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Global DNA methylation and risk of subclinical atherosclerosis in young adults: The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study

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Abstract

Objective—The association between hepatic global DNA methylation measured using pyrosequencing technology and the risk of subclinical atherosclerosis was examined in the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. PDAY is a bi-racial investigation of the natural history of atherosclerosis and its risk factors involving 3013 individuals aged 15–34 years who underwent autopsy after dying of unrelated causes in 1987–1994.

Methods—Raised atherosclerotic lesions were defined as the sum of the percentages of intimal surface area detected in the right coronary artery and left half of the abdominal and thoracic aorta harboring fibrous plaques, complicated lesions, and calcified lesions during a postmortem pathological examination. To conduct the case–control study, 300 cases selected with the highest raised lesion scores were paired with 300 controls without raised lesions after matching for age, race, and gender.

Results—Global DNA methylation was not associated with disease risk in the study population considered as a whole using conditional logistic regression models to analyze matched pairs. Since the estimation of the risk of atherosclerosis associated with inter-individual variation in DNA methylation was similar if unconditional logistic regression was used, subgroup analyses were carried out after adjusting for matching variables. A modest association with methylation levels below the median value was found in white but not in African-American study participants (odds ratio = 1.59, 95% confidence interval = 1.02–2.49, $p = 0.04$).

Conclusions—Hepatic global DNA methylation does not appear to be a definitive determinant of atherosclerosis burden in a postmortem sample of young adults.

Keywords

Coronary heart disease; Epigenetics; DNA methylation; Molecular epidemiology

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Conflicts of interest

The authors declare no conflicts of interest.

1. Introduction

Coronary heart disease and stroke are the first and third leading causes of death in developed countries and can be largely attributed to deposition and rupture of atherosclerotic plaques in the coronary and carotid arteries [1]. Several lines of evidence suggest that alterations in global rather than gene-specific DNA methylation patterns may be implicated in atherosclerosis. A significant reduction in global 5-methylcytosine content was found in advanced atherosclerotic lesions from patients with coronary heart disease, in smooth muscle cells, and in mice lacking apolipoprotein E (*Apoe*), a widely used animal model of atherosclerosis due to defective lipid metabolism [2–5]. More recently, a higher level of global DNA methylation in the peripheral blood leukocytes of patients with prevalent coronary heart disease when compared to controls has been observed in two studies, while lower methylation of LINE-1 repeats has been shown to predict both baseline and incident ischemic heart disease and stroke in a cohort of elderly individuals from the Boston-area Normative Aging Study [6–8].

In this study, hepatic global methylation status was examined using a case–control design in which 600 subjects were selected from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. PDAY is a bi-racial investigation of the development and progression of subclinical atherosclerosis involving 3013 individuals aged 15–34 who underwent autopsy within 48 h following violent death [9,10]. The PDAY study assessed the relationship between pathologically defined atherosclerotic lesions and postmortem risk factors for coronary heart disease including serum lipoprotein levels, smoking, hypertension, glycohemoglobin levels, and obesity. The sum of the percentages of intimal surface area detected in the right coronary artery and left half of the abdominal and thoracic aorta harboring fibrous plaques, complicated lesions, and calcified lesions was defined as a raised lesion. All of the aortas and approximately half of the coronary arteries examined in individuals 15–19 years of age had fatty streaks or raised lesions, increasing to about 75% in those 30–34 years of age, suggesting that the pathological processes leading to atherosclerosis are initiated in childhood and early adolescence [11]. For the case–control study, 300 cases with the highest raised lesion scores were paired with 300 controls free of raised lesions after matching for age, race, and gender. Global DNA methylation content was measured in genomic liver DNA derived from the PDAY study participants by a high throughput luminometric methylation assay (LUMA) using pyrosequencing technology [12].

2. Materials and methods

2.1. Pathobiological Determinants of Atherosclerosis in Youth study

The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study is a bi-racial investigation of the development and progression of atherosclerosis involving 3013 individuals aged 15–34 who underwent autopsy within 48 h following death from external causes including accidents, homicides, and suicide [9]. The PDAY study, initiated in 1987, assessed the relationship between established risk factors for coronary heart disease and pathologically defined atherosclerotic lesions. Fifteen cooperating centers in eight communities adopted standardized procedures to collect tissue specimens and blood from individuals autopsied in the cooperating medical examiners' laboratories. Three pathologists independently evaluated each right coronary artery and left half of the abdominal and thoracic aorta collected from each study subject, and visually estimated the percent of intimal surface occupied by fatty streaks, fibrous plaques (firm elevated lesion covered by a fibrous cap), complicated lesions (plaque with hemorrhage, thrombosis, or ulceration), and calcified lesions. The sum of the percentages of intimal surface area harboring fibrous plaques, complicated lesions, and calcified lesions after adjusting for age, age squared, race, and gender was defined as a "raised lesion" and is the phenotype analyzed in this study. The

cases selected for the nested case–control study were 300 individuals with the highest raised lesion scores for whom a DNA sample was available for analysis. The control group was chosen from subjects with no raised lesions after matching for age, race, and gender (n = 300). The Institutional Review Board of each participating center approved the PDAY study. The current study was approved by the Committee for the Protection of Human Subjects, the Institutional Review Board for the University of Texas Health Science Center at Houston.

2.2. Clinical and laboratory measurements

The risk factors for atherosclerosis examined in the PDAY study included serum lipoprotein levels, smoking, hypertension, glycohemoglobin levels, and obesity. All of these covariates were found to be determinants of the transition from fatty streaks to fibrous plaques, and were also associated with lesion features [10]. Total serum cholesterol and high density lipoprotein (HDL) cholesterol were measured enzymatically and the level of non-HDL cholesterol was calculated by subtraction. Smoking status was defined by the presence or absence of a serum thiocyanate level ≥ 90 mol/L. Hypertension was defined as a mean arterial blood pressure ≥ 110 mmHg as indicated by the intimal thickness of small renal arteries. Individuals with a body mass index (BMI) ≥ 30 kg/m^2 were considered obese. Red blood cell glycated hemoglobin ≥8% was a measure of hyperglycemia.

2.3. DNA isolation and quantification

DNA from participants in the PDAY study was isolated using liver samples collected at autopsy. The liver samples (50 g) were frozen in liquid nitrogen before shipment for storage at −80 °C. DNA was extracted from liver specimens using a salting out procedure [13]. In brief, nuclei were prepared and treated with 5% dodecyl sulfate and proteinase K (100 μg/ml) overnight at 55 °C. Proteins were removed by precipitation in 2.5 M NaCl, and the DNA was precipitated in ethanol. The DNA was then harvested using glass pipettes prior to resuspension in water at a final concentration of 1 mg/ml. All tubes were barcoded during the isolation process. After DNA extraction, the samples were quantified using the PicoGreen assay (Invitrogen, Carlsbad, CA), aliquoted, and normalized to appropriate concentrations for downstream applications.

2.4. Methylation analysis by pyrosequencing

Global DNA methylation content was measured using genomic liver DNA derived from PDAY study participants by a high throughput luminometric assay using pyrosequencing technology [12]. For the LUMA (Luminometric Methylation Assay) procedure, 0.2 μg of genomic DNA was divided into two separate aliquots for restriction enzyme digestion with one of a pair of isoschizomers that is either sensitive (*Hpa*II) or insensitive (*Msp*I) to the methylation status of an internal CpG dinucleotide in their common recognition site (5′-C/CGG-3′). *Eco*RI (5′-G/AATTC-3′) is also included in all reactions as an internal reference for DNA input. Following incubation for four hours at 37 °C, the extent of cleavage is measured in a polymerase extension assay in which dNTPs are provided in four successive steps: (1) dATP; (2) dGTP + dCTP; (3) dTTP; and (4) dGTP + dCTP. Addition of a given dNTP to an *Hpa*II-*Eco*RI or *Msp*I-*Eco*RI restriction fragment produces inorganic pyrophosphate that is used as a substrate for conversion to ATP by ATP sulfurylase. Luciferin present in the reaction mixture is subsequently converted to oxyluciferin by ATP and luciferase to produce light that is proportional to the amount of pyrophosphate released and that can be detected by a charge coupled device camera. Global DNA methylation status was assessed by the *Hpa*II-*Eco*RI/*Msp*I-*Eco*RI ratio which ranges from a maximum of 1.0 if the DNA sample is completely unmethylated to a minimum of 0.0 if it is totally methylated. All assays were performed in duplicate for each DNA sample using a PSQ 96 HQ pyrosequencing instrument (QIAGEN Inc., Valencia, CA). There was no significant

difference between replicate measurements as assessed by Student's paired *t*-test ($p = 0.19$). Unmethylated pBlue-script II plasmid DNA (Agilent Technologies, Santa Clara, CA) with an expected *Hpa*II-*Eco*RI/*Msp*I-*Eco*RI ratio of 1.0 was included as a control for all measurements.

2.5. Statistical analysis

All subjects in the nested case control study were matched for age (±1 year), race, and gender. Conditional logistic regression was used as a statistical method to evaluate the relative risk or odds of disease case status that allows for adjustment for potential confounders and covariates in a matched pairs analysis. For analysis of subclinical atherosclerosis, the sum of percent intimal surface area of the abdominal aorta, thoracic aorta, and right coronary artery that is involved with raised atherosclerotic lesions was used to discriminate between cases and controls. In secondary analyses, the risk of atherosclerosis was estimated using unconditional logistic regression models adjusted for the three matching variables. The application of conditional and unconditional logistic regression models to evaluate the effect of global DNA methylation gave similar results, and both are presented in this report. The proportions, means, and standard deviations for covariates included in the analyses were calculated for the case and comparison groups. Global DNA methylation was modeled both as a continuous independent variable, and as a categorical variable with high global methylation index defined as a LUMA *Hpa*II-*Eco*RI/*Msp*I-*Eco*RI ratio at or above the median for the study population and low global methylation index defined as a LUMA *Hpa*II-*Eco*RI/*Msp*I-*Eco*RI ratio below the median. Stata 9 software (StataCorp, College Station, TX) was used to perform the statistical analyses. A p-value < 0.05 was considered statistically significant for all tests.

3. Results

The clinical and demographic characteristics of the PDAY study participants selected for the nested case–control study are presented in Table 1. Since cases and controls were matched on age, gender, and ethnicity, there was no variation in the distribution of these variables between cases and controls. Cases had a significantly higher frequency of hypertension, obesity defined as a BMI ≥ 30, and smoking prevalence when compared to the controls, and also had a higher mean level of non-HDL cholesterol. Global DNA methylation status was modeled both as a continuous variable and as a categorical variable with a high global DNA methylation index defined as a LUMA methylation ratio that was greater than the median value observed for the study population (LUMA ratio ≥ 0.52). There was no difference in either the mean LUMA methylation ratio, or in the proportion of individuals with either a high or low global DNA methylation ratio, when cases and controls were compared.

The application of conditional and unconditional logistic regression models to evaluate global DNA methylation as a predictor of the risk of subclinical atherosclerosis (odds ratio (OR) conditional logistic regression = 1.22, 95% confidence interval (CI) = 0.90–1.66, $p = 0.21$; OR unconditional logistic regression = 1.24, 95% CI = 0.90–1.71, $p = 0.19$) gave similar results. All further analyses of the association between global DNA methylation and subclinical atherosclerosis were carried out using unconditional logistic regression models adjusted for the matching variables and are displayed in Table 2. In these analyses, the risk of subclinical atherosclerosis was estimated in subgroups defined by race and gender. A high global DNA methylation index was found to be a significant determinant of risk of atherosclerosis in white but not in African-American study participants. There was no difference between groups observed when the association was evaluated by gender (Table 2), or when methylation was considered as a continuous variable in the regression models (data not shown).

4. Discussion

Global DNA methylation status was determined in 600 participants in the PDAY study to evaluate whether variation in hepatic DNA methylation was associated with subclinical atherosclerosis in young adults matched for age, gender, and ethnicity. The liver is the site of synthesis of endogenous cholesterol and lipoproteins as well as acute-phase response reactants to inflammation such as C-reactive protein that have been shown to predict the risk of cardiovascular disease when their levels are elevated [14,15]. Experiments performed in mouse models of atherosclerosis suggest that gene expression changes in the liver with disease progression [16], and modification of either global or locus-specific DNA methylation could serve as a possible regulatory mechanism.

In this context, global DNA methylation has been shown to decrease with age in human peripheral blood leukocytes [17]. In addition, Bjornsson et al. have demonstrated that global DNA methylation status can change over time within a single individual. When peripheral blood leukocytes from participants in the population-based AGES (Aging Gene-Environment Susceptibility)-Reykjavik study were investigated, 29% showed a change of 10% or more over an 11-year period [18]. Although to date all of the published reports of epigenetic differences between affected and unaffected individuals for complex diseases including diabetic neuropathy and preeclampsia have been confined to cross-sectional analyses, an important implication of this work is that longitudinal alterations in DNA methylation that depend on a specific environment or metabolic status may lead to disease-specific transcriptional changes [19,20]. Alterations in gene-specific methylation have also been reported for human atherosclerotic aortas *in vivo*. In an early study, Post et al. demonstrated that the estrogen receptor-α (*ESR1*) CpG island was more highly methylated in coronary atherosclerotic plaques when compared to normal samples taken from the proximal aorta, possibly contributing to abnormal proliferation of smooth muscle cells within these lesions [21]. In contrast, when the methylation status of 10,367 CpG islands in 45 arteries from individuals with atherosclerosis and 16 normal controls was interrogated using DNA methylation microarrays, a subset were found to be consistently hypermethylated in control arteries but largely unmethylated and expressed in atherosclerotic tissues, including regions adjacent to genes encoding transcription factors implicated in atherogenesis [22].

In this study, variation in hepatic global DNA methylation was not associated with disease risk in the study population considered as a whole using conditional logistic regression models to analyze matched pairs. Since the estimation of the risk of atherosclerosis associated with inter-individual variation in DNA methylation was similar if unconditional logistic regression was used, subgroup analyses were carried out after adjusting for the matching variables. An association of borderline significance with methylation levels below the median for the study population was found in white but not in African-Americans study participants.

There have been inconsistent results reported in previous studies of global methylation in peripheral blood leukocytes using a variety of laboratory methods in ethnically diverse populations. Higher global methylation of Alu and satellite 2 repeats determined by real-time polymerase chain reaction was found in males but not females aged 45–74 years with a history of cardiovascular disease among 286 individuals enrolled in the Singapore Chinese Health Study [7]. Similarly, in 287 Indian subjects who were 30–75 years of age, global DNA methylation was higher in blood from patients with angiographically confirmed coronary artery disease than in controls as assessed by a cytosine extension assay [6]. In contrast, lower methylation of LINE-1 repeats measured by pyrosequencing has been shown to predict both baseline and incident ischemic heart disease and stroke in a cohort of 712 elderly individuals from the Boston-area Normative Aging Study [8].

While the modest association between high global DNA methylation index and susceptibility to subclinical atherosclerosis in whites in the PDAY study may be due to chance, differences in the prevalence of cardiovascular risk factors between African-American and white children as well as differences in susceptibility to cardiovascular disease have been documented in other studies. For example, there was a significantly greater prevalence of high BMI-for-age at three different cut-points for non-Hispanic black girls when compared to non-Hispanic white girls among individuals included in the 2007–2008 Health and Nutrition Examination Survey, a representative sample of the United States population [23]. In the multiethnic SEARCH for Diabetes in Youth Study, newly diagnosed type 2 diabetes was more common in African-American than in non-Hispanic white adolescents 10–19 years of age, and was combined with a higher incidence rate [24]. Similarly, African-American children aged 5–14 years of age had higher blood pressure than did white children, and more extensive fatty streaks were found in the aortas from African-American than from white adolescents and young adults in the community-based Bogalusa Heart Study [25,26].

There is precedent for differences in DNA methylation patterns between racial and ethnic groups in two recent studies that were not related to the etiology of cardiovascular disease. In an analysis of the association between global methylation levels measured in middle age and epidemiologic risk factors including early life exposures, DNA methylation in peripheral blood leukocytes was lower in African-Americans and higher in Hispanics when compared to non-Hispanic whites in a birth cohort of women born between 1959 and 1963 in New York City [27]. There were also significant differences between African-Americans and whites in the level of methylation present at birth for 13.7% of 26,485 CpG dinucleotides interrogated by microarray in umbilical cord blood from 201 infants [28]. In these studies as well as the PDAY study, variation in genetic or environmental factors such as diet or the level of homocysteine and other compounds involved in one-carbon metabolism that could contribute to differences in DNA methylation may underlie the observed racial disparities. DNA methyltransferases use S-adenosyl methionine (SAM) as a methyl group donor to modify CpG dinucleotides after a series of reactions that depend on the transmethylation of homocysteine to methionine. If the supply of methyl groups is inadequate, the abnormally high level of homocysteine leads to an elevation in the amount of S-adenosylhomocysteine (SAH), a direct inhibitor of methylatransferases. Hyperhomocysteinemia has been associated with an increased risk of cardiovascular disease in two independent meta-analyses [29,30]. Though homocysteine levels have not been measured in PDAY study participants, evaluation of their impact on DNA methylation in other multiethnic cohorts may be warranted.

Although important caveats in interpreting the study results include the possibility that the methylation measurements in autopsied specimens may reflect changes immediately prior to death, and that the LUMA assay is limited to analysis of only a restricted set of enzyme cleavage sites across the genome, hepatic global DNA methylation does not appear to be a definitive determinant of subclinical atherosclerosis in this postmortem sample of young adults. Replication in other study populations will be required to confirm the modest association between global DNA methylation status and disease risk identified in a subgroup of white study participants.

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design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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Table 1

Clinical and demographic characteristics. PDAY study.

	Subclinical atherosclerosis (*N* = 300)			Matched controls (*N* = 300)			*p*
	N	*N* (%) or mean	SD	*N*	*N* (%) or mean	SD	
Matching variables							
Male		241 (80.3)			241 (80.3)		
White		161 (53.7)			161 (53.7)		
African-American		139 (46.3)			139 (46.3)		
Age (years)		27.1	4.6		27.1	4.6	
Atherosclerotic lesions							
FS, thoracic aorta (%)	300	25.4	14.8	300	19.5	13.5	
RL, thoracic aorta (%)	300	1.8	5.8	300	0.01	0.1	
FS, abdominal aorta (%)	300	32.6	19.2	300	25.4	19.5	
RL, abdominal aorta (%)	300	15.2	15.8	300	0.07	0.3	
FS, right coronary artery (%)	300	9.5	12.8	300	3.6	7.9	
RL, right coronary artery (%)	300	11.8	17.6	300	0.1	0.4	
Clinical variables							
Hyperglycemia	266	16 (6.0)		270	9 (3.3)		0.14
BMI (kg/m^2)	300	25.8	5.7	299	25.0	4.4	0.07
Obese (BMI ≥ 30 kg/m^2)	300	56 (18.7)		299	36 (12.0)		0.02
Smoking	153	98 (64.0)		169	70 (41.4)		<0.001
HDL chol., mg/dL	153	54.2	22.1	169	54.8	24.6	0.82
Non-HDL chol., mg/dL	148	162.4	66.5	164	132.5	48.5	<0.001
Global DNA methylation							
LUMA methylation ratio	300	0.53	0.12	300	0.52	0.13	0.37
LUMA methylation ratio (<0.52)	300	146 (48.7)		300	162 (54.0)		0.19
LUMA methylation ratio (≥0.52)	300	154 (51.3)		300	138 (46.0)		
High LUMA methylation ratio (≥0.52)		OR (95% CI):1.22 (0.9, 1.66)					0.21*

N, number; SD, standard deviation; p, p-value; FS, fatty streak; RL, raised lesion; BMI, body mass index; chol, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LUMA, luminometric methylation assay; OR, odds ratio; CI, confidence interval.

**p*-value for conditional logistic regression.

Table 2

Global DNA methylation and subclinical atherosclerosis stratified by race and gender.

Subgroups for stratified logistic regression analysis	*N*	OR (95% CI)	*p*
African-American			
High LUMA methylation ratio (adjusted for age and gender)	278	0.94 (0.59, 1.51)	0.81
White			
High LUMA methylation ratio (adjusted for age and gender)	322	1.59 (1.02, 2.49)	0.04
Male			
High LUMA methylation ratio (adjusted for age and race)	482	1.12 (0.78, 1.61)	0.52
Female			
High LUMA methylation ratio (adjusted for age and race)	118	1.92 (0.90, 4.09)	0.09

N, number; OR, odds ratio; CI, confidence interval; *p*, *p*-value; LUMA, luminometric methylation assay.