Endocr (2009) 35:204–210
DOI 10.1007/s12020-009-9153-7

ORIGINAL PAPER

Peripheral blood leukocyte distribution and body mass index are associated with the methylation pattern of the androgen receptor promoter

Sofia Movérare-Skrtic · Dan Mellström · Liesbeth Vandenput · Mathias Ehrich · Claes Ohlsson

Received: 14 October 2008 / Accepted: 26 November 2008 / Published online: 6 February 2009

Abstract Methylation of CpG sites in the promoter region can affect gene transcription. DNA derived from peripheral blood leukocytes (PBL) from the well-characterized clinical cohorts might be useful to study the influence of environmental factors on DNA methylation. However, these studies could be confounded by the heterogeneous nature of PBL. The aims of this study were to determine the impact of PBL distribution on methylation status of the androgen receptor (AR) promoter, and determine the associations between PBL distribution-adjusted methylation status of the AR promoter and AR-related phenotypes. PBL differential count analyses were performed at the time of blood sampling for DNA preparation in 170 elderly men. The DNA was bisulfite treated, and the methylation status of five CpG units in the AR promoter was analyzed using a high-throughput technique based on MALDI-TOF mass spectrometry. The degree of methylation of all the five investigated CpG units was strongly positively associated with the percent of lymphocytes in the PBL ($r_s = 0.17$–0.49, $P < 0.05$). Furthermore, the PBL distribution-adjusted methylation status of a specific CpG unit in the AR promoter was significantly associated with body mass index ($r_s = 0.24$) and other measures reflecting fat mass in elderly men. In conclusion, adjustment for PBL distribution needs to be done to be able to use DNA from whole blood for methylation analysis of the AR promoter and most likely also when investigating other promoters.

Keywords Androgen receptor · Epigenetics · Methylation · Mass spectrometry · BMI

S. Movérare-Skrtic (✉) · D. Mellström · L. Vandenput · C. Ohlsson
Division of Endocrinology, Departments of Internal Medicine and Geriatrics, Center for Bone Research at the Sahlgrenska Academy (CBS), University of Gothenburg, Gröna Stråket 8, 413 45 Gothenburg, Sweden
e-mail: sofia.moverare@medic.gu.se

M. Ehrich
SEQUENOM Inc., San Diego, CA, USA

Introduction

Epigenetic regulations, which are heritable changes in gene expression that occur in the absence of alterations in DNA sequences, may be involved in the complex gene-by-environment interactions that can lead to human diseases. DNA methylation is an epigenetic modification of the genome, whereby a methyl group is covalently linked at position 5 of the cytosine pyrimidine ring, typically occurring in a 5′-CpG-3′dinucleotide context. It may be induced spontaneously in response to environmental factors, or in response to the presence of a particular allele. The DNA methylation state of CpG site(s) can affect the chromatin structure and transcriptional activity of the associated gene [1]. The CpG sites are often concentrated in distinct areas of the genome called CpG islands, which are regions of more than 500 bp in size and with GC content >55%. These stretches of DNA are located within the promoter region of about 40% of mammalian genes and, when hyper-methylated they can cause stable heritable reduction of gene transcription [2].

Alterations in DNA methylation are not only linked to normal developmental processes, but also to many human diseases including cancer [3, 4]. However, the impact of DNA methylation on complex human diseases such as cardiovascular diseases, diabetes, obesity, and osteoporosis is largely unclear, and the role of environmental factors for DNA methylation of specific CpG sites is not much

investigated. The relative impact of the average methylation and the CpG site-specific methylation has been extensively studied in cancer research, demonstrating that the site-specific methylation is more informative than average methylation of a larger DNA region [5]. Thus, the methylation of a specific CpG site might affect an important regulatory gene sequence, and this information would be missed by analyses of the average methylation.

One reason for the limited information of the impact of environmental factors on site-specific DNA methylation has been the lack of accurate and reproducible high-throughput techniques for analyses of individual CpG sites. We have recently developed a novel approach for sensitive, specific, high-throughput analyses of DNA methylation based on a base-specific cleavage reaction combined with mass spectrometric analyses [6].

Another reason for the lack of information of the impact of environmental factors is that the CpG site-specific DNA methylation probably differs between different types of cells, and it is difficult to sample a homogenous cell population/tissue for DNA methylation analyses from large human cohorts. DNA derived from peripheral blood leukocytes (PBL) from whole blood samples is already prepared from several well-characterized human cohorts and has been used with success for the evaluation of the impact of genetic variations for several different human phenotypes/diseases. PBL-derived DNA has in some recent studies been used for analyses of DNA methylation [7, 8], but these studies might have been confounded by the fact that PBL are a heterogeneous population, consisting of several different cell populations (i.e., mainly lymphocytes, neutrophils, monocytes, and eosinophils), which might differ in methylation status, and it is known that there are clear inter-individual variations in PBL distribution.

There is a CpG island in the androgen receptor (AR) promoter which extends from −500 through exon 1 [9]. It has previously been published that PBL cells express AR mRNA [10]. Several studies have reported on the affected DNA methylation of the AR promoter in DNA derived from prostate cancer tumor tissues or cell lines [11–13]. Recently, Vottero et al. [7] demonstrated that age was inversely related to AR methylation of DNA derived from PBL in a small cohort of children (1–12 years of age), but no adjustment for PBL distribution was done in that study. Furthermore, it has been shown that treatment with testosterone increased the degree of methylation of the AR promoter in the mouse brain [14].

The aims of this study were to determine the impact of PBL distribution on the CpG site-specific methylation status of the AR promoter, and determine the associations between PBL distribution-adjusted methylation status of the AR promoter and AR-related phenotypes in a well-characterized male cohort.

Results

Description of the CpG units analyzed in the AR promoter

The analyzed region of the CpG island of the human AR promoter was a 329-bp long sequence located between −297 and +32 (Fig. 1). During the bisulfite treatment of the genomic DNA, methylated cytosine at CpG sites is unchanged, whereas unmethylated cytosine at CpG sites is changed to thymine on the forward PCR strand. This C → T change on the forward strand is represented as G → A change on the reverse strand. G and A have a mass difference of 16 Da, which is detected in the mass spectrometry analysis. The subsequent U specific cleavage reaction of the reverse strand causes cleavage products with different masses. If there is no U nucleotide at the reverse strand between two CpG sites, these sites could not be separated by the mass spectrometry. Thus, each cleavage product encloses either one CpG site or an aggregate of multiple CpG sites and, therefore, these sites are regarded as one CpG unit. Five CpG units (I–V, Fig. 1) were, in this study, analyzed in PBL-derived DNA from 170 elderly subjects. The degree of methylation for these five CpG units as well as the characteristics of the investigated cohort of elderly men is shown in Table 1.

Fig. 1 The nucleotide sequence of the analyzed region (−297 to +32) of the AR promoter. The location of the five analyzed CpG units in this cohort of 170 elderly men is indicated by Roman numerals (I–V). CpG sites within these CpG units are in *bold*. Each CpG unit cover between 1 and 3 CpG sites

Table 1 Characteristics of the elderly men investigated

	Means ± SD (n = 170)
Age (years)	78.4 ± 2.0
BMI (kg/m^2)	26.0 ± 3.3
BMI at 25 years of age (kg/m^2)	22.7 ± 1.9
BMI change from 25 years of age (kg/m^2)	3.3 ± 3.2
Serum leptin (ng/ml)	19.6 ± 17.6
Fat mass (kg)	18.7 ± 5.5
Percent fat (%)	24.0 ± 4.7
Serum testosterone (ng/ml)	4.2 ± 1.9
Smoking status (%)	7
Glucocorticoid therapy (%)	1
PBL distribution	
Neutrophils (%)	57.0 ± 9.1
Lymphocytes (%)	29.8 ± 7.9
Monocytes (%)	9.4 ± 2.3
Eosinophils (%)	3.8 ± 2.1
Degree of AR promoter methylation (%)	
AR CpG unit I	8 ± 4
AR CpG unit II	9 ± 6
AR CpG unit III	13 ± 6
AR CpG unit IV	13 ± 4
AR CpG unit V	4 ± 3

Values are given as means ± SD

BMI body mass index, *AR* androgen receptor

Table 2 The impact of PBL distribution on methylation status of CpG units in the AR promoter of elderly men

A

	Neutrophils	Lymphocytes	Monocytes	Eosinophils
CpG unit I	−0.21**	0.26***	−0.02	0.03
CpG unit II	−0.44***	0.49***	0.04	0.03
CpG unit III	−0.24**	0.31***	−0.15*	0.02
CpG unit IV	−0.17*	0.19*	0.01	0.01
CpG unit V	−0.15	0.17*	0.05	0.08

Correlation analyses of methylation status in individual AR CpG units versus PBL distribution (percent neutrophils, lymphocytes, monocytes, or eosinophils). Spearman rank correlation coefficient (r_s) is given, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (n = 170)

B

	0% lymphocytes	100% lymphocytes
CpG unit I (%)	2	20
CpG unit II (%)	0	35
CpG unit III (%)	9	24
CpG unit IV (%)	9	21
CpG unit V (%)	3	6

Calculated value of the degree of methylation (%) for each of the five CpG units (I–V) in a theoretical PBL population devoid of lymphocytes (0% lymphocytes) and in a pure lymphocyte population (100% lymphocytes), using linear regression models

The impact of PBL distribution for methylation of CpG units in the AR promoter

The PBL is a heterogeneous population consisting of several different cell types, whereof neutrophils and lymphocytes are the main sub-populations. As there are known inter-individual variations in PBL distribution and the degree of methylation could differ between different cell populations, we investigated the impact of the different major cell populations (neutrophils, lymphocytes, monocytes, and eosinophils) on the degree of methylation of PBL-derived DNA from elderly men. As expected, neutrophils (57%) and lymphocytes (30%) were the most abundant cell types in the PBL of these elderly men (Table 1). Univariate correlation analyses demonstrated that the percent of lymphocytes was clearly positively associated with the degree of methylation of CpG units in the AR promoter, whereas the percent of neutrophils was negatively associated with the degree of methylation of CpG units in the AR promoter (Table 2A). These data strongly suggest that lymphocytes are the PBL sub-population with the highest degree of methylation. The rather strong association between the percent lymphocytes and the degree of methylation of CpG unit II in the AR promoter is illustrated in Fig. 2 and for this CpG unit the percent of lymphocytes explained as much as 27% of the variance (r^2) in the degree of methylation (Fig. 2). These data indicate that most of the methylation of CpG units in the heterogeneous PBL population is found in lymphocytes, whereas the degree of methylation of neutrophils is rather low.

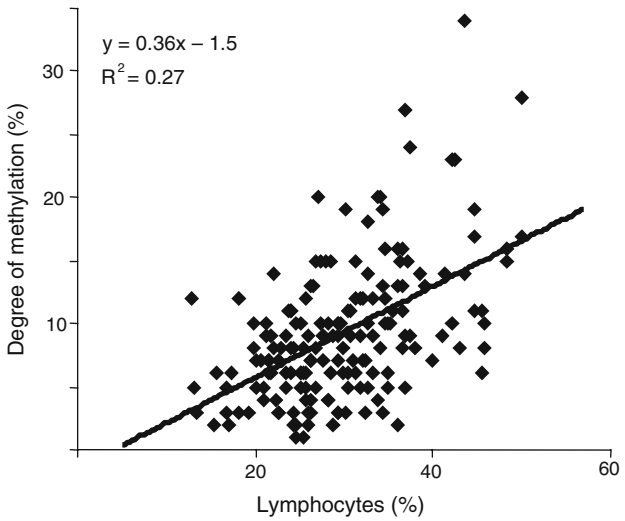

Fig. 2 Scatter plot of the association between the percent of lymphocytes and the degree of methylation at CpG unit II in the AR promoter. r^2 for the association is given in the figure. $P < 0.001$

In order to get an estimate of the degree of methylation in lymphocytes in relation to non-lymphocytes in the heterogeneous PBL population, the degree of methylation in a theoretical pure PBL population (100% lymphocytes) and in a theoretical population devoid of lymphocytes (0% lymphocytes) was calculated, using linear regression models (Table 2B). These analyses supported the notion that most of the methylation of CpG units in PBL is found in lymphocytes, while the degree of methylation of non-lymphocyte PBL cells is rather low (Table 2B). The data demonstrate that adjustment for PBL distribution, analyzed at the time of blood sampling for DNA preparation, has to be done to be able to use DNA from whole blood for methylation analysis of the AR promoter. In regression models including both PBL distribution and absolute white blood cell count, PBL distribution ($P < 0.05$) but not absolute white blood cell count (non-significant) was independently associated with the degree of methylation of the different investigated CpG units in the AR promoter. Therefore, in all subsequent analyses, the methylation status of the different CpG units was first adjusted for PBL distribution using linear regression models.

Correlation of the methylation status between different CpG units in the AR promoter

In order to determine if the different CpG units in the AR promoter displayed a similar methylation pattern, univariate correlation analyses were performed (Table 3). The methylation status of most of the CpG units in the AR promoter was significantly associated with each other but the variance explained (r^2) ranged between 2 and 18%.

Body mass index is associated with the PBL distribution-adjusted methylation status of the AR promoter

Age has previously been described to be inversely related to AR promoter methylation in PBL from children [7], and testosterone treatment was in an experimental animal study associated with increased AR promoter methylation in the mouse brain [14]. Furthermore, we have recently demonstrated that there is a clear inverse association between serum androgens and body mass index (BMI) in this population of elderly men [15]. Therefore, we next evaluated the associations between PBL distribution-adjusted methylation status of the AR promoter and age, serum testosterone and BMI (Table 4A). Serum testosterone was not associated with the PBL distribution (data not shown) or the PBL distribution-adjusted methylation status of any of the investigated CpG units in the AR promoter (Table 4A). Within the present cohort of elderly men with an age span of 69–80 years, age was inversely related to methylation status of CpG unit V, but after Bonferroni correction for multiple comparisons this association did not remain significant. Interestingly, BMI was significantly associated with the degree of methylation of CpG unit III in the AR promoter (Table 4A). When subjects were divided into tertiles according to the methylation status of this CpG unit, it was seen that subject in the lowest tertile had lower BMI (25.1 ± 3.4 kg/m^2) than subjects in the highest tertile (27.0 ± 3.2 kg/m^2), whereas the BMI in the middle tertile (25.7 ± 3.0 kg/m^2) was intermediate ($P < 0.01$ ANOVA).

Explorative sub-analyses demonstrated that the degree of methylation of CpG unit III was significantly associated with several parameters reflecting fat mass (total body fat, total body fat percent, and serum leptin levels, Table 4B). Furthermore, BMI change from 25 years of age but not BMI

Table 3 Correlation of the methylation status between different CpG units in the AR promoter of elderly men

CpG unit	I	II	III	IV	V
I	1.00	0.42***	0.17*	0.15*	0.21**
II		1.00	0.43***	0.19*	0.26**
III			1.00	0.16*	0.14
IV				1.00	0.30***
V					1.00

Spearman rank correlation coefficient (r_s) is given, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 170$)

Table 4 BMI is associated with the methylation status of the AR promoter

A

	CpG units				
	I	II	III	IV	V
Age	−0.05	−0.10	−0.04	−0.02	−0.16
BMI	0.00	0.04	0.24*	−0.05	0.13
Serum testosterone	−0.06	0.03	−0.11	−0.09	−0.08

The association between phenotypes and methylation status of CpG units (I–V) in the AR promoter. Spearman rank correlation coefficient (r_s) is given. *BMI* body mass index. * $P < 0.05$ after Bonferroni correction for multiple comparisons

B

	r_s
Fat mass	0.20*
Percent fat	0.16*
Serum leptin	0.19*
BMI at 25 years of age (kg/m^2)	0.13
BMI change from 25 years of age (kg/m^2)	0.21*

Explorative sub-analyses of the association between the methylation status of CpG unit III of the AR promoter and different measures of body composition

at 25 years of age was significantly associated with methylation status of CpG unit III in the AR promoter in this cohort of elderly men (Table 4B). Neither glucocorticoid therapy nor smoking status was associated with the degree of methylation in the AR promoter (data not shown).

Discussion

The importance of epigenetics for the regulation of gene expression is becoming increasingly apparent. Environmental factors might affect gene transcription through the altered site-specific DNA methylation, but the impact of environmental factors on DNA methylation has been difficult to determine. Using a recently developed high-throughput methodology, we herein demonstrate that adjustment for PBL distribution, analyzed at the time of whole blood sampling for DNA preparation, should be done to be able to use DNA from whole blood for methylation analysis of the AR promoter and most likely also when investigating other promoters. In addition, using PBL distribution-adjusted methylation status, we demonstrate that the site-specific AR promoter methylation was associated with BMI and other parameters reflecting fat mass in elderly men.

In this study, we found that the proportion of lymphocytes in PBL was clearly positively associated with the degree of methylation of all investigated CpG units in the AR promoter when evaluated in a rather large cohort of men and that a lymphocyte-enriched PBL population had a higher degree of methylation than a neutrophil-enriched PBL population. These data indicate that most of the methylation of CpG units in the heterogeneous PBL population is found in lymphocytes, while the degree of methylation of neutrophils is rather low. One might speculate that the higher degree of methylation in lymphocytes than in neutrophils is caused by the fact that AR expression is more influenced by epigenetic regulation in lymphocytes than in neutrophils. Alternatively, DNA methylation might occur more frequently in cell populations with relatively longer life span such as lymphocytes (weeks–year) than in cell populations with rather short life span, such as neutrophils (hours–days; [16]). Thus, previous studies investigating the methylation status in PBL have most probably been confounded by inter-individual variations in PBL distribution [7, 8]. Importantly, our data suggest that when evaluating environmental/phenotype associations not only with genetic but also with epigenetic variations, one should consider PBL distribution analyses at the time of PBL sampling for DNA preparation.

The regulation of the degree of methylation of the different CpG sites in a promoter region might be both uniform and CpG site specific. Even though most of the CpG units in the AR promoter, in this present study, were significantly associated with each other, supporting a uniform regulation, most of the variation of the degree of methylation for each individual CpG unit was independent of the methylation pattern of the other analyzed CpG units in the AR promoter, supporting a CpG site-specific regulation. This indicates that it is not enough to determine the average degree of methylation of the whole promoter region as one then might lose CpG site-specific information.

There was a non-significant (significant before but not after Bonferroni correction for multiple comparisons) tendency of an inverse relation between age and methylation status of the AR promoter in this study including a large cohort of older men, whereas Vottero et al. [7] demonstrated that age was rather strongly inversely related to AR promoter methylation in children. There are two main differences between the study by Vottero et al. and this study: (i) the average age of the study subjects differed and (ii) PBL distribution adjustment was performed in this study but not in the study by Vottero et al. Further studies including a high number of subjects of different ages and both sexes are required to determine if AR promoter methylation is slightly reduced by age in PBL.

We have recently shown that serum levels of androgens and androgen metabolites are strongly associated with BMI/body composition in this cohort of elderly men [15]. Interestingly, in this study, the PBL distribution-adjusted methylation status of a specific CpG unit in the AR promoter was significantly associated with BMI and several other parameters reflecting fat mass. The significant association with current BMI and BMI change from 25 years of age but not with BMI at 25 years of age would suggest that it is the age-dependent fat mass accumulation that is associated with the methylation status of the AR promoter. One might speculate that alterations in methylation pattern of the AR promoter affect AR expression and thereby the androgenic response, resulting in affected body composition. Thus, it is possible that environmental factors might affect the degree of the methylation of CpG unit III in the AR promoter and thereby AR expression and the androgenic response, which in turn might regulate the amount of fat mass.

There are several possible reasons why serum testosterone was not significantly associated with the methylation pattern of the AR promoter in this study: (i) It might be a power problem as not more than 170 subjects were included in this study. (ii) It might be due to that the androgenic response is affected in peripheral tissues such as fat but not in cells responsible for the negative feedback regulation of serum testosterone as a consequence of cell-specific expression of co-regulatory proteins. (iii) Finally, one cannot exclude that the variation in methylation status of the AR promoter does not substantially affect AR expression. A limitation of this study is that information

regarding the ongoing infectious diseases, which might affect the number and distribution of PBL, is not available.

As the methylation status most probably is promoter dependent and differs between cell populations, one should emphasize that the present AR promoter methylation pattern found in PBL cannot be directly extrapolated to be valid for other promoters and/or cell populations.

In conclusion, adjustment for PBL distribution, analyzed at the time of blood sampling for DNA preparation, needs to be done to be able to use DNA from whole blood for methylation analysis of the AR promoter, and most likely also when investigating other promoters. We propose that PBL distribution-adjusted DNA methylation status of CpG units in DNA derived from whole blood could be used to screen the impact of environmental factors on epigenetic regulations. Furthermore, BMI is associated with the methylation pattern of the AR promoter in elderly men.

Materials and methods

Study subjects

The MrOS (osteoporotic fractures in men) study is a multicenter study including elderly men in Sweden (3,014), Hong Kong (1,999), and the United States (5,995). In this study, the degree of methylation at different CpG units in the AR promoter was investigated in a subpopulation of the Gothenburg part of MrOS Sweden ($n = 170$) with PBL differential count analyses performed simultaneously with the sampling of the whole blood for DNA preparation. Study subjects (men aged 69–80) were randomly identified using national population registers, contacted and asked to participate. To be eligible for the study, the subjects had to be able to walk without aids. There were no other exclusion criteria [17]. The study was approved by the ethics committee at the University of Gothenburg. Informed consent was obtained from all study participants. Information about smoking status, glucocorticoid therapy, and young adult BMI (25 years of age) was taken from the MrOS Sweden questionnaire (Table 1).

PBL differential count

The blood differential count was analyzed on EDTA-stabilized whole blood using the CELL-DYN Sapphire™ (Abbott Diagnostics, Abbott Park, IL).

Assessment of testosterone and leptin in serum

Serum levels of testosterone were analyzed as previously described [18, 19]. Briefly, the validated gas chromatography-mass spectrometry (GC-MS) system was used for the analysis of testosterone [limit of detection 0.05 ng/ml, interassay coefficient of variation (CV) 3.4%]. The GC-MS uses a 50% phenyl-methylpolysiloxane capillary column with helium as carrier gas. The analytes and internal standard were detected using a HP5973 quadrupole mass spectrometer equipped with a chemical ionization source. In addition to the mass detected for the analyte, the retention time of the analyte, which corresponds to the retention time and mass of the synthetic standard, ensures the specificity of the method. Leptin was analyzed in serum samples using a commercially available kit (Diagnostic Systems Laboratories Inc., Webster, TX; interassay CV 5.3%).

Dual energy X-ray absorptiometry

Whole body fat mass and percent fat were assessed using the Hologic QDR 4500/A-Delphi DXA (Hologic, Waltham, MA).

DNA isolation and epigenetic analyses

Genomic DNA was isolated from EDTA-stabilized whole blood using the FlexiGene DNA purification kit (Qiagen, Valencia, CA). Bisulfite treatment was performed on 1 μg of the genomic DNA according to a protocol from Paulin et al. [20].

PCR and in vitro transcription

A 329-bp long region of a CpG island located in the promoter region of the AR was PCR amplified from the bisulfite-treated genomic DNA by using two primer pairs with different forward primers but the same reverse primer. The two different primer pairs (different forward primers but the same reverse primer in the two assays) were included as a control mechanism of the assay used. To be included in the final analyses, the analyzed CpG units should have the same methylation degree when the two different primer pairs were used. The sequence of the forward primer of primer pair one was 5′-TGG TTT TTA GGA AAT TTG GAG TTT TG-3′ and of primer pair two 5′-AGG AAG TAG GGG TTT TTT AGG GTT AGA GT-3′. A T7 [5′-CAG TAA TAC GAC TCA CTA TAG GGA GA] promoter tagged reverse primer (5′-CTC TCC CAA CAA ACT AAC TCC CC-3′) was identical for the two primer pairs. The PCR reactions were carried out in a total volume of 5 μl by using 1 pmol of each primer/40 μM dNTP/0.1 units of Hot Star *Taq*DNA polymerase (Qiagen)/1.5 mM $MgCl_2$/buffer supplied with the enzyme (final concentration 1×). The reaction mix was pre-activated for 15 min at 95°C. The reactions were amplified in 45 cycles of 95°C for 20 s, 62°C for 30 s, and 72°C for 30 s, followed by 72°C for 3 min. Unincorporated dNTPs were

dephosphorylated by adding 1.7 μl H_2O/0.3 units of shrimp alkaline phosphatase (SAP). The reaction was incubated at 37°C for 20 min, and SAP was then heat inactivated for 10 min at 85°C.

Typically, 2 μl of the PCR reaction was directly used as template in a 4 μl transcription reaction. Twenty units of T7 R&DNA polymerase (Epicentre, Madison, WI) was used to incorporate non-cleavable dCTP in the transcripts. Ribonucleotides were used at 1 mM and the dNTP substrate at 2.5 mM; other components in the reaction were as recommended by the supplier. After the in vitro transcription, RNase A was added to cleave the in vitro transcript. The mixture was then further diluted with H_2O to a final volume of 27 μl. Conditioning of the phosphate backbone before MALDI-TOF MS was achieved by the addition of 6 mg CLEAN Resin (SEQUENOM, Inc., San Diego, CA).

Mass spectrometry measurements

15 nl of the cleavage reactions was robotically dispensed onto silicon chips preloaded with matrix (SpectroCHIP; SEQUENOM, Inc.). Mass spectra were collected by using a MassARRAY mass spectrometer (Bruker-SEQUENOM). Spectra were analyzed by using proprietary peak picking and spectra interpretation tools. CpG units, clearly identified both when using primer pair one and when using primer pair two in the PCR reactions, were evaluated in the further association studies and for these analyses the average of duplicate methylation measurements was used.

Statistical analysis

Spearman rank correlation assessed univariate associations among variables. Values are given as means ± SD. We performed statistical analyses with SPSS for Windows (version 13.0, SPSS, Chicago, IL).

Acknowledgments This study was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, The ALF/LUA research grant in Gothenburg, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation, the Novo Nordisk Foundation, the Magnus Bergvall Foundation, and the Tore Nilson Foundation.

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