 1.20
P value	0.01		0.73		0.001		0.038	
Vit. B_{12} (pmol/L ± SE)	324.64 ± 11.84	337.55 ± 12.30	338.33 ± 18.88	375.28 ± 33.99	324.22 ± 15.54	326.24 ± 14.92	314.02 ± 27.50	324.60 ± 20.41
P value	0.49		0.69		0.92		0.40	

control group. There was no significant ethnic difference in the tHcy levels between the Malay [control: (10.27 ± 2.55) μmol/L; stroke: 11.23 ± 2.40 μmol/L], Chinese [control: (10.45 ± 3.09) μmol/L; stroke: (11.44 ± 3.14) μmol/L] and Indian [control: (10.40 ± 2.60) μmol/L, stroke: (11.30 ± 2.35) μmol/L] ethnic groups in either the controls (P = 0.864) or the stroke group (P = 0.936).

The mean and median for serum folate levels in the control group was 28.09 nmol/L and (26.40 ± 0.96) nmol/L while for the stroke group, the mean and median was 23.91 nmol/L and (22.55 ± 0.74) nmol/L. All subjects had folate levels within normal range except for 2 subjects. In the control group, the Chinese had the highest folate levels while the Malays had the lowest, the stroke group, the Chinese still had the highest folate levels, the Indians had the lowest. A significant difference between the 3 ethnicities was found only in the control group (P = 0.034), and post-hoc analysis showed significant difference only in the folate levels between Malays and Chinese, with a mean difference of 5.51 nmol/L (P = 0.01). A weak negative correlation was found between tHcy levels with serum folate (ρ = –0.19, P = 0.001), between serum vitamin B_{12} levels and tHcy levels, a moderate negative correlation was seen (ρ = –0.43, P = 0.001).

The genotype and allelic frequencies of the polymorphism studied are summarised in Table 2. The TT genotype was also compared against TC pooled with CC genotypes, as in the recessive model between control and ischaemic stroke group. On the whole, the TT genotype distribution was significantly higher in the ischaemic stroke group (P = 0.03), with an OR of 2.41 (95% CI, 1.06-5.45). The TT genotype frequencies were not found to be significantly different among the Malay and Indian ethnic groups, and of borderline significance in the Chinese. All genotype frequencies were in Hardy-Weinberg equilibrium.

The data were also analysed to find out if the genotypes were in any way associated with tHcy levels, as summarised in Table 3. The results showed increasing tHcy levels with addition of the T polymorphic allele and this was seen in both the control and stroke groups. Post-hoc analysis in the control group showed significant difference between tHcy levels of CC and CT (P = 0.019), CT and TT (P <0.001), as well as CC and TT (P <0.001). In the ischaemic stroke group, there was a significant difference in the tHcy levels among the CC and CT, CT and TT as well as CC and TT with all P values <0.001.

The mean serum folate levels (± SE) for the control subjects in the CC, CT and TT genotype was (27.6 ± 1.26) pmol/L, (29.54 ± 1.65) pmol/L and (24.84 ± 3.20) pmol/L, respectively and the difference was not significant (P = 0.46). In the ischaemic stroke group, the means were (25.32 ± 1.25) pmol/L, (22.72 ± 1.06) pmol/L and (25.32 ± 1.25) pmol/L respectively for the CC, CT and TT genotype and the difference was also not significant. Comparisons were also made between control and ischaemic stroke subjects of the same genotypes, but no significant difference was found.

Table 3. Overall tHcy Levels with Different MTHFR C677T Genotypes

tHcy levels with different MTHFR C677T genotypes (μmol/L ± SE)	Control (n = 142)	Stroke (n = 150)
CC	9.72 ± 0.24	10.19 ± 0.24
CT	10.58 ± 0.32	11.31 ± 0.27
TT	15.66 ± 1.52	15.28 ± 0.64
P value	<0.001	<0.001

Discussion

This study found that the tHcy was significantly higher in the stroke group, which was consistent with previous studies from Malaysia and Singapore,[18,19] and other epidemiological studies including meta-analyses.[9,20,21] No significant difference in tHcy levels were found between the Malay, Chinese and Indian ethnic groups, in tandem with findings from previous studies.[18,22-24] Evidence from experimental studies also suggest that hyperHcy contributed to ischaemic strokes by accelerating atherogenesis and thrombogenesis through direct endothelial injury, causing endothelial dysfunction.[25] The underlying mechanism behind endothelial dysfunction includes induction of NADPH oxidase by Hcy and nitric oxide synthase activity, contributing to increased superoxide radicals production[26] and decreasing bioavailability of nitric oxide (NO).

The increasing tHcy with T allele relationship seen in this study has been demonstrated in other studies,[27-29] showing that the T allele is an important predictor for tHcy. The T allele, which causes an amino acid change from alanine to valine, is associated with 50% reduction of the activity of MTHFR,[30] producing an increase of between 20% to 35% higher tHcy levels.[29,31] When this occurs, Hcy will be diverted from the remethylation pathway to the transsulfuration pathway, which is unable to handle the increased Hcy burden, resulting in accumulation of Hcy intracellularly, and subsequently, hyperHcy.[32] Our findings support the findings of many other studies which found a higher frequency of the T allele in ischaemic stroke patients and a positive correlation between the TT genotype with ischaemic stroke including a large Chinese,[27] Singaporean[19] and an Indian study.[28] However, the frequency of the TT genotype is variable across different populations, between 7.7% to 27.7%.[7,33-36] The TT genotype frequency in our ischaemic stroke population was 15%, higher than that found

in the Singaporean population from 2 previous studies of 9.2% and 8.4%.[19,29]

B vitamins supplementation has been shown to decrease tHcy levels between 2.0 and 3.8 μmol/L in major clinical studies, but failed to show any benefit of tHcy reduction to prevent vascular events.[37-40] These findings have contributed to the argument that tHcy is an epiphenomenon in the pathogenesis of atherosclerosis.[31] A substudy of the VITATOPS trial done in Singaporeans[29] showed an increase in magnitude of tHcy reduction in the TT genotype (–4.7 μmol/L) compared to the CC (–3.2 μmol/L) and CT (–2.7 μmol/L) genotypes of the MTHFR C677T polymorphism subjects treated with B vitamins, although the difference was not significant. However, the sample size for the subjects with the TT genotype was relatively small (n = 23), apart from the fact that the TT genotype frequency seen in the Singaporeans was lower. A greater reduction in tHcy levels, by further risk stratifying ischaemic stroke patients by the TT genotype, may yield significant clinical benefits from the B vitamin and folate therapy.

We did not find any significant difference in folate levels between the different ethnic groups from the stroke patients. This was not consistent with the data from a Singaporean study[24] which had the same ethnic composition, but were predominantly Chinese. This study showed that the Malays had significantly lower folic acid levels compared to the other ethnic groups. Interestingly, the Chinese and Indians in our study showed a significant difference in the folate levels between the control and stroke group. This probably indicates that these ethnic groups would further benefit more from folate supplementation to decrease the tHcy levels.[41] Our study findings are limited due to the fact that dietary history estimating folate intake was not done. In humans, folate is solely obtained from dietary sources, as they are not able to synthesise it in vivo.[42]

To our knowledge, this is the first study to examine the association between MTHFR genotype with tHcy and ischaemic stroke in Malaysians. However, further studies looking at different populations from different parts of the country, taking into account dietary status, as well as looking at specific stroke subtypes would add value to the available data. In this present study, a possibility that asymptomatic strokes can be missed out using recall method exists, and this would hypothetically contribute to a higher tHcy levels in the control group. Despite this, we still found a significant difference in the tHcy levels between the group of controls and ischaemic stroke patients.

Conclusion

The findings from this study indicate that there is a significant association between MTHFR C677T polymorphism with ischaemic stroke. This was demonstrated in the significant difference in the genotype as well as allelic frequencies between the control and the ischaemic stroke groups. The TT genotype was found to be significantly higher in the stroke group. The tHcy levels were significantly higher in the ischaemic stroke group when compared to the control group, and increasing levels of tHcy were also seen with each addition of the T allele in this study. This demonstrated that the TT genotype is an important determinant of tHcy levels. Further studies focusing on the selection of ischaemic stroke patients with the TT genotype for B vitamin and folate supplementation may achieve greater levels of tHcy reduction and produce significant clinical benefits.

REFERENCES

1. Wolfe CD. The impact of stroke. Br Med Bull 2000;56:275-86.
2. Jamrozik K, Broadhurst RJ, Anderson CS, Stewart-Wynne EG. The role of lifestyle factors in the etiology of stroke. A population-based case–control study in Perth, Western Australia. Stroke 1994;25:51-9.
3. Goldstein LB. Novel risk factors for stroke: homocysteine, inflammation, and infection. Curr Atheroscler Rep 2000;2:110-4.
4. Heinecke JW, Rosen H, Suzuki LA, Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem 1987;262:10098-103.
5. Tsai JC, Perrella MA, Yoshizumi M, Hsieh CM, Haber E, Schlegel R, et al. Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. Proc Natl Acad Sci U S A 1994;91:6369-73.
6. Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. J Thromb Haemost 2005;3:1646-54.
7. Li CM, Zhang C, Lu XL, Feng HY, Su QX, Zeng Y, et al. [Relationship between methylenetrahydrofolate reductase gene and ischemic stroke]. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 2006;18:264-7.
8. Bhagwat VR, Yadav AS, Rathod IM. Homocysteine, lipid indices and antioxidants in patients with ischaemic heart disease from Maharashtra, India. Singapore Med J 2009;50:418-24.
9. Kelly PJ, Rosand J, Kistler JP, Shih VE, Silveira S, Plomaritoglou A, et al. Homocysteine, MTHFR 677C->T polymorphism, and risk of ischemic stroke: results of a meta-analysis. Neurology 2002;59:529-36.
10. Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, et al. Gene structure of humn and mouse methylenetetrahydrofolate reductase (MTHFR) Mamm Genome 1998;9:652-6.
11. From the NIH: New Standards for classification and diagnosis of diabetes. JAMA 1980;243:2296-7.
12. Carretero OA, Oparil S. Essential hypertension : Part I: definition and etiology. Circulation 2000;101:329-35.
13. Expert Panel on Detection, Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001;285:2486-97.

14. Lee TH, Hsu WC, Chen CJ, Chen ST. Etiologic study of young ischemic stroke in Taiwan. Stroke 2002;33:1950-5.
15. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 1993;39:1764-79.
16. Schmitz C, Lindpaintner K, Verhoef P, Gaziano JM, Buring J. Genetic polymorphism of methylenetetrahydrofolate reductase and myocardial infarction. A case-control study. Circulation 1996;94:1812-4.
17. Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, et al. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 1997;277:1775-81.
18. Tan KS, Lee TC, Tan CT. Hyperhomocysteinaemia in patients with acute ischaemic stroke in Malaysia. Neurol J Southeast Asia 2001;6:113-9.
19. Moe KT, Woon FP, De Silva DA, Wong P, Koh TH, Kingwell B, et al. Association of acute ischemic stroke with the MTHFR C677T polymorphism but not with NOS3 gene polymorphisms in a Singapore population. Eur J Neurol 2008;15:1309-14.
20. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002;325:1202.
21. Grabowski EF, Buonanno FS, Krishnamoorthy K. Prothrombotic risk factors in the evaluation and management of perinatal stroke. Semin Perinatol 2007;31:243-9.
22. Lopez JB, Peng CL. Plasma homocysteine reference values of adult Malaysians from three ethnic groups. Clin Chim Acta 2004;340:235-8.
23. Hughes K, Ong CN. Homocysteine, folate, vitamin B12, and cardiovascular risk in Indians, Malays, and Chinese in Singapore. J Epidemiol Community Health 2000;54:31-4.
24. Kasiman K, Eikelboom JW, Hankey GJ, Lee SP, Lim JP, Lee JH, et al. Ethnicity does not affect the homocysteine-lowering effect of B-vitamin therapy in Singaporean stroke patients. Stroke 2009;40:2209-11.
25. Kaul S, Zadeh AA, Shah PK. Homocysteine hypothesis for atherothrombotic cardiovascular disease: not validated. J Am Coll Cardiol 2006;48:914-23.
26. Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, et al. Increased superoxide production in coronary arteries in hyperhomocysteinaemia: role of tumor necrosis factor - alpha, NAD(P) H oxidase and inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol 2003;23:418-24.
27. Li Z, Sun L, Zhang H, Liao Y, Wang D, Zhao B, et al. Elevated plasma homocysteine was associated with hemorrhagic and ischemic stroke, but methylenetetrahydrofolate reductase gene C677T polymorphism was a risk factor for thrombotic stroke: A Multicenter Case-Control Study in China. Stroke 2003;34:2085-90.
28. Biswas A, Ranjan R, Meena A, Akhter MS, Yadav BK, Munisamy M, et al. Homocystine levels, polymorphisms and the risk of ischemic stroke in young Asian Indians. J Stroke and Cerebrovasc Dis 2009;18:103-10.
29. Ho GY, Eikelboom JW, Hankey GJ, Wong CR, Tan SL, Chan JB, et al. Methylenetetrahydrofolate reductase polymorphisms and homocysteine-lowering effect of vitamin therapy in Singaporean stroke patients. Stroke 2006;37:456-60.
30. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.
31. Brattstrom L, Wilcken DE. Homocysteine and cardiovascular disease: cause or effect? Am J Clin Nutr 2000;72:315-23.
32. Medina M, Urdiales JL, Amores-Sanchez MI. Roles of homocysteine in cell metabolism: old and new functions. Eur J Biochem 2001;268:3871-82.
33. Kohara K, Fujisawa M, Ando F, Tabara Y, Niino N, Miki T, et al. MTHFR gene polymorphism as a risk factor for silent brain infarcts and white matter lesions in the Japanese general population: The NILS-LSA Study. Stroke 2003;34:1130-5.
34. Al-Allawi NA, Avo AS, Jubrael JM. Methylenetetrahydrofolate reductase C677T polymorphism in Iraqi patients with ischemic stroke. Neurol India 2009;57:631-5.
35. Kawamoto R, Kohara K, Oka Y, Tomita H, Tabara Y, Miki T. An association of 5,10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism and ischemic stroke. J Stroke and Cerebrovasc Dis 2005;14:67-74.
36. Brattstrom L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. Circulation 1998;98:2520-6.
37. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Eng J Med 2006;354:1567-77.
38. Bønaa KH, Njølstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Eng J Med 2006;354:1578-88.
39. Spence JD, Bang H, Chambless LE, Stampfer MJ. Vitamin Intervention for Stroke Prevention Trial: An Efficacy Analysis. Stroke 2005;36:2404-9.
40. Hankey GJ, Eikelboom JW, Baker RI, Gelavis A, Hickling SC, Jamrozik K, et al. B vitamins in patients with recent transient ischaemic attack or stroke in the VITAmins TO Prevent Stroke (VITATOPS) trial: a randomised, double-blind, parallel, placebo-controlled trial. Lancet Neurol 2010;9:855-65.
41. Selhub J. Public health significance of elevated homocysteine. Food Nutr Bull 2008;29:S116-25.
42. Iyer R, Tomar SK. Folate: A functional food constituent. J Food Sci 2009;74:R114-22.