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Plasma *S*-adenosylmethionine, *DNMT* polymorphisms, and peripheral blood LINE-1 methylation among healthy Chinese adults in Singapore

Maki Inoue-Choi[1*], Heather H Nelson[1,2], Kim Robien[1,2,3], Erland Arning[4], Teodoro Bottiglieri[4], Woon-Puay Koh[5,6] and Jian-Min Yuan[7,8]

Abstract

Background: Global hypomethylation of repetitive DNA sequences is believed to occur early in tumorigenesis. There is a great interest in identifying factors that contribute to global DNA hypomethylation and associated cancer risk. We tested the hypothesis that plasma *S*-adenosylmethionine (SAM) level alone or in combination with genetic variation in DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) was associated with global DNA methylation extent at long interspersed nucleotide element-1 (LINE-1) sequences.

Methods: Plasma SAM level and LINE-1 DNA methylation index were measured using stored blood samples collected from 440 healthy Singaporean Chinese adults during 1994-1999. Genetic polymorphisms of 13 loci in *DNMT1*, *DNMT3A* and *DNMT3B* were determined.

Results: LINE-1 methylation index was significantly higher in men than in women ($p = 0.001$). LINE-1 methylation index was positively associated with plasma SAM levels ($p \leq 0.01$), with a plateau at approximately 78% of LINE-1 methylation index (55 nmol/L plasma SAM) in men and 77% methylation index (50 nmol/L plasma SAM) in women. In men only, the T allele of *DNMT1* rs21124724 was associated with a statistically significantly higher LINE-1 methylation index ($p_{trend} = 0.001$). The *DNMT1* rs2114724 genotype modified the association between plasma SAM and LINE-1 methylation index at low levels of plasma SAM in men.

Conclusions: Circulating SAM level was associated with LINE-1 methylation status among healthy Chinese adults. The *DNMT1* genetic polymorphism may exert a modifying effect on the association between SAM and LINE-1 methylation status in men, especially when plasma SAM level is low. Our findings support a link between plasma SAM and global DNA methylation status at LINE-1 sequences.

Background

Inter-individual variation in DNA methylation extent has been associated with increased risk for many chronic diseases including cancer [1-4]. Global DNA hypomethylation, the genome-wide loss of methylcytosine, has been observed in malignant and benign tumors and normal tissues surrounding tumors, indicating that global DNA hypomethylation may be one of the early molecular events in carcinogenesis [5-7]. This reduced global DNA methylation impacts repetitive DNA sequences rich in CpG dinucleotides such as long interspersed nucleotide element-1 (LINE-1). Methylation extent at LINE-1 sequences, as a surrogate marker of global DNA methylation status, varies by gender, age and environmental and lifestyle factors [3,8-14].

Methylation of DNA requires the methyl donor *S*-adenosylmethionine (SAM), a key metabolite in one-carbon metabolism (OCM). DNA methyltransferase (DNMT) enzymes transfer a methyl moiety from SAM to the 5th carbon of the cytosine pyrimidine ring at target CpG dinucleotides. A number of epidemiologic studies

* Correspondence: inou0021@umn.edu
[1]Division of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minneapolis, USA
Full list of author information is available at the end of the article

have evaluated the association between OCM nutrients, such as folate, and the risk of cancer [15-17]. The molecular mechanism for the OCM-cancer association is not completely understood. One hypothesis is that an altered balance in OCM metabolites results in an insufficient supply of methyl moieties for DNMT-catalyzed reactions, resulting in global hypomethylation at DNA sequences [18,19].

In addition to nutrient-related inter-individual variation in the supply of methyl moieties, genetic variation that impacts the activity level of the DNMT enzyme may influence global DNA methylation. DNMT1 is the primary enzyme for maintenance of DNA methylation, whereas DNMT3A and DNMT3B function primarily (but not exclusively) as *de novo* methyltransferases which are responsible for the establishment of DNA methylation patterns in early embryonic development [20-25]. Genetic variation in these *DNMT*s might influence DNA methylation levels and modify the association between SAM status and global DNA methylation.

To advance our understanding of how OCM contributes to cancer susceptibility, there is a need for the establishment of a direct link of plasma SAM and other OCM metabolites to DNA methylation status. In this study, we tested the hypothesis that plasma SAM level, alone or in combination with *DNMT1*, *DNMT3A* and *DNMT3B* genetic variation, was associated with methylation levels at LINE-1 sequences in a healthy human population.

Methods

Study subjects

The Singapore Chinese Health Study (SCHS) is a population-based prospective cohort investigation of diet and the risk for cancer and other chronic diseases. The detailed study design of the SCHS has been described previously [26]. In brief, Chinese men and women aged 45 – 74 years who were permanent residents in Singapore were invited to participate in the study from April 1993 through December 1998. A total of 63,257 participants (85% of the eligible individuals) were enrolled. Baseline information including demographic and lifestyle factors, medical history, family history of cancer and usual dietary intake was collected through in-person interviews at recruitment.

A 3% random sample of the cohort was contacted to donate blood or urine samples starting in 1994. By the end of cohort participant enrollment in 1999, 1,194 subjects donated blood (n = 906) or buccal cells (n = 288). Two 10-mL tubes of blood were drawn from each cohort participant and immediately placed on ice during the transportation to the National University of Singapore. At the laboratory, one tube blood was processed and separated into plasma, buffy coat, and red blood cells, and the other for serum. All blood components were stored in a liquid nitrogen tank at -180°C until 2001, when they were moved to -80°C freezers for long term storage. The present study was based

on the subjects who were included in a nested study on plasma homocysteine initiated in 1996-1997 [26]. By that time, 509 subjects had donated blood samples. These subjects had somewhat higher education, lower prevalence of smoking, and higher prevalence of alcohol intake than the overall SCHS participants, but otherwise were comparable to the whole cohort participants in terms of age, height, body weight, and BMI. This study was approved by the Institutional Review Boards at the University of Minnesota and the National University of Singapore. Prior to study participation, written informed consent was obtained from participants.

SNP selection and genotype determinations

We selected common single nucleotide polymorphisms (SNPs) of *DNMT1*, *DNMT3A* and *DNMT3B* with a minor allele frequency (MAF) ≥20%, given the relatively small sample size of the present study. Six SNPs were chosen based on their reported association with cancer [27,28]: rs2114724, rs2241531, rs1863771, rs1699593, and rs75616428 for *DNMT1* and rs1550117 for *DNMT3A*. Additional 12 SNPs were selected for haplotype tagging using Han Chinese (CHB) data in the International HapMap Project database (Tagger Pairwise method, HapMap Data Rel 27 Phase II + III, Feb09, on NCBI B36 assembly, dbSNP b126): rs2228611, rs2288350 and rs7253062 for *DNMT1*; rs6722613, rs7575625, rs7581217, rs7587636, rs12987326, rs12999687, rs13036246 and rs34048824 for *DNMT3A*; and rs2424908 and rs6141813 for *DNMT3B*. One SNP of *DNMT1* (rs1863771) failed in the Sequenom assay design, and 2 SNPs of *DNMT1* (rs1699593 and rs75616428) did not display genetic variation. Two SNPs of *DNMT3A* (rs12987326 and rs12999687) were excluded from the analysis because they were not in Hardy-Weinberg equilibrium ($p < 0.05$). As a result, we report a total of 13 SNPs (4 SNPs of *DNMT1*, 7 SNPs of *DNMT3A*, and 2 SNPs of *DNMT3B*).

DNA was extracted from stored buffy coats using a Qiagen QIAmp 96 DNA Blood Kit (Qiagen Inc.), and genotype determination was performed in multiplex using the Sequenom MALDI-TOF mass spectrometry system (Sequenom Inc.) by the University of Minnesota BioMedical Genomics Center (BMGC). Each 96-well plate contained positive and negative controls.

Laboratory measurements

We used stable-isotope dilution liquid chromatography-electrospray ionization (ESI) tandem mass spectrometry (LC-ESI-MS/MS) to determine SAM and *S*-adenosylhomocysteine (SAH) concentrations in plasma. Calibrators and internal standards ($^2H_3$-SAM and $^2H_4$-SAH) were included in each analytical run for calibration. One-mM stock solutions of each standard were diluted in distilled water to perform a 5-point

calibration curve (Table 1). Sample preparation involved ultrafiltration utilizing microcentrifugal filter units, Microcon YM-10, 10 kDa NMWL (Millipore). Samples were prepared by the addition of 100 μL mobile phase A containing 10 – 50 μmol/L labeled-isotope internal standards to 30 μl of standard or plasma. Sample filtrate was transferred to a microtiter plate for analysis. Chromatographic separation was achieved on an EZ-faast 250 × 2.0 mm 4 μ AAA-MS analytical column (Phenomenex) maintained at 36°C at a flow of 250 μL/min with a binary gradient with a total run time of 12 minutes. Solvents for HPLC were: (A) 4 mM ammonium acetate, 0.1% formic acid and 0.1% heptafluorobutyric acid (pH = 2.5); and (B) 100% methanol and 0.1% formic acid. The compounds were detected by multiple reaction monitoring (MRM) using positive-ESI. Sample separation and injection was performed by a Shimadzu Prominence LC System interfaced with a 4000 Q TRAP® LC-MS/MS (ABSciex). All data were collected using Analyst software version 1.4.2. The inter-assay precision (CV%) was 6.2 – 9.2% for SAM and 5.9 – 10.4% for SAH.

LINE-1 DNA methylation was quantified using the quantitative bisulfite PCR pyrosequencing method developed by Yang et al [29]. Genomic DNA from peripheral lymphocytes was sodium bisulfite treated using the EZ-96 DNA Methylation Kit, converting non-methylated cytosine residues into uracil, according to the manufacturer's protocol (Zymo Research). Bisulfite converted DNA was PCR amplified using Hotstar Taq Polymerase (Qiagen Inc.). For pyrosequencing, the PCR product was purified using a biotin-labeled primer and Streptavadin Sepharose beads (GE Healthcare). The bead immobilized PCR product was purified, washed, denatured using a NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc.). PCR amplifications were done in triplicate and the extent of methyl cytosine relative to the total cytosine and thymine (%) at each of 4 CpG sites was measured. The average of methylation across the four CpG sites was computed for each replicate, and the average of three replicate measurements of LINE-1 DNA methylation was used as LINE-1 methylation index (%) for each sample in the analysis.

Statistical analysis
Twenty-nine subjects with missing values of LINE-1 methylation index were excluded. Of the remaining 480 subjects, 16 subjects who had missing values (n = 14) or extremely high values (n = 2) of plasma SAM level, and 24 subjects whose serum creatinine values were missing (n = 23) or extremely high (n = 1) were also excluded. As a result, a total of 440 subjects were included in the analysis of plasma SAM level and LINE-1 methylation index. In addition, 8 subjects with missing values for 2 or more genotypes were omitted in the analysis of *DNMT1*, *DNMT3A*, and *DNMT3B* genotypes and haplotypes and LINE-1 methylation index.

All analyses were conducted in men and women separately, given the difference in LINE-1 methylation index between sexes [30-32]. Spline curves were created to visualize the association between plasma SAM and LINE-1 methylation index and to determine acut-off value of low LINE-1 methylation index. Using cut points identified in the spline curves, LINE-1 methylation index was compared across plasma SAM categories (<55 nmol/L and ≥55 nmol/L for men and <50 nmol/L, 50 – 90 nmol/L and ≥90 nmol/L for women) using multiple linear regression modeling with age at blood draw and serum creatinine level as covariates. We adjusted the analysis for serum creatinine level because it has been associated with the SAM-SAH ratio [33,34], and was associated with both plasma SAM level and LINE-1 methylation index in our study population. Similarly, LINE-1 methylation index was compared across genotypes of each *DNMT*. Haplotypes with a 5% or more frequency were constructed. Logistic regression modeling was used to calculate the odds of being in the low LINE-1 methylation index group (<78% for men and <77% for women) for each haplotype relative to the most frequently observed haplotype. A series of spline curves for the relationship between plasma SAM and LINE-1 methylation index by *DNMT* genotypes were created to evaluate a potential modifying effect of *DNMT* genotypes on the association between plasma SAM level and LINE-1 methylation index.

Haplotype analysis was performed using R version 2.13.2, haplostat package. All other analyses were conducted using SAS version 9.2 (SAS Institute Inc.). All reported p values are two-sided, and those that were < 0.05 were considered to be statistically significant. A significance level was adjusted as $p < 0.0038$ (0.05/13 SNPs) for multiple testing of *DNMT* SNPs and LINE-1 methylation index using the Bonferroni correction method.

Results
The average age of the 440 study subjects was 58.1 y (standard deviation (SD), 7.8 y; range, 46 – 77 y).

Table 1 Mass transitions and method statistics for the determination of plasma SAM and SAH

Analyte	Analyte MRM (m/z)	Labeled isotope	Labeled isotope MRM (m/z)	Retention time (minutes)	LOQ (nmol/L)	Calibration curve	Inter-assay precision	
							Level 1 (CV%)	Level 2 (CV%)
SAM	399 → 250	$^2H_3$-SAM	402 → 250	7.1	5	400 – 25 nmol/L	9.2	6.2
SAH	385 → 136	$^2H_4$-SAH	389 → 138	6.8	5	400 – 25 nmol/L	10.4	5.9

Approximately 72% of the study subjects had body mass index (BMI) below 24 while only 4% had BMI of 28 or above. Men had slightly higher education level than women; 40% of men had secondary school or some college education, while 25% of women attained the same level of education. About 56% of men and 7% of women were past or current smokers. Approximately 30% of men and 7% of women consumed alcoholic beverages on a regular basis. The mean plasma SAM level was higher in men (69.7 nmol/L; range, 26.6 – 156.0 nmol/L) than women (63.3 nmol/L; range, 17.2 – 149.0 nmol/L); however, the geometric mean of plasma SAM level after adjustment for age at blood draw and serum creatinine level was comparable (63.3 nmol/L in men and 63.9 nmol/L in women). Plasma SAM level was higher with higher BMI and never smoking status in men, and with older age in women in our study population [35]. Plasma SAM level was not different by education level, alcohol intake, and menopausal status (in women). Plasma SAM level was not associated with methylenetetrahydrofolate reductase (*MTHFR*) C677T genotype or plasma total homocysteine (tHcy) level [35]. Comorbid conditions that may affect the absorption of nutrients in OCM (stomach or duodenum ulcer, partial removal of stomach, and polyps of intestine) were not associated with plasma SAM level or LINE-1 methylation index (data not shown).

LINE-1 methylation index was normally distributed ranging from 68.7% to 83.4% with an average of 77.7% in all subjects. Table 2 shows the mean levels of LINE-1 methylation index across different groups of age, BMI, level of education, smoking and alcohol consumption for men and women separately. The mean LINE-1 methylation index was statistically significantly higher in men (78.1%) than in women (77.3%) ($p = 0.001$). LINE-1 methylation index was positively associated with age in men, but not in women. High BMI was associated with slightly lower LINE-1 methylation index in women only. There was no statistically significant difference in LINE-1 methylation index by level of education, smoking status or alcohol consumption, in both sexes.

Table 2 LINE-1 methylation index (%) by demographic and lifestyle factors in the Singapore Chinese health study

	Men			Women		
	n	Mean (95% CI)[a]	p_{trend}	n	Mean (95% CI)[a]	p_{trend}
LINE-1 methylation (%)	192	78.1 (77.8 – 78.4)[b]	-	248	77.3 (77.1 – 77.6)[b]	-
Age						
<50	25	77.8 (77.0 – 78.7)	0.04	41	77.0 (76.3 – 77.8)	0.82
50 – 59	79	77.8 (77.3 – 78.3)		123	77.6 (77.1 – 78.0)	
60 – 69	71	78.2 (77.7 – 78.7)		55	77.2 (76.5 – 77.8)	
≥70	17	79.3 (78.2 – 80.3)		29	77.1 (76.3 – 78.0)	
BMI						
<20	23	78.3 (77.4 – 79.2)	0.31	38	77.3 (76.5 – 78.1)	0.08
20 – <24	112	77.8 (77.4 – 78.3)		143	77.6 (77.2 – 78.0)	
24 – <28	49	78.4 (77.7 – 79.0)		56	76.9 (76.3 – 77.5)	
≥28	8	78.9 (77.4 – 80.4)		11	76.1 (74.7 – 77.6)	
Level of education						
No formal school	27	77.8 (76.9 – 78.6)	0.28	93	77.0 (76.5 – 77.6)	0.19
Primary school	89	77.9 (77.5 – 78.4)		94	77.5 (77.0 – 78.0)	
Secondary school	53	78.5 (77.9 – 79.1)		52	77.6 (76.9 – 78.3)	
Some college or above	23	78.2 (77.2 – 79.1)		9	77.6 (76.0 – 79.2)	
Smoking status						
Never	84	78.2 (77.7 – 78.7)	0.60	234	77.4 (77.1 – 77.7)	0.21
Past	49	78.0 (77.4 – 78.7)		2	78.5 (75.1 – 81.9)	
Current	59	78.0 (77.4 – 78.6)		12	76.4 (74.9 – 77.8)	
Alcohol intake						
None	135	78.1 (77.7 – 78.5)	0.98	231	77.4 (77.1 – 77.7)	0.12
<7 drinks/week	42	78.1 (77.4 – 78.8)		13	77.1 (75.7 – 78.4)	
≥7 drinks/week	15	78.0 (76.9 – 79.2)		4	75.3 (72.9 – 77.7)	

[a]Adjusted for age at blood draw.
[b]*p* for difference between men and women was 0.001.

Figure 1 shows spline curves of LINE-1 methylation index by plasma SAM level. LINE-1 methylation index was positively associated with plasma SAM level, with a plateau at approximately 78% methylation (55 nmol/L plasma SAM) in men and 77% methylation (50 nmol/L plasma SAM) in women, with a second rise in methylation at 90 nmol/L plasma SAM for women.

Men with plasma SAM level at 55 nmol/L or above had statistically significantly higher LINE-1 methylation index than men with low plasma SAM level ($p = 0.01$) (Table 3). Similarly, in women, there was a statistically significant positive trend in LINE-1 methylation index across the three categories of plasma SAM level ($p_{trend} = 0.005$).

The associations between *DNMT* SNPs and LINE-1 methylation index are presented in Table 4. There was no statistically significant association between any of the 13 SNPs and LINE-1 methylation index in women. In men, there was a statistically significant positive association between the number of variant allele (T) of the *DNMT1* rs2114724 and LINE-1 methylation index ($p_{trend} = 0.001$). In contrast, the number of variant allele (T) of the *DNMT3A* rs758127 was inversely associated with LINE-1 methylation index ($p_{trend} = 0.008$). However, this association did not reach the significance level after taking into account for multiple comparisons. LINE-1 methylation index was not associated with any other
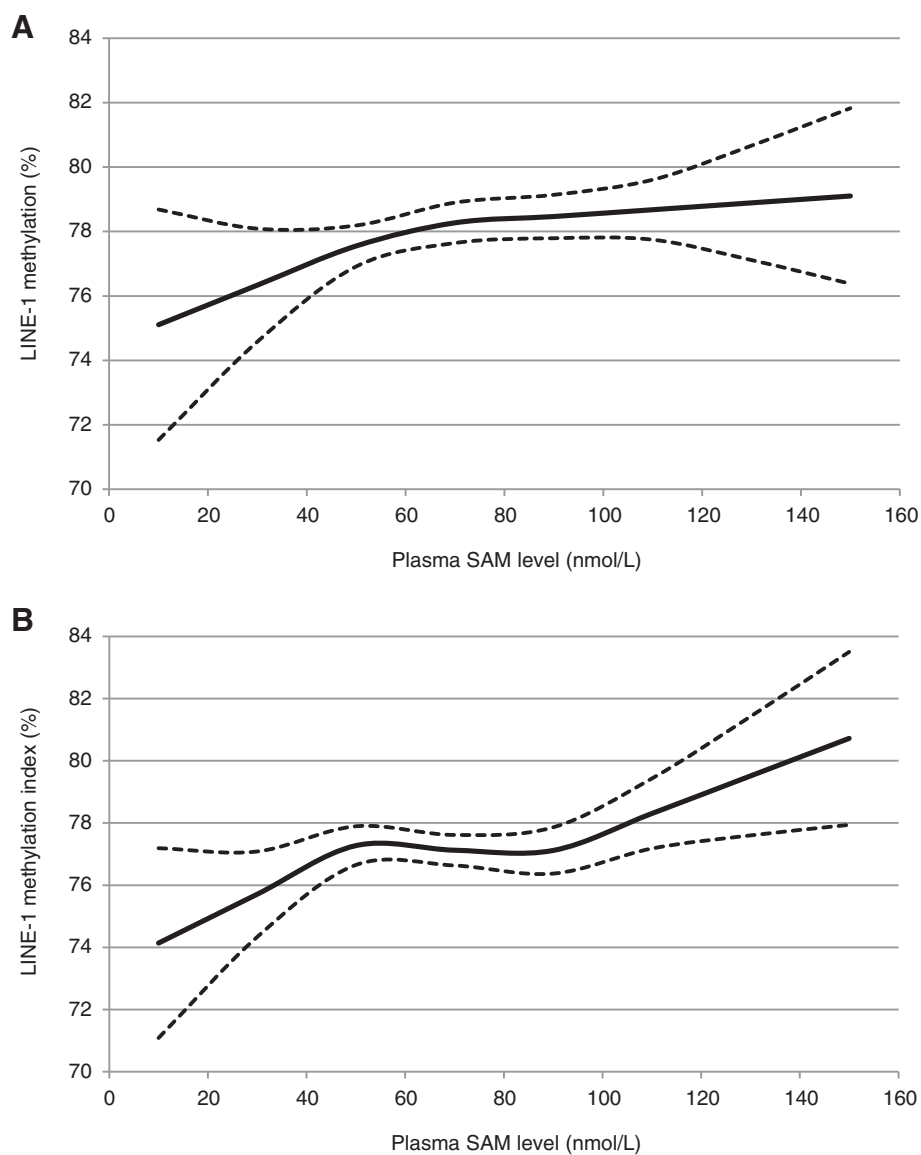

Figure 1 LINE-1 methylation and plasma SAM level in men (A) and women (B). * Adjusted for age at blood draw and serum creatinine level. ** Solid lines: mean LINE-1 methylation index corresponding to the mean plasma SAM level; dashed lines: upper and lower 95% confidence intervals.

Table 3 LINE-1 methylation index (%) by plasma SAM level in the Singapore Chinese health study

Men				Women			
SAM (nmol/L)	n	LINE-1 methylation[a]	p	SAM (nmol/L)	n	LINE-1 methylation[a]	p_{trend}
< 55	38	77.3 (76.6 – 78.0)	0.01	< 50	48	76.6 (75.9 – 77.2)	0.005
≥ 55	154	78.3 (77.9 – 78.6)		50 – < 90	180	77.5 (77.1 – 77.8)[b]	
				≥ 90	20	78.3 (77.2 – 79.4)[c]	

[a]Mean (95% CI) adjusted for age at blood draw and serum creatinine level.
[b]p = 0.02 compared to plasma SAM levels <50 nmol/L.
[c]p = 0.01 compared to plasma SAM levels <50 nmol/L.

SNPs examined (Table 4) or haplotypes of individual *DNMT*s (data not shown).

Finally, we examined whether the *DNMT1* rs2114724 genotype modified the association between plasma SAM level and LINE-1 methylation index (Figure 2). Below the threshold of 78% LINE-1 methylation index (55 nmol/L plasma SAM), plasma SAM level was positively associated with LINE-1 methylation index in men carrying the CC genotype of *DNMT1* rs2114724, while there was no association between plasma SAM and LINE-1 methylation index in men possessing the CT or TT genotype. Among men carrying variant genotypes, LINE-1 methylation index was constantly at or above 78% regardless of plasma SAM level. Such an effect modification by the *DNMT1* rs2114724 genotype was not observed among women. Other SNPs of *DNMT1*, *DNMT3A* and *DNMT3B* did not modify the SAM-LINE-1 methylation association (data not shown).

Discussion

Inter-individual variation in DNA methylation, specifically global hypomethylation of DNA in circulating lymphocytes, has been associated with risk for several diseases including cancer. Our results showed that LINE-1 methylation index, as a surrogate marker of global DNA methylation status, was positively associated with plasma SAM level, and this dose-response relationship plateaued at approximately 78% methylation for men (at 55 nmol/L SAM) and 77% methylation for women (at 50 nmo/L SAM). These findings support the hypothesis that circulating SAM level influences global DNA methylation. In addition, we have described genetic variation in *DNMT1* that influences LINE-1 methylation and possibly modifies the association between plasma SAM and LINE-1 methylation in men.

One possible explanation for the observed plateau in LINE-1 methylation with increasing SAM is an active inhibition of DNA methylation by SAH. After providing a methyl moiety for methylation, SAM is converted to SAH, an established competitive inhibitor of DNMTs [36-38]. We have previously demonstrated a significant positive association between plasma levels of SAM and SAH in this study population [35]. However, neither plasma SAH level nor the SAM:SAH ratio was

associated with LINE-1 methylation index (data not shown). Future studies are needed to firmly establish the presence of this plateau and the biologic drivers of its formation.

Methyltransferase enzymes are necessary to establish and maintain the epigenome, and in mice, *DNMT1* mutation results in global DNA hypomethylation [39,40]. DNMT1 enzyme is expressed in proliferating cells and somatic tissues with a major function as a maintenance methyltransferase, copying the existing parental-strand DNA methylation pattern onto the daughter strand after DNA replication [20,21]. In contrast, DNMT3A and DNMT3B are enzymes that are highly expressed in embryonic cells, early embryos, and germ cells, where *de novo* DNA methylation occurs, but are downregulated in somatic tissues [41].

One striking observation across studies is consistently lower LINE-1 methylation among women [11,30,31]. The mechanism of this sex difference is not established. However, LINE-1 elements are believed to be involved in X chromosome inactivation [42] and the X chromosome is enriched nearly 2-fold for LINE-1 elements compared with autosomes [43]. A recent study reported that the major group of LINE-1 promoter regions were significantly hypomethylated on inactive X chromosomes compared with active X chromosomes [30]. They also reported that while total LINE-1 methylation extent was lower in women compared with men, methylation at specific LINE-1 elements on autosomes was not differentially methylated by gender. This differential biologic pressure for LINE-1 methylation on the sex chromosome may underlie the gender difference observed in our and other epidemiologic studies.

The present study has several limitations. One concern is the possible degradation of SAM in stored plasma samples during prolonged storage. The median plasma SAM level in the present study (63.3 nmol/L; interquartile range, 54.5 – 76.0 nmol/L) was somewhat lower compared with the reference values in populations in Europe (70 – 128 nmol/L) [44]. However, the wide range of plasma SAM value (50 – 150 nmol/L) in various studies might be due to different study populations and laboratory methods [36,45-50]. In our study population, we measured plasma tHcy level in the same subjects at

Table 4 LINE-1 methylation index (%) by genotypes of *DNMT1*, *DNMT3A* and *DNMT3B* in the Singapore Chinese health study

	Men				Women			
	n[a]	Mean (95% CI)[b]	*p*	*p*trend	n[a]	Mean (95% CI)[b]	*p*	*p*trend
DNMT1								
rs2114724								
CC	90	77.7 (77.2 – 78.1)		0.001	126	77.2 (76.8 – 77.6)		0.38
CT	71	78.3 (77.8 – 78.8)	0.06		98	77.6 (77.1 – 78.1)	0.25	
TT	21	79.3 (78.4 – 80.3)	0.002		20	77.4 (76.3 – 78.5)	0.75	
rs2241531								
GG	44	77.9 (77.2 – 78.5)		0.11	61	77.1 (76.5 – 77.7)		0.71
CG	94	78.1 (77.6 – 78.5)	0.60		121	77.5 (77.1 – 78.0)	0.29	
CC	46	78.6 (78.0 – 79.2)	0.11		63	77.3 (76.7 – 77.9)	0.70	
rs2288350								
CC	55	78.5 (77.9 – 79.1)		0.05	74	77.4 (76.8 – 78.0)		0.57
CT	93	78.1 (77.6 – 78.6)	0.31		115	77.5 (77.0 – 77.9)	0.85	
TT	38	77.6 (76.9 – 78.3)	0.05		55	77.1 (76.5 – 77.8)	0.53	
rs7253062								
GG	109	78.3 (77.9 – 78.7)		0.12	149	77.3 (76.9 – 77.7)		0.92
GA	72	77.8 (77.3 – 78.3)	0.13		83	77.5 (76.9 – 78.0)	0.71	
AA	4	77.6 (75.4 – 79.8)	0.54		11	77.1 (75.7 – 78.6)	0.78	
DNMT3A								
rs1550117								
CC	113	78.3 (77.8 – 78.7)		0.10	146	77.5 (77.1 – 77.9)		0.30
CT	58	78.0 (77.4 – 78.5)	0.39		92	77.1 (76.6 – 77.6)	0.24	
TT	15	77.3 (76.2 – 78.4)	0.12		8	77.3 (75.6 – 79.0)	0.83	
rs6722613								
GG	93	78.0 (77.6 – 78.5)		0.87	101	77.4 (76.9 – 77.9)		0.69
GA	69	78.2 (77.7 – 78.8)	0.58		112	77.4 (76.9 – 77.8)	0.93	
AA	24	78.0 (77.1 – 78.9)	0.93		33	77.2 (76.3 – 78.0)	0.65	
rs7575625								
AA	115	78.2 (77.8 – 78.6)		0.29	148	77.3 (76.9 – 77.7)		0.79
AG	59	78.2 (77.7 – 78.8)	0.86		83	77.3 (76.8 – 77.8)	0.98	
GG	11	77.0 (75.7 – 78.2)	0.08		15	77.6 (76.4 – 78.9)	0.66	
rs7581217								
CC	59	78.5 (78.0 – 79.1)		0.008	83	77.5 (77.0 – 78.0)		0.92
CT	94	78.1 (77.7 – 78.6)	0.27		114	77.1 (76.7 – 77.6)	0.26	
TT	33	77.2 (76.4 – 77.8)	0.006		49	77.6 (76.9 – 78.3)	0.90	
rs7587636								
GG	95	78.1 (77.6 – 78.5)		0.89	116	77.2 (76.8 – 77.7)		0.95
GA	68	78.2 (77.6 – 78.7)	0.86		103	77.6 (77.1 – 78.0)	0.32	
AA	22	78.1 (77.2 – 79.1)	0.94		27	77.0 (76.0 – 77.9)	0.57	
rs13036246								
CC	102	78.0 (77.5 – 78.4)		0.26	129	77.5 (77.1 – 77.9)		0.14
CT	68	78.2 (77.7 – 78.7)	0.47		106	77.3 (76.8 – 77.7)	0.48	
TT	15	78.6 (77.5 – 79.7)	0.31		10	76.1 (74.6 – 77.6)	0.08	
rs34048824								
TT	116	78.1 (77.7 – 78.5)		0.61	150	77.3 (76.9 – 77.7)		0.70
TC	58	78.3 (77.8 – 78.9)	0.59		77	77.3 (76.8 – 77.9)	0.99	
CC	10	77.1 (75.8 – 78.5)	0.18		18	77.7 (76.5 – 78.8)	0.57	

Table 4 LINE-1 methylation index (%) by genotypes of *DNMT1*, *DNMT3A* and *DNMT3B* in the Singapore Chinese health study (Continued)

DNMT3B							
rs2424908							
CC	61	77.9 (77.3 – 78.4)		0.83	88	77.4 (76.9 – 77.9)	0.88
CT	79	78.4 (77.9 – 78.9)	0.18		121	77.3 (76.9 – 77.8)	0.88
TT	44	77.9 (77.2 – 78.6)	0.96		37	77.3 (76.5 – 78.1)	0.91
rs6141813							
AA	82	78.0 (77.5 – 78.5)		0.76	103	77.2 (76.8 – 77.7)	0.74
AG	73	78.3 (77.8 – 78.8)	0.35		115	77.4 (77.0 – 77.9)	0.56
GG	30	78.0 (77.2 – 78.8)	0.99		27	77.3 (76.3 – 78.2)	0.94

[a]Subjects with missing data on a genotype of each SNP were excluded from an analysis.
[b]Adjusted for age at blood draw.
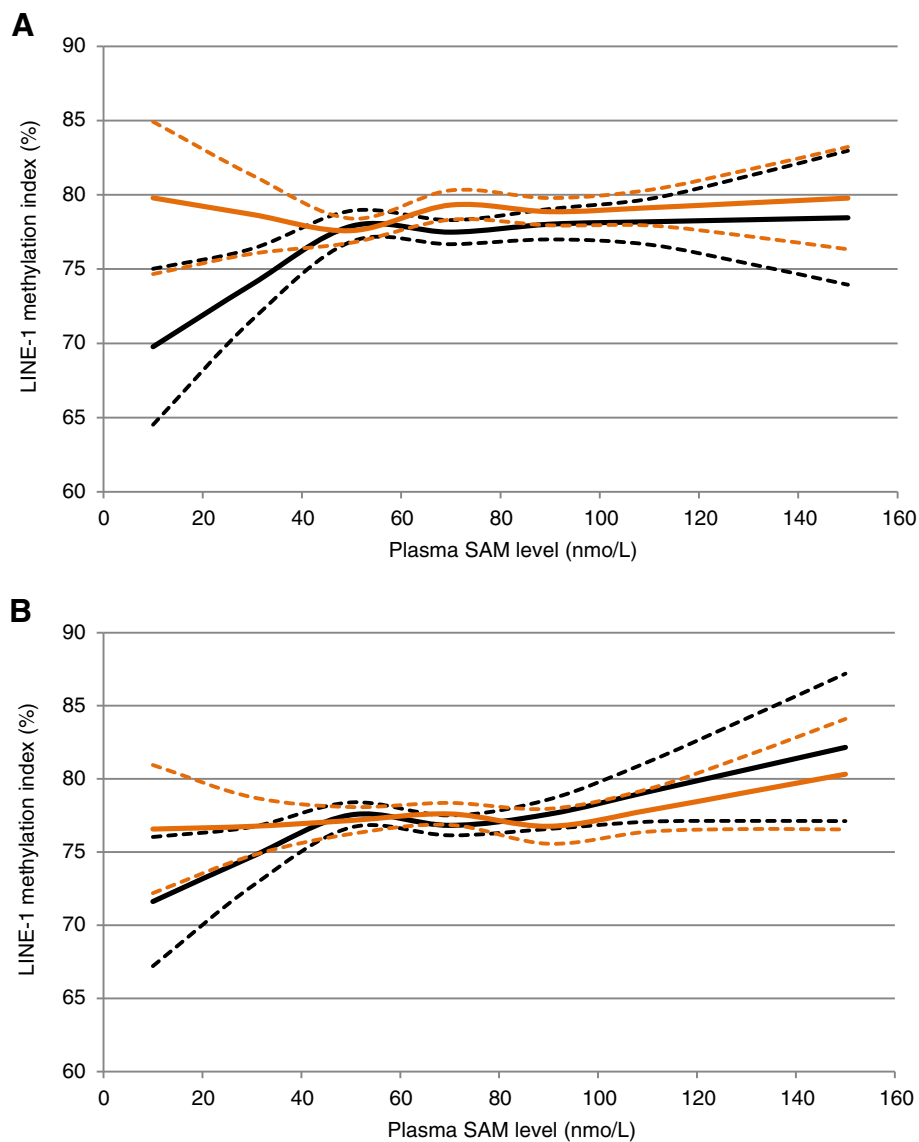
Figure 2 LINE-1 methylation and plasma SAM by *DNMT1* rs2114724 genotype in men (A) and women (B). [*] Adjusted for age at blood draw and serum creatinine level. [**] Black line: the wild type genotype (CC) (n = 90 for men and n = 126 for women); red line: variant genotypes (CT and TT) (n = 93 for men and n = 118 for women); dashed lines: upper and lower 95% confidence intervals.

two different times with more than 10 years apart (in 1996 – 1997 and in 2010). High correlation between the two tHcy measurements (r, 0.70) indicates that the degradation of Hcy, and possibly SAM, might have been minimal. Furthermore, degradation of plasma SAM would not result in the observed associations, given that it occurred in a non-differential manner. LINE-1 is the most common family of retrotransposons in the human genome and accounts for at least 17% of human DNA, but methylation extent at LINE-1 is not a direct measurement of global DNA methylation [51,52]. LINE-1 is dispersed across the genome; however, because there is a mix of both active and inactive LINE-1 elements present in the genome, it cannot be viewed as either a passive dosimeter of methylation processes or a reflection of methylation processes at active chromatin. Therefore, we are limited in our interpretation of LINE-1 methylation index in examining exposures and disease risk. Whether the methylation status of other surrogate markers of global DNA methylation across the genome are associated with plasma SAM levels remains to be investigated in future studies. Another limitation of the present study is potentially limited variability in LINE-1 methylation because all of the study subjects were cancer-free, healthy individuals. A relatively small sample size is also a limitation. We did not have enough power to test an interaction between plasma SAM, *DNMT* genetic polymorphisms and the LINE-1 methylation index. Lastly, our study did not investigate other factors that may affect the SAM-SAH ratio, such as glucose-6-phosphate dehydrogenase deficiency, folate transport deficiency, and other genetic factors (e.g., glycine *N*-methyltransferase: *GNMT*), as cofounders. Nonetheless, our data suggest that genetic variation in *DNMT* may influence LINE-1 methylation index in peripheral blood in a Chinese population in South Asia.

Conclusion
Our findings provide supporting data for the association between circulating SAM level and DNA methylation at LINE-1 sequences in peripheral blood in healthy Chinese men. While preliminary, our data also suggest that this link between plasma SAM level and global DNA methylation at LINE-1 sequences in men may be modified by *DNMT1* genetic variation.

Abbreviations
BMI: Body mass index; DNMT: DNA methyltransferase; ESI: Electrospray ionization; HPLC: High performance liquid chromatography; LINE-1: Long interspersed nucleotide element-1; MAF: Minor allele frequency; MS: Mass spectrometry; OCM: One-carbon metabolism; PCR: Polymerase chain reaction; SAH: *S*-adenosylhomocysteine; SAM: *S*-adenosylmethionine; SCHS: Singapore Chinese Health Study; SNP: Single nucleotide polymorphism.

Competing interests
None of the authors had a conflict of interest to declare.

Author details
[1]Division of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minneapolis, USA. [2]Masonic Cancer Center, University of Minnesota, Minneapolis, USA. [3]Department of Epidemiology and Biostatistics, George Washington University, Washington, USA. [4]Institute of Metabolic Disease, Baylor Research Institute, Dallas, USA. [5]Saw Swee Hock School of Public Health, National University of Singapore, Singapore, Singapore. [6]Duke-NUS Graduate Medical School, Singapore, Singapore. [7]Cancer Control and Population Sciences, University of Pittsburgh Cancer Institute, Pittsburgh, USA. [8]Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, USA.

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