A potential epigenetic marker mediating serum folate and vitamin B_{12} levels contributes to ischemic stroke risk

Keat Wei Loo[1,2], Lyn R Griffiths[2], Heidi Sutherland[2], Larisa M Haupt[2], Shalini Bhaskar[3], Siew Hua Gan[1]

[1]Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, 16150, Kelantan, Malaysia.

[2]Genomics Research Centre, Institute of Health and Biomedical Innovation Queensland University of Technology, Musk Ave, Kelvin Grove, QLD, 4059, Australia.

[3]Medical Department, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia.

Abstract

Background and Purpose

Stroke is a multifactorial disease that may be associated with aberrant DNA methylation profiles. We investigated epigenetic dysregulation for the *MTHFR* gene among ischaemic stroke patients.

Methods

Cases (n=297) and controls (n=110) were recruited after obtaining signed written informed consent, following a screening process against the inclusion/exclusion criteria. Serum vitamin metabolites (folate, vitamin B_{12} and homocysteine) were determined using immunoassaysand methylation profiles for CpGs A and B in the MTHFR gene were determined using bisulfite-pyrosequencing method.

Results

Methylation of *MTHFR* significantly increased the susceptibility risk for ischemic stroke. In particular, CpG A outperformed CpG B in mediating folate and vitamin B_{12} levels to increase ischemic stroke susceptibility risks by 4.73 fold. CpGs A and B were not associated with either serum homocysteine levels or ischemic stroke severity.

Conclusion

CpG A is a potential epigenetic marker in mediating serum folate and vitamin B_{12} to contribute to ischemic stroke.

Introduction

Stroke is a multifactorial disease with 90% of cases being classified as ischemic stroke while hemorrhagic stroke makes up the remaining (Matouk *et al*., 2012). Emerging lines of evidencehave suggested that aberrant DNA methylation may affect the vulnerability of central nervous system injury responses following ischemia (Qureshi and Mehler, 2010, Matouk *et al*., 2012). Despite its active involvement in generating S-adenosylmethionine for the methylation process, to datethe methylation profiles of MTHFR have not been fully elucidated. We aim to investigate potential epigenetic dysregulation of the *MTHFR* gene in ischaemic stroke patients.

Methods

This study was approved by the Institutional Ethics Review Committee of Universiti Sains Malaysia (reference no: USMKK/PPP/JEPeM [231.1.(08)]) which complies with the Declaration of Helsinki. Signed written informed consent forms were obtained from each participant prior to their enrollments and subjects following screening against the inclusion and exclusion criteria (Supplementary Table 1, Supplementary Figure 1).

Fasting blood samples (6 ml) were collected in clot activator gel tube (Becton Dickinson) for the determination of serum vitamin metabolites (serum homocysteine, folate and vitamin B_{12} levels). Serum homocysteine levels were determined using fluorescence polarization immunoassay (Architect ci8200 Abbott, Illinois) while serum folate and vitamin B_{12} levels were determined using competitive immunoassay of direct chemiluminescence technology respectively (ADVIA Centaur XP immunoassay system, Siemens Healthcare). Venous blood samples (2 mL) were collected for DNA methylation profiling. DNA extraction bisulphite treatment and PCR-pyrosequencing were performed to determine methylation levels of CpGs A and B (Supplementary Figure 2, Loo *et al*. unpublished data).

Statistical analysis was performed using R packages version 3.0.3 (New Jersey, USA). The demographic characteristics of the study subjects were analyzed using t-test for continuous variables while chi-squared (χ^2) test was used for categorical variables. Pearson correlation coefficient (*r*) was used to establish the correlation between methylation and serum vitamin metabolites. Logistic regression models were used to estimate the odds ratios (ORs) and 95% confidence intervals (CI) for determining possible high risk methylation profiles for ischemic stroke and its severity. Age, gender and serum vitamin metabolites were controlled in multivariate analysis.

Results

Cases and controls demonstrated similar age groupings (Table 1, all $p>0.05$) while CpG A demonstrated higher ratios of females amongst cases when compared to controls (Table 1, $p<0.05$). Correlation analysis demonstrated that the methylation levels in CpG A were positively correlated with serum folate ($r=0.106$, $p=0.032$) and vitamin B_{12} ($r=0.114$, $p=0.022$) levels but not with those in CpG B. Methylation profiles in both CpGs A and B were not significantly

correlated with serum homocysteine levels. Following multivariate adjustment, CpG A methylation levels were shown to confer a significantly higher risk (by 4.73-fold) of ischemic stroke (95% CI 2.56-8.75, $p<0.001$). The susceptibility risk for CpG B levels was however non-significantly (Table 2) associated with ischemic stroke risk. In addition, neitherCpG levels were significantly associated with ischemic stroke severity following multivariate analysis (Table 3, $p>0.05$).

Discussion

To our knowledge, this is the first study to investigate *MTHFR* methylation profiles among Asians ischemic stroke patients. Similar to most epidemiological studies utilising DNA from peripheral blood cells (Relton et al., 2012, Lin et al., 2014), our study supports the notion that DNA is an informative determinant for epigenetic variation especially when brain cells are inaccessible (Relton et al., 2012). We demonstrated that CpG A but not CpG B methylation levels were significantly associated with increasedsusceptibility risk for ischemic stroke. There is some support for a role of epigenetics in ischemic stroke risk but to most studies (Baccarelli et al 2010; Lin et al., 2014) have focued on increased susceptibility risk of ischemic stroke and long interspersed element-1 methylation.

MTHFR methylation profiles at CpG A were shown to be associated with serum folate and vitamin B_{12} levels and also associated with thigher risks of ischemic stroke when compared to CpG B. Computational biology analysis have indicated that low density lysine 4 histone H3 tri-methylation and incomplete RNA polymerase II in CpG B (as observed from our unpublished *in silico* data) may affect the transcriptional activity of *MTHFR*. Increased lysine 4 histone H3 methylation is an epigenetic mark for euchromatin, often accompanied by the presence of RNA polymerase II to act as an active promoter (Majocchi et al., 2014).

Several studies have demonstrated alink between vitamin metabolites and DNA methylation indicating the pertinent role of one carbon metabolism in modulating DNA methylation (Fernàndez-Roig et al., 2012; Batra and Devasagayam, 2012). One carbon metabolism has been reported to be the main channel for methyl group donation at cellular levels (Johansson et al., 2011) where a depletion of vitamin B_{12} or folate can reduce the bioavailability of S-adenosylmethionine (Batra and Devasagayam, 2012). Decreased bioavailability of S-adenosylmethionine can hamper the genome wide methylation process (Baccarelli et al., 2010) and favour the reduction of lysine 4 of histone H3 methylation (Batra and Devasagayam, 2012).

Howeverwe have not established any correlation of *MTHFR* methylation with serum homocysteine levels suggesting that 1) an intergenic methylation of *MTHFR* may not play a major role in affecting the serum homocysteine levels or 2) serum homocysteine levels may not be an essential proxy for gene expression changes. In addition, the non-significant correlation may also explain the non-significant correlation between *MTHFR* methylation and ischemic stroke severity. This is supported by the fact that homocysteine is actively involved in oxidative stress events which can affect vascular structure (Pezzini *et al*., 2007) to worsen ischemic stroke severity (**Okubadejo et al., 2008).**

A recent report on the association between hemi-methylated *MTHFR* gene with silenced *MTHFR* gene expression among end-stage renal disease patients (Ghattas et al., 2014), has suggested a significant role of *MTHFR* promoter methylation on its gene expression levels. Therefore, future studies which investigate the effects of *MTHFR* methylation on gene expression changes is warranted for a better understanding of the epigenetic mechanisms of *MTHFR* in mediating serum vitamin metabolites to contribute to ischemic stroke.

Conclusion

Methylation of *MTHFR* significantly increases susceptibility risk for ischemic stroke but does not affect ischemic stroke severity. *MTHFR* CpG A outperforms CpG B in mediating folate and vitamin B_{12} levels to increase ischemic stroke susceptibility by 4.73 fold; however, neither CpG level was correlated with serum homocysteine levels.

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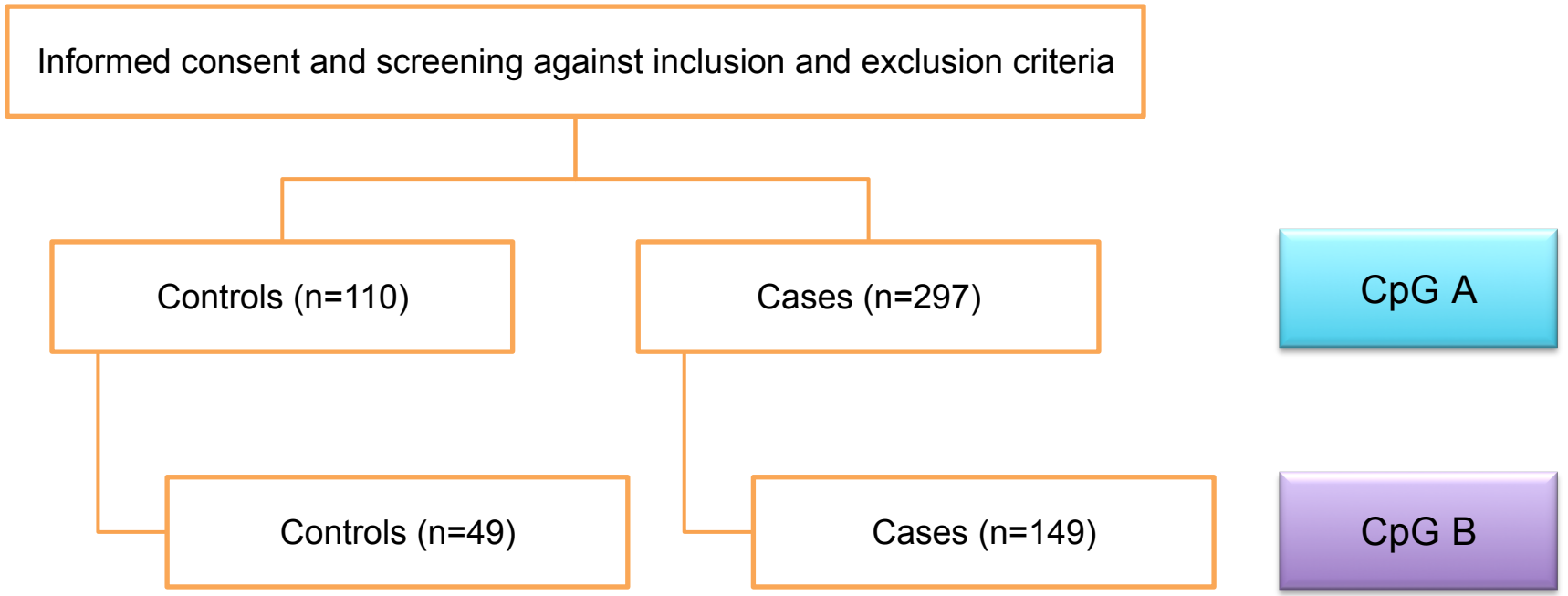
Conflicts of interest: None declared.



Supplementary Figure 1 An overview of the study design.

Supplementary Table 1 Inclusion and exclusion criteria for study subjects

	Inclusion criteria	Exclusion criteria
Controls	1. Three generations of Malays 2. Healthy subjects aging between 18 and 70 years old. 3. Normal findings on medical history and physical examination	1. Confirmed to have medical illness that require treatments 2. Having family history of stroke 3. Subjects on any form of vitamins B supplementation six months prior to the study
Cases	1. Three generations of Malays 2. Ischaemic stroke patients aged between 18 and 70 3. Occlusion, stenosis and/or lesion	1. Participation in any on-going clinical trial study 2. Subjects on any form of vitamins B supplementation six months prior to the study 3. Intra-cerebral haemorrhage confirmed by

confirmed by brain imaging (computerised tomography scan or magnetic resonance imaging)	brain imaging (computerised tomography scan or magnetic resonance imaging)
4. Stroke severity assessment based on Modified Rankin Scale	4. Having brain tumours or other forms of cancer

Table 1 Demographic characteristic of study subjects

	CpG A		CpG B	
Parameters	Controls (n=110)	Cases (n=297)	Controls (n=49)	Cases (n=149)
Age (years)	52.74 ± 7.69	52.62 ± 8.83	51.12 ± 7.29	52.08 ± 8.21
Gender (male:female)	1.16	1.83*	0.96	1.48

Values are the mean ± standard deviation from t-test for continuous variables and χ^2 test for categorical variables. *p <0.05 versus controls.

Table 2 Susceptible risk of DNA methylation on ischemic stroke

	Crude			Adjusted		
CpGs	β	95% CI	p value	β	95% CI	p value[a]
A	3.85	2.72-5.45	<0.001	4.73	2.56-8.75	<0.001
B	1.38	1.08-1.77	0.011	0.90	0.56-1.45	NS

Abbreviations: NS: not significant.

[a]Multivariate analysis adjusted for age, genders and serum vitamin metabolites.

Table 3 Influence of DNA methylation on ischemic stroke severity

CpGs	Crude			Adjusted		
	β	95% CI	p value	β	95% CI	p value[a]
A	-0.09	-0.15 to 0.13	NS	-0.02	-0.16 to 0.12	NS
B	0.02	-0.25 to 0.31	NS	0.02	-0.71 to 0.85	NS

Abbreviations: NS: not significant.

[a]Multivariate analysis adjusted for age, genders and serum vitamin metabolites.