Original Article

LINE-1 Hypomethylation is Associated with the Risk of Coronary Heart Disease in Chinese Population

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Abstract

Background: Global methylation level in blood leukocyte DNA has been associated with the risk of coronary heart disease (CHD), with inconsistent results in various populations. Similar data are lacking in Chinese population where different genetic, lifestyle and environmental factors may affect DNA methylation and its risk relationship with CHD.

Objectives: To examine whether global methylation is associated with the risk of CHD in Chinese population.

Methods: A total of 334 cases with CHD and 788 healthy controls were included. Global methylation in blood leukocyte DNA was estimated by analyzing LINE-1 repeats using bisulfite pyrosequencing.

Results: In an initial analysis restricted to control subjects, LINE-1 level reduced significantly with aging, elevated total cholesterol, and diagnosis of diabetes. In the case-control analysis, reduced LINE-1 methylation was associated with increased risk of CHD; analysis by quartile revealed odds ratios (95%CI) of 0.9 (0.6-1.4), 1.9 (1.3-2.9) and 2.3 (1.6-3.5) for the third, second and first (lowest) quartile ($P_{trend} < 0.001$), respectively, compared to the fourth (highest) quartile. Lower (<median) LINE-1 methylation was associated with a 2.2-fold (95%CI = 1.7-3.0) increased risk of CHD. The lower LINE-1-related CHD risk estimates tended to be stronger among subjects with the highest tertile of homocysteine ($P_{interaction} = 0.042$) and those with diagnosis of hypertension ($P_{interaction} = 0.012$).

Conclusion: LINE-1 hypomethylation is associated with the risk of CHD in Chinese population. Potential CHD risk factors such as older age, elevated total cholesterol, and diagnosis of diabetes may have impact on global DNA methylation, whereby exerting their effect on CHD risk. (Arq Bras Cardiol. 2014; 102(5):481-488)

Keywords: Epigenetics; Coronary Heart Disease; Global Methylation; LINE-1; Blood Leukocyte DNA

Introduction

Coronary heart disease (CHD) constitutes 90-95% of all cases with cardiovascular diseases, which rank the leading cause of death in the world[1,2]. Age, sex, diets, cigarette smoking, hypertension, diabetes, dyslipidemia, homocysteine, obesity, family history of CHD and genetic factors have been characterized to play a major role in the CHD etiology[2-4].

Epigenetic modification, especially aberrant global DNA methylation pattern, is increasingly recognized as a key factor in the development of CHD. Global DNA hypomethylation may induce genomic instability and deregulate gene transcription, thereby contributing to the development of various human diseases including CHD[5]. In animal models, global DNA hypomethylation has been associated with aortic lipid deposition, a predictor of future atherosclerosis[6,7]. Further, an association between global DNA hypomethylation and susceptibility to subclinical atherosclerosis has been reported in young adults[8]. In addition, in healthy subjects, global methylation level measured in blood leukocyte DNA has been associated with risk factors for CHD, such as aging, cigarette smoking, folate deficiency, hyperhomocysteinemia, higher levels of low density lipoprotein and lower levels of high density lipoprotein[9-14]. Several epidemiological studies have reported that global methylation level could be a determinant risk factor for CHD, but the results were inconsistent in various populations[15-17]. Similar data are lacking in Chinese population where different genetic, lifestyle and environmental factors may affect DNA methylation and its risk relationship with CHD[18, 19].

About 55% of human genome consists of repetitive elements[20], including approximately 500,000 LINE-1 repeats which represent approximately 17% of the human genome[21]. Because of high representation throughout the genome and heavy methylation in normal tissue, LINE-1 has been used as a surrogate marker for estimating global DNA methylation levels[22,23].

In the present study, we have investigated the relationship between LINE-1 methylation and the risk of CHD in a Chinese

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population. We also examined the association between LINE-1 methylation and potential risk factors for CHD including age, sex, body mass index (BMI), total cholesterol, triglyceride, homocysteine, smoking status, hypertension and diabetes, as well as their modifying effect on the LINE-1-related risk estimate for CHD.

Methods

Study participants

This case-control study enrolled 334 patients with CHD and 788 control subjects from the Fourth Affiliated Hospital, Harbin Medical University, Harbin, China from March 2007 to September 2010. All participants were unrelated individuals from the Chinese Han population, and the controls were frequency-matched to the cases by age (± 5 years) and sex. The diagnosis of CHD was established by angiographic evidence of ≥70% stenosis of 1 major coronary artery, or ≥ 50% of the left main coronary artery. The controls filled a regular health survey and their detailed examination by history analysis, physical examination, electrocardiography, and echocardiography revealed absence of CHD or other heart diseases. For both CHD and control groups, subjects with spastic angina pectoris, peripheral artery disease, and any kind of autoimmune-related disease or cancers were excluded. Information on age, sex, height, weight, cigarette smoking, and diagnosis of hypertension and diabetes was obtained by using a structured questionnaire through in-person interviews. An ever-smoker was defined as a smoker of at least 1 cigarette per day for at least 6 months. BMI was calculated using the formula: body weight in kilograms divided by the square of body height in meters (kg/m^2). Information on serum total cholesterol, triglyceride and plasma homocysteine was collected on the basis of medical records. Written informed consent was obtained from each participant. The study protocol was approved by the ethics review committee of the Institutional Review Board of the participant hospital.

DNA methylation analysis of LINE-1 repetitive elements

Genomic DNA was extracted from blood leukocytes using the QIAamp DNA Blood Extraction kit (Qiagen, Shanghai, China), and then modified by treatment with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo, CA, USA), following the manufacturer's protocol. A modified method of PCR-based pyrosequencing originally described by Yang et al[22] was performed to quantitate methylation of the LINE-1 repetitive elements. In brief, PCR was carried out in a 50-μl reaction volume containing 25 μl of GoTaq Green Master mix (Promega, WI, USA), 1 pmol of forward primer (TTT TGA GTT AGG TGT GGG ATA TA), 1 pmol of biotinylated reverse primer (biotin-AAA ATC AAA AAA TTC CCT TTC), and 50 ng of bisulfite-treated genomic DNA. PCR cycling conditions were 95°C for 30 s, 50°C for 30 s and 72°C for 30 s for 40 cycles. PCR product was bound to streptavidin sepharose beads (Amersham Biosciences, Uppsala, Sweden). These sepharose beads containing the bound PCR product were purified, washed, denatured and washed again. Then, 0.3-μM pyrosequencing primer (AGT TAG GTG TGG GAT ATA GT) was annealed to the purified single-stranded PCR product. Pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System. For all assays we used built-in controls to verify bisulfite conversion. Each sample was assayed in duplicate and their average was used in final analysis. The degree of LINE-1 methylation was expressed as % 5-methylated cytosines (%5mC) over total (methylated + unmethylated) cytosines.

Statistical analysis

Chi-square test was used to examine the differences in the distributions of categorical variables and Student's *t*-test for the differences in means of continuous variables between CHD cases and control subjects. Linear regression models were used to evaluate LINE-1 differences among controls in relation to age, sex, BMI, total cholesterol, triglyceride, homocysteine, smoking status, hypertension and diabetes. Unconditional logistic regression was used to estimate odds ratio (OR) for CHD and 95% confidence interval (CI). Quartile and median cut-points were based on LINE-1 distributions among controls. All models were adjusted for age (as a continuous variable), sex, and smoking status (ever-smoker: yes vs no). Further adjustment by other potential confounding variables, including BMI, total cholesterol, triglyceride, homocysteine, hypertension and diabetes, did not materially alter the risk estimates. Therefore, these variables were not included in the final models. Effect modification by individual covariates was assessed using likelihood-ratio tests. All tests were two-sided and a *P* value of <0.05 was considered significant. Statistical analyses were conducted using the Stata 10.1 (Stata Corporation, College Station, TX).

Results

The potential risk factors of study subjects by case and control status are shown in Table 1. No significant differences between CHD cases and control subjects were found in the distributions of age, sex, BMI, or triglyceride. When compared with the controls, CHD cases were more likely to be ever-smoker, and tended to have higher levels of total cholesterol and homocysteine. Cases also were likely to have diagnosis of hypertension and diabetes.

The effects of potential risk factors on LINE-1 methylation level among control subjects are shown in Table 2. As expected, LINE-1 level reduced significantly with increasing age ($p = 0.016$). We also observed that total cholesterol ($p < 0.001$) and diagnosis of diabetes ($p < 0.001$) were inversely associated with LINE-1 methylation levels. No statistically significant relationships were observed between LINE-1 methylation level and sex, smoking status, BMI, triglyceride, homocysteine, or diagnosis of hypertension.

CHD cases had significantly reduced LINE-1 methylation level than controls (mean (standard deviation, SD): 80.96 (2.40) vs 81.67 (2.46), $p < 0.001$) (Table 1). Analyses of LINE-1 in quartiles, based on the distribution in controls, show that the LINE-1 methylation level was inversely associated with the risk of CHD (Table 3). Relative to subjects in the fourth (highest) quartile of LINE-1 methylation, ORs

Table 1 – Potential risk factors of study subjects

Potential risk factors	Cases, n (%)	Controls, n (%)	p value*
Age (tertile), years			
< 59	96 (28.7)	266 (33.8)	
60-65	119 (35.6)	236 (29.9)	
> 65	119 (35.6)	286 (36.3)	0.12
Sex			
Female	66 (19.8)	150 (19.0)	
Male	268 (80.2)	638 (81.0)	0.78
Ever-smoker			
No	156 (47.4)	590 (75.4)	
Yes	173 (52.6)	193 (24.6)	< 0.001
Body mass index (tertile), kg/m²			
< 22.0	97 (29.1)	262 (33.2)	
22.0-25.4	122 (36.5)	264 (33.6)	
> 25.4	115 (34.4)	262 (33.2)	0.36
Total cholesterol (tertile), mmol/L			
< 2.9	57 (17.1)	262 (33.2)	
2.9-4.8	123 (36.8)	263 (33.4)	
> 4.8	154 (46.1)	263 (33.4)	< 0.001
Triglyceride (tertile), mmol/L			
< 1.1	113 (33.8)	265 (33.7)	
1.1-1.6	111 (33.2)	258 (32.8)	
> 1.6	110 (33.0)	263 (33.5)	0.98
Homocysteine (tertile), µmol/L			
< 9	70 (21.6)	241 (32.3)	
9-11	106 (32.7)	252 (33.7)	
> 11	148 (45.7)	254 (34.0)	< 0.001
Hypertension			
No	128 (38.3)	520 (66.0)	
Yes	206 (61.7)	268 (34.0)	< 0.001
Diabetes			
No	212 (63.5)	638 (81.0)	
Yes	122 (36.5)	150 (19.0)	< 0.001
Mean LINE-1 (SD)	80.96 (2.40)	81.67 (2.46)	< 0.001†

*SD: standard deviation. *P value obtained from a X^2-test comparing cases and controls. †P value obtained from a Student's t-test comparing cases and controls.*

for CHD were 0.9 (95% CI, 0.6-1.4), 1.9 (95% CI, 1.3-2.9) and 2.3 (95% CI, 1.6-3.5) for the subjects with methylation in the third, second and first (lowest) quartile ($P_{trend} < 0.001$), respectively. When using the alternative cut-point based on the median, individuals with lower (<median) LINE-1 methylation had a 2.2-fold (95% CI, 1.7-3.0) increased risk of CHD compared with subjects with higher (> median) LINE-1 methylation.

When stratified by potential risk factors, the lower LINE-1-related CHD risk estimates tended to be stronger among subjects with the highest tertile of homocysteine (OR = 3.2, 95% CI, 3.2-5.2) and those with diagnosis of hypertension (OR = 3.1, 95 %CI, 2.1-4.8) (Table 4). Statistically significant interactions in relation to CHD risk were observed between LINE-1 methylation and homocysteine level ($P_{interaction} = 0.042$), and between

Table 2 – Relation of potential CHD risk factors to LINE-1 methylation levels among control subjects

Potential risk factors	n	LINE-1, mean (95% CI)*	P value*
Age (tertile), years†			
< 59	266	81.58 (81.33-81.83)	
60-65	236	81.47 (81.22-81.72)	
> 65	286	81.21 (80.97-81.45)	0.016
Sex‡			
Female	150	81.47 (81.14-81.81)	
Male	638	81.40 (81.24-81.56)	0.46
Ever smoker§			
No	590	81.59 (81.41-81.76)	
Yes	193	81.05 (80.80-81.31)	0.35
Body mass index (tertile), kg/m²			
< 22.0	262	81.41 (81.16-81.66)	
22.0-25.4	264	81.24 (80.99-81.48)	
> 25.4	262	81.58 (81.34-81.83)	0.54
Total cholesterol (tertile), mmol/L			
< 2.9	262	81.83 (81.57-82.10)	
2.9-4.8	263	81.57 (81.34-81.81)	
> 4.8	263	80.95 (80.72-81.18)	< 0.001
Triglyceride (tertile), mmol/L			
< 1.1	265	81.39 (81.15-81.64)	
1.1-1.6	258	81.43 (81.18-81.68)	
> 1.6	263	81.42 (81.18-81.67)	0.42
Homocysteine (tertile), µmol/L			
< 9	241	81.53 (81.26-81.80)	
9-11	252	81.37 (81.12-81.63)	
> 11	254	81.41 (81.17-81.65)	0.91
Hypertension			
No	520	81.53 (81.34-81.72)	
Yes	268	81.25 (81.03-81.47)	0.59
Diabetes			
No	638	81.59 (81.43-81.75)	
Yes	150	80.85 (80.56-81.14)	< 0.001

*CHD: Coronary heart disease; CI: Confidence interval. *Adjusted for age, sex, and smoking status. †Only adjusted for sex and smoking status. ‡Only adjusted for age and smoking status. §Only adjusted for age and sex.*

LINE-1 methylation and hypertension ($P_{interaction}$ = 0.012). Stratification by other potential risk factors including age (tertile), sex (female, male), smoking status (ever-smoker: no, yes), BMI (tertile), total cholesterol (tertile), triglyceride (tertile), and diagnosis of diabetes (no, yes), produced comparable risk estimates (data not shown). The interactions between LINE-1 methylation and these factors on CHD risk were not statistically significant (data not shown).

Discussion

In the present study, we demonstrated a statistically significant, inverse relationship between LINE-1 methylation level and CHD risk in the Chinese population. Homocysteine level and diagnosis of hypertension modified this inverse relationship. Considering that CHD is one of the most common diseases, along with its severity[24], the risk factor

Table 3 – LINE-1 methylation levels in relation to CHD risk

LINE-1 (%5mC)	Cases, *n* (%)	Controls, *n* (%)	OR (95% CI)*
Quartile†			
Q4(> 82.73)	55 (16.5)	197 (25.0)	1.0 (reference)
Q3(81.52-82.72)	49 (14.7)	194 (24.6)	0.9 (0.6-1.4)
Q2(80.17-81.51)	101 (30.2)	199 (25.3)	1.9 (1.3-2.9)
Q1(< 80.16)	129 (38.6)	198 (25.1)	2.3 (1.6-3.5)
			P_{trend} < 0.001
Median†			
High(≥ 81.52)	104 (31.1)	391 (49.6)	1.0 (reference)
Low(< 81.52)	230 (68.9)	397 (50.4)	2.2 (1.7-3.0)

*%5mC: % 5-methylated cytosines; CHD: coronary heart disease; CI: confidence interval; OR: odds ratio.*Adjusted for age, sex, and smoking status. †The quartiles and the median of LINE-1 measures were based on values among control subjects.*

Table 4 – LINE-1 level in relation to CHD risk, by homocysteine level and diagnosis of hypertension

Potential risk factors	LINE-1*	Cases, *n* (%)	Controls, *n* (%)	OR (95% CI)†
Homocysteine, µmol/L				
< 9	High	24 (34.3)	126 (52.3)	1.0 (reference)
	Low	46 (65.7)	115 (47.7)	2.2 (1.2-4.0)
9-11	High	41 (38.7)	115 (45.6)	1.0 (reference)
	Low	65 (61.3)	137 (54.4)	1.4 (0.9-2.3)
> 11	High	36 (24.3)	135 (53.1)	1.0 (reference)
	Low	112 (75.7)	119 (46.9)	3.2 (2.0-5.2)
Hypertension				
No	High	51 (39.8)	251 (48.3)	1.0 (reference)
	Low	77 (60.2)	269 (51.7)	1.5 (1.0-2.3)
Yes	High	53 (25.7)	140 (52.2)	1.0 (reference)
	Low	153 (74.3)	128 (47.8)	3.1 (2.1-4.8)

*CHD: coronary heart disease; CI: confidence interval; OR: odds ratio. *The median of LINE-1 measure was based on values among control subjects. High: ≥ 81.52 (%5mC); Low: < 81.52 (%5mC). †Adjusted for age, sex, and smoking status.*

of global hypomethylation could have a sizable impact on public health. Because DNA methylation is a reversible epigenetic mechanism, this blood-based marker could offer exciting new opportunities for population-based CHD prevention as well as risk assessment[25].

Global DNA methylation levels, assessed in repeat regions from leukocyte-derived DNA, have been reported to be associated with risk of CHD in American, Singapore, and Indian populations, with inconsistent observations[15-17]. In a cohort of 712 elderly men from American population, LINE-1 hypomethylation was associated with increased risk for ischemic heart disease in both cross-sectional and longitudinal analyses[15]. This is consistent with the findings in the present study. However, in our data, there were also significant relationships among women as well as in different age groups between LINE-1 methylation and CHD risk, which provide further data to what was previously observed in the American population[15]. On the other hand, in a Singapore population, higher methylation levels of Alu/Sat2 repeats were reported in males with a history of myocardial infarction or stroke (n = 8) than in control males (n = 121)[16]; In an Indian population, higher methylation levels of CCGG sequences were reported in cases with coronary artery disease (n = 137) than in controls (n = 150)[17]. The different lifestyle, environmental exposures, and genetic backgrounds among the populations, and differences in sample size and repetitive elements of DNA targeted for measuring global hypomethylation levels[26-28], might contributed to the different findings across these studies.

The modifying effect of age on global DNA methylation levels, consistent with previous data[29], may reflect age-related cumulative effects of enviromental exposures to risk factors for CHD. Global hypomethylation in leukocyte DNA has been

associated with exposure to multiple types of enviromental pollutants such as polycyclic aromatic hydrocarbons, particulate air pollution, black carbon and sulfates[30-32]. The present study also showed that two recognized CHD risk factors, elevated total cholesterol and diagnosis of diabetes, were related to reduced LINE-1 methylation levels, further supporting a role of DNA hypomethylation in the development of CHD.

In our data, there was a lack of association between LINE-1 methylation and homocysteine level or diagnosis of hypertension, which differs somewhat from what was observed previously[10,33]. However, we observed that both homocysteine level and diagnosis of hypertension modified the inverse relationship between LINE-1 methylation and CHD risk. Further studies are warranted to replicate these findings and to elucidate the underlying mechanism of homocysteine level and diagnosis of hypertension on their modifying role in the association between global hypomethylation and CHD risk.

Our study had the advantages of being based on a relatively large sample size, diagnosis of CHD by angiographic evidence, and accurate quantitative analysis using pyrosequencing methodology, which is suitable for measuring subtle changes in DNA methylation. The chief limitation of the present study was the retrospective nature of the study design, i.e., the collection of blood samples from CHD patients took place after their CHD diagnosis. If the diagnosis of CHD had any direct or indirect impact on global DNA methylation through changing subject's lifestyle or environmental exposure, we could have observed a confounded or biased association between LINE-1 methylation and CHD risk. However, similar risk estimates were observed when the logistic regression analysis was restricted to 212 newly diagnosed CHD cases as compared to controls (data not shown). Moreover, our finding that LINE-1 methylation levels were reduced in control subjects with CHD risk factors such as older age, elevated total cholesterol, and diagnosis of diabetes, indicates that reduced LINE-1 methylation in high-risk control subjects may result from exposures related to the risk of CHD. In addition, in the longitudinal analyses in the study by Baccarelli et al[31], lower LINE-1-related increased risk of ischemic heart disease has been observed.

Conclusion

The findings of the present study support global DNA hypomethylation measured in LINE-1 repeats is associated with the risk of CHD in the Chinese population. Potential CHD risk factors such as older age, elevated total cholesterol and diagnosis of diabetes may have impact on global DNA methylation, whereby exerting their effect on CHD risk.

Author contributions

Conception and design of the research and Analysis and interpretation of the data: Wei L, Liu S, Su Z, Cheng R, Bai X, Li X; Acquisition of data: Wei L, Su Z, Cheng R; Statistical analysis: Wei L, Bai X, Li X; Writing of the manuscript: Wei L, Liu S, Su Z, Cheng R, Bai X; Critical revision of the manuscript for intellectual content: Li X.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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