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Original Article

Predictors of white blood cell interleukin-6 DNA methylation levels in healthy subjects

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Abstract: DNA methylation at gene regulatory sequences has been associated with risk of human diseases. Interleukin-6 (IL-6) methylation is a candidate biomarker for predicting disease risk, but factors that influence IL-6 methylation in healthy individuals remain largely unknown. The aim of this study was to identify predictors of IL-6 methylation in white blood cells (WBCs) from 673 healthy individuals. DNA methylation of IL-6 regulatory sequences in peripheral WBCs was quantified by PCR-pyrosequencing. Individuals with higher body mass index (BMI) (≥23.6 kg/m^2) had higher IL-6 methylation levels (β = 2.08%, 95% confidence interval (95% CI): 0.59% to 3.58%), compared with subjects with lower BMI. Serum triglyceride also correlated with IL-6 methylation, and an interquartile increase in triglyceride was associated with a 0.69% (95% CI: 0.02% to 1.36%) increase in IL-6 methylation. No associations were found with age, gender, alcohol consumption, smoking, diabetes, hypertension, total serum cholesterol or plasma homocysteine. The total number of WBCs and interquartile plasma C-reactive protein levels were inversely associated with IL-6 methylation. These findings need to be considered in designing future epidemiological investigations aimed at identifying associations between IL-6 methylation and health outcomes.

Keywords: Interleukin-6, white blood cell, DNA methylation, epigenetics

Introduction

DNA methylation is a well-defined epigenetic mechanism of gene regulation involved in a wide variety of biological processes, and dysregulated DNA methylation has been implicated in human disease [1]. Due to the growing interest in DNA methylation and the increasing availability of methylation analyses technology, the number of epidemiological investigations focused on DNA methylation has increased rapidly in recent years [2, 3]. Gene-specific methylation, which occurs in specific regions of a gene such as regulatory sequences, can either silence or activate expression [4]. There is increasing evidence that aberrant promoter methylation is a risk factor for human diseases [5, 6]. Interleukin-6 (IL-6) is a multifunctional cytokine with pleiotropic effects that plays an important role in inflammation, immunity and disease [7]. A functional polymorphism in the IL-6 promoter has been associated with a genetic susceptibility to many pathological conditions [8-11]. Furthermore, previous studies have demonstrated that promoter methylation is an essential epigenetic mechanism of IL-6 regulation [12-14] and that the IL-6 promoter is hypomethylated in white blood cells (WBCs) from systemic lupus erythematosus, rheumatoid arthritis and chronic periodontitis patients [15-17]. In healthy populations, inter-individual variations in IL-6 methylation from WBCs have been associated with risk factors for ischemic heart disease (IHD) and pulmonary disorders [18, 19]. These data suggest that altered IL-6 methylation is a potential biomarker for predicting disease risk, but little is known about what controls IL-6 methylation in WBCs, which is relevant for designing effective statistical analyses of epidemiological investigations.

In this study, we sought to identify predictors of WBC DNA methylation at the regulatory sequence downstream of the IL-6 promoter through associations with subject characteristics including age, gender, body mass index (BMI), smoking status, alcohol consumption, history of hypertension or diabetes, total serum

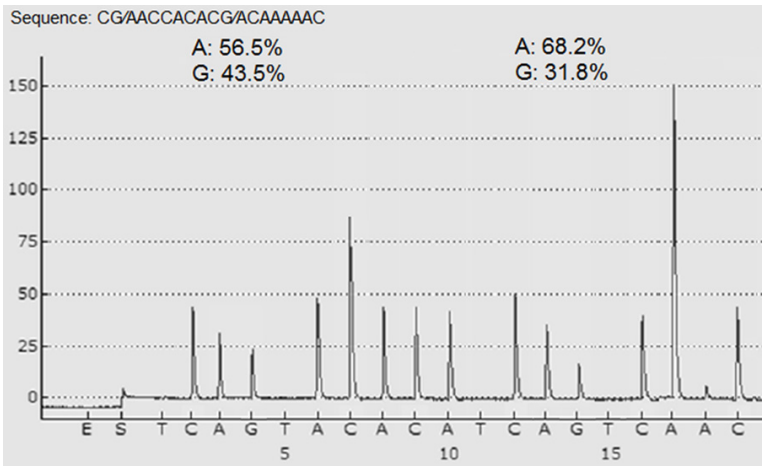

Figure 1. Representative image of IL-6 methylation pyrosequencing analysis.

cholesterol and triglyceride, and plasma homocysteine. Because DNA methylation was measured in WBCs and IL-6 is a major inflammatory mediator, whether differences in WBC counts and plasma C-reactive protein (CRP) (inflammation markers) were associated with IL-6 methylation was also explored.

Materials and methods

Study participants

The study consisted of 673 control subjects from an epidemiologic case-control study that was conducted at the Fourth Affiliated Hospital, Harbin Medical University, Harbin, China between March 2011 and November 2013 to screen for genetic and epigenetic markers that are associated with IHD development. All subjects received a general health survey and those with IHD, peripheral atherosclerotic arterial disease, autoimmune disease or cancers were excluded. Age, gender, height, weight, alcohol consumption, smoking status and history of hypertension and diabetes were obtained via a structured questionnaire given through in-person interviews. Subjects who had smoked more than 100 cigarettes in their lifetime were defined as smokers. A drinker was defined as a person who reported drinking alcoholic beverages at least once a week for 6 months or longer. BMI was calculated using the formula: weight divided by height squared (kg/m^2). WBC counts, total serum cholesterol and triglyceride, and plasma homocysteine and CRP were collected from medical records. Written informed consent was obtained from each participant. This study was approved by the Institutional Review Board of the Fourth Affiliated Hospital, Harbin Medical University, Harbin, China.

IL-6 methylation analysis

IL-6 methylation analysis was performed on bisulfite-treated DNA using a quantitative PCR-pyrosequencing-based analysis. Genomic DNA was extracted from WBCs using the QIAamp DNA Blood kit (QIAGEN, Hilden, Germany), and then bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The target region in IL-6 was PCR-amplified with biotinylated forward primer (5'-biotin-TAT TTT AGT TTT GAG AAA GGA GGT G-3') and reverse primer (5'-CAA TAC TCT AAA ACC CAA CAA AAA C-3'). PCR cycling conditions were 5 min at 95°C followed by 45 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min followed by a final elongation for 5 min at 72°C. Pyrosequencing was performed on the PSQ HS 96 Pyrosequencing System (QIAGEN) using the sequencing primer (5'-TCC TAA TAC AAA CAA CCC C-3'), according to the manufacturer's protocol. Non-CpG cytosine residues were used to verify bisulfite conversion. The degree of methylation for each CpG was expressed as percent 5-methylated-cytosine (%5mC), which was defined as the number of methylated cytosines divided by the sum of methylated and unmethylated cytosines. Total IL-6 methylation was calculated by averaging the results of the two CpGs analyzed (**Figure 1**).

Statistical analysis

Linear regression models were used to evaluate IL-6 methylation levels in relation to subject characteristics (age, gender, BMI, alcohol consumption, smoking status, diabetes, hypertension, total cholesterol, triglyceride and homocysteine) and inflammation markers in WBC counts (total WBC numbers, and percent neutrophils, lymphocytes, monocytes, eosinophils and basophils) and plasma CRP levels. The regression equation was as follows: $y = a + bx$; where: y = IL-6 methylation; x = predictive variable; a = constant; b = regression coefficient (β). To evaluate whether differences in total

Table 1. Association of study characteristics with IL-6 methylation

Variable	N	Univariate linear regression model			Multivariate linear regression model[c]		
		%5mC (95% CI)	β (95% CI)	*P* value	%5mC (95% CI)	β (95% CI)	*P* value
Age (years)[a]							
<62	335	43.5 (42.5-44.5)			43.5 (42.4-44.6)		
≥62	338	42.7 (41.6-43.8)	-0.79 (-2.29 to 0.71)	0.304	42.7 (41.7-43.8)	-0.79 (-2.29 to 0.71)	0.301
Gender							
Female	173	43.4 (41.9-44.9)			43.4 (41.9-44.9)		
Male	500	43.0 (42.2-43.9)	-0.38 (-2.10 to 1.34)	0.663	43.0 (42.1-43.9)	-0.38 (-2.10 to 1.34)	0.663
Body mass index (kg/m^2)[a]							
<23.6	339	42.1 (41.0-43.2)			42.0 (41.0-43.1)		
≥23.6	334	44.2 (43.1-45.2)	2.08 (0.59 to 3.58)	0.006	44.2 (43.1-45.3)	2.20 (0.68 to 3.73)	0.005
Smoking status							
Nonsmoker	360	43.0 (42.0-44.1)			43.0 (41.9-44.0)		
Smoker	313	43.2 (42.2-44.3)	0.20 (-1.31 to 1.70)	0.797	43.3 (42.1-44.4)	0.31 (-1.26 to 1.87)	0.703
Alcohol consumption							
Nondrinker	483	43.5 (42.6-44.4)			43.5 (42.6-44.4)		
Drinker	190	42.2 (40.9-43.5)	-1.29 (-2.96 to 0.37)	0.128	42.2 (40.8-43.6)	-1.28 (-2.96 to 0.41)	0.137
Hypertension							
No	475	43.0 (42.1-43.9)			43.0 (42.1-43.9)		
Yes	198	43.4 (41.9-44.8)	0.38 (-1.27 to 2.02)	0.655	43.4 (42.0-44.8)	0.38 (-1.30 to 2.05)	0.657
Diabetes							
No	633	43.0 (42.2-43.7)			43.0 (42.2-43.7)		
Yes	40	45.4 (43.0-47.9)	2.45 (-0.72 to 5.62)	0.130	45.5 (42.4-48.5)	2.49 (-0.69 to 5.67)	0.125
Total cholesterol (mmol/L)[a]							
≤4.1	338	43.3 (42.2-44.4)			43.3 (42.3-44.4)		
>4.1	335	42.9 (41.9-43.9)	-0.44 (-1.95 to 1.06)	0.562	42.9 (41.8-44.0)	-0.44 (-1.96 to 1.07)	0.564
Triglyceride (mmol/L)[b]							
≤0.92	167	42.2 (40.6-43.8)			42.2 (40.7-43.7)		
0.93-1.30	169	42.8 (41.2-44.3)			42.8 (41.3-44.3)		
1.31-1.75	169	42.9 (41.5-44.4)			42.9 (41.4-44.5)		
≥1.76	168	44.5 (43.1-45.9)			44.5 (43.0-46.0)		
Trend statistics			0.69 (0.02 to 1.36)	0.044		0.69 (0.02 to 1.37)	0.045
Homocysteine (μmol/L)[a]							
≤10.2	305	43.4 (42.3-44.5)			43.4 (42.3-44.5)		
>10.3	294	42.5 (41.3-43.6)	-0.97 (-2.55 to 0.61)	0.227	42.5 (41.3-43.6)	-0.97 (-2.57 to 0.64)	0.237

%5mC, percentage of 5-methyl-cytosine; CI, confidence interval. [a]Graded by the median of the variable. [b]Graded by the quartile of the variable. [c]Adjusted for age (as a continuous variable) and gender.

WBC numbers and plasma CRP confounded the associations between subject characteristics and IL-6 methylation, multivariate linear regression models were fitted. Statistical analyses were conducted using STATA 13.0 (STATA Corporation, College Station, TX, USA). *P* values less than 0.05 in two-sided analyses were considered statistically significant.

Results

A total of 673 healthy subjects were included in this study (Supplementary Table). IL-6 methylation in WBC DNA was approximately normally distributed. The median and mean levels of IL-6 methylation were 43.2% (range: 11.8%-72.9%) and 43.1% (standard deviation (SD) = 9.9%), respectively. Both serum triglyceride and plasma CRP showed skewed distributions (both $P<0.001$, Skewness and Kurtosis test for normality).

Associations between subject characteristics and IL-6 methylation are shown in **Table 1**. In univariate analyses, individuals with higher BMI (≥ median (23.6 kg/m^2)) had higher IL-6 methylation levels (β = 2.08%, 95% confidence interval (95% CI): 0.59% to 3.58%, P = 0.006), compared with subjects with lower BMI (< median). Additionally, serum triglyceride was significantly correlated with IL-6 methylation (P = 0.044), and an interquartile range increase in serum

Table 2. Effect of white blood cell count and plasma C-reactive protein on IL-6 methylation

Variable	N	Univariate linear regression model		Multivariate linear regression model[b]	
		β (95% CI)	*P* value	β (95% CI)	*P* value
White blood cell count					
Total numbers (10^3 cell/mm^3)	668	-0.23 (-0.44 to -0.01)	0.037	-0.23 (-0.44 to -0.01)	0.037
Neutrophils (%)	664	-0.02 (-0.10 to 0.07)	0.704	-0.02 (-0.10 to 0.07)	0.697
Lymphocytes (%)	664	0.03 (-0.06 to 0.11)	0.558	0.03 (-0.06 to 0.11)	0.555
Monocytes (%)	664	0.10 (-0.24 to 0.45)	0.552	0.11 (-0.24 to 0.46)	0.533
Eosinophils (%)	664	-0.17 (-0.51 to 0.17)	0.315	-0.18 (-0.52 to 0.16)	0.310
Basophils (%)	664	0.88 (-0.62 to 2.37)	0.251	0.87 (-0.63 to 2.37)	0.257
C-reactive protein (mg/L)[a]					
≤0.73	163				
0.74-1.44	160				
1.45-2.90	158				
≥2.91	161				
Trend statistics		-0.78 (-1.46 to -0.09)	0.026	-0.78 (-1.47 to -0.10)	0.025

CI, confidence interval. [a]Graded by the quartile of the variable. [b]Adjusted for age (as a continuous variable) and gender.

Table 3. Effects of body mass index and serum triglyceride on IL-6 methylation in multivariate models

Variable	N	%5mC (95% CI)[c]	β (95% CI)[c]	*P* value[c]
Body mass index (kg/m^2)[a]				
<23.6	339	42.2 (41.1-43.3)		
≥23.6	334	44.2 (43.1-45.3)	1.90 (0.34 to 3.46)	0.017
Triglyceride (mmol/L)[b]				
≤0.92	167	42.0 (40.5-43.6)		
0.93-1.30	169	43.1 (41.6-44.7)		
1.31-1.75	169	42.9 (41.3-44.4)		
≥1.76	168	44.7 (43.1-46.2)		
Trend statistics			0.76 (0.06 to 1.46)	0.033
C-reactive protein (mg/L)[b]				
≤0.73	163	44.6 (43.1-46.2)		
0.74-1.44	160	43.5 (41.9-45.0)		
1.45-2.90	158	43.0 (41.4-44.5)		
≥2.91	161	41.6 (40.0-43.1)		
Trend statistics			-0.98 (-1.68 to -0.29)	0.005
WBC number (10^3 cell/mm^3)	668	43.2 (42.4-43.9)	-0.25 (-0.46 to -0.04)	0.021

%5mC, percentage of 5-methyl-cytosine; CI, confidence interval; WBC, white blood cell. [a]Graded by the median of the variable. [b]Graded by the quartile of the variable. [c]Adjusted for white blood cell number (as a continuous variable), C-reactive protein (≤0.73, 0.74-1.44, 1.45-2.90, ≥2.91 mg/L), body mass index (<23.6, ≥23.6 kg/m^2) and serum triglyceride (≤0.92, 0.93-1.30, 1.31-1.75, ≥1.76 mmol/L).

triglyceride levels was associated with a 0.69% (95% CI: 0.02% to 1.36%) increase in IL-6 methylation. IL-6 methylation was not associated with age, gender, alcohol consumption, smoking, diabetes, hypertension, total serum cholesterol or plasma homocysteine. In multivariate analyses (**Table 1**), no major differences from the results reported above were found, nor were there changes in their statistical significance.

IL-6 is a key inflammatory mediator and DNA methylation was measured in WBCs; therefore, we next examined whether differential WBC counts and plasma CRP levels were associated with IL-6 methylation (**Table 2**). Total WBC count

was inversely associated with IL-6 methylation (β = -0.23%, 95% CI: -0.44% to -0.01%, P = 0.037). Plasma CRP levels were also significantly associated with IL-6 methylation (P = 0.026). One interquartile range increase in plasma CRP was associated with a decrease in IL-6 methylation by 0.78% (95% CI: -1.46% to -0.09%). Further multivariate linear regression models produced comparable results (**Table 2**). No associations between the neutrophil, lymphocyte, monocyte, eosinophil, or basophil percentages and IL-6 methylation were found in either univariate or multivariate analyses (**Table 2**). Adding total WBC number and plasma CRP levels into the multivariate linear regression models produced no major difference in the effects of BMI and serum triglyceride on IL-6 methylation (**Table 3**).

Discussion

In this study, we showed that both BMI and serum triglyceride were positively associated with IL-6 methylation levels in WBCs from healthy subjects. We also showed that total WBC count and plasma CRP levels modified IL-6 methylation, but did not confound the associations of BMI and serum triglyceride.

Studies investigating the association between BMI and WBC IL-6 methylation are limited. Based on a relatively small study cohort, Zhang *et al*. reported no association between BMI and IL-6 methylation in a middle-aged cancer-free population [20]. In another small-scale case-control study, IL-6 methylation levels in WBCs were not significantly different between obese (BMI>30 kg/m^2) and lean subjects (BMI≤25 kg/m^2) [18]. However, in the latter study, methylation levels of distinct CpG sites in the IL-6 promoter were positively correlated with body weights for both the obese and lean groups [18]. Here we show for the first time that BMI was positively associated with WBC IL-6 methylation levels. Given that the BMI-related IL-6 methylation alteration was small in our analysis, particularly compared with the inter-individual variabilities in IL-6 methylation, the lack of the association with BMI in previous studies [18, 20] could be partially due to smaller sample sizes, insufficient BMI ranges and/or differences in the CpG sites analyzed. It has been reported that increased BMI was associated with reduced long interspersed element-1 (LINE-1) methylation, a surrogate for global DNA methylation [21], and that LINE-1 methylation was inversely correlated with IL-6 methylation [22]. Notably, we also observed that serum triglyceride, which is strongly positively associated with BMI in healthy subjects [23], was positively associated with IL-6 methylation. Both obesity and serum triglyceride are significantly positively associated with red blood cell folate, an essential factor in the metabolic pathways that facilitate biological methylation and nucleotide synthesis [24]. Furthermore, animal models have demonstrated that DNA methyltransferase 3a expression is markedly upregulated in adipose tissue of obese mice [25]. Further studies are warranted to clarify whether these associations exist in human WBCs, and if so, to what extent they affect IL-6 methylation.

IL-6 is a prominent pro-inflammatory cytokine and the key inducer of CRP [26]. In this study, we demonstrated for the first time that both total WBC number and plasma CRP were inversely correlated with IL-6 methylation levels. Further analysis showed that the addition of total WBC numbers and plasma CRP levels into multivariate regression models did not modify the associations between the subject characteristics we evaluated and IL-6 methylation levels. However, the associations we observed of total WBC number and plasma CRP level with IL-6 methylation indicate that findings from epidemiological studies based on methylation analyses from blood DNA might be confounded by shifts in inflammation markers. This would be a concern for disease states that are associated with inflammatory responses that may produce changes in total WBC number and plasma CRP levels [27].

Our data also showed that age, gender, alcohol consumption, smoking, diabetes, hypertension, serum total cholesterol and plasma homocysteine were not associated with IL-6 methylation. Consistent with this data, other studies investigating associations between WBC IL-6 methylation and age, gender, alcohol consumption and diabetes also reported negative results [18, 20]. No data on the effects of hypertension, total serum cholesterol or plasma homocysteine on IL-6 methylation have been previously reported. Several studies have examined associations between smoking and IL-6 methylation, with most studies reporting no associations [22, 28]. Only one study based

on elderly men from an American population reported that former smokers, rather than current smokers, had higher WBC IL-6 methylation compared with never-smokers [19]. In our data, no association was found between smoking and IL-6 methylation. Given that our data included mostly current smokers, additional studies on the effect of past smoking on IL-6 methylation are required.

The strong points of this study were that it included a large sample size and used a quantitative pyrosequencing-based analysis that is suitable for measuring subtle methylation changes at more than one CpG site and thus, more accurately detects DNA methylation in the region [29]. Several limitations should be noted; first, while our results demonstrated that subject characteristics including BMI, serum triglyceride, total WBC number and plasma CRP levels were associated with IL-6 methylation levels in WBC DNA, whether the methylation pattern is a cause or consequence of the changes in these characteristics cannot be determined in our analysis. Second, other variables such as diet and environmental exposures, which may also influence IL-6 methylation levels and potentially confound the associations between the subject characteristics and IL-6 methylation were not investigated [19, 30]. Finally, subjects with IHD, peripheral atherosclerotic arterial disease, autoimmune disease and/or cancers were excluded from the analysis, and thus the extent to which our conclusions apply to the general population remains to be determined.

In conclusion, our analysis showed that clinical data routinely collected in epidemiological investigations, including BMI and serum triglyceride, are predictors of WBC IL-6 methylation levels. We also showed that total WBC number and plasma CRP levels influenced IL-6 methylation. These findings provide valuable information that can be used in planning future epidemiological studies.

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Disclosure of conflict of interest

None.

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