Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 492–496
BBRC
www.elsevier.com/locate/ybbrc

Quantitative methylation-sensitive arbitrarily primed PCR method to determine differential genomic DNA methylation in down syndrome

Abalo Chango [a,b,*], Latifa Abdennebi-Najar [a], Frederic Tessier [a], Séverine Ferré [a], Sergio Do [c], Jean-Louis Guéant [b], Jean Pierre Nicolas [b], Francis Willequet [a]

[a] *ISAB, Department of Animal Science and Nutritional Science and Health, Beauvais, France*
[b] *INSERM U724, Vandœuvre lès nancy, France*
[c] *Laboratory of Medical Biochemistry, Vandoeuvre-Lès-Nancy, France*

Received 28 July 2006
Available online 15 August 2006

Abstract

Relative levels of DNA hypermethylation were quantified in DS individuals using a new method based on a combination of methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP-PCR) and quantification of DNA fragments with the Agilent 2100 bioanalyzer. Four of the DS individuals had low plasma total homocysteine (tHcy) level (4.3 ± 0.3 μmol/l) and 4 other had high-tHcy level (14.1 ± 0.9 μmol/l). Eight healthy control individuals were matched to the DS cases for age, sex, and tHcy levels. We have identified and quantified six hypermethylated fragments. Their sizes ranged from 230-bp to 700-bp. In cases and controls, low-tHcy did not affect methylation level of identified fragments, mean methylation values were $68.0 \pm 39.7\%$ and $52.1 \pm 40.3\%$, respectively. DNA methylation in DS individuals did not change significantly ($59.7 \pm 34.5\%$) in response to high-tHcy level in contrast to controls ($23.4 \pm 17.7\%$, $P = 0.02$). Further, the quantitative MS-AP-PCR using this microfludic system is a useful method for determining differential genomic DNA methylation.

Keywords: Arbitrarily primed PCR; DNA methylation; Homocysteine; Down syndrome

There is evidence that individuals with trisomy 21 (Down syndrome) have abnormal one carbon (folate/homocysteine) metabolism resulting in altered DNA methylation. Recently, we demonstrated that plasma homocysteine, S-adenosylmethionine (AdoMet), and *S*-adenosylhomocysteine (AdoHcy) levels were significantly decreased in children with Down syndrome (DS). These alterations were associated with lymphocyte genomic DNA hypermethylation relative to normal siblings [1]. The low level of methionine metabolites is consistent with an over expression of the cystathionine beta-synthase (CBS) gene which is located on chromosome 21. The associated 157% increase in CBS activity is thought to deplete homocysteine and its metabolic precursors [2,3].

Methylation of specific cytosines in promoter region CpG islands is a major mechanism for regulation of gene expression [4]. DNA methylation is necessary for normal embryonic development and may affect gene expression during embryonic development and differentiation [5]. The increase in global DNA methylation in lymphocytes of children with DS observed in our previous study was unexpected in the context of reduced AdoMet and AdoHcy levels [1]. It is possible that the DNA hypermethylation reflected the presence of three highly methylated sequences of chromosome 21 [6–8]. The ability to detect methylation changes in DS is of critical importance to determine whether alterations in DNA methylation contribute to the observed down-regulation of certain genes.

* Corresponding author. Fax: +33344062526.
E-mail address: abalo.chango@isab.fr (A. Chango).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.038

Given the fact that DNA methylation patterns can vary among individuals, we have developed a sensitive and quantitative method to determine relative levels of DNA methylation in individuals with DS as compared to age and sex-matched controls according to their plasma total homocysteine level. Hypermethylated DNA fragments were detected by methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP-PCR) as described by Gonzalgo et al., [9] and Liang et al., [10]. As in their method quantitative information cannot be obtained from visual inspection of restriction patterns, we combined the MS-AP-PCR and DNA fragments quantification capability with the Agilent 2100 bioanalyzer. The bioanalyzer is a novel type of capillary electrophoresis instrument for nucleic acid separation based on microfluidic technology [11,12]. It utilizes lab-on-a-chip to perform electrophoresis. The 2100 expert software allowed real-time analysis of the data.

Subjects and methods

Subjects and DNA samples. To demonstrate the application MS-AP-PCR with bioanalyzer quantification, we selected 8 DNA samples from individuals with full trisomy 21 (4 with low tHcy level, 4 with high tHcy level), and 8 DNA samples from healthy controls (4 with low tHcy level, 4 with high tHcy level) with an age of 18 ± 2 years. Frozen samples from an earlier study were used [13]. The protocol was approved by the Ethics Committee for studies with human subjects of CHU de Nancy and volunteers gave their informed consent for the investigation. Fasting blood from participants was collected by venipuncture and immediately placed on ice. All samples were processed by centrifugation for 20 min at 2000*g* and aliquots were stored at −20 °C until analysis. Fasting tHcy concentration, total amount of protein and non-protein bound homocysteine were determined by HPLC [14]. Red blood cell (RBC) folate and serum folate were measured by immunoassay (Abbott, Rungis, France). Genomic DNA was isolated from white blood cells (WBC) with the use of the NucleoSpin Blood Quick Pure (Machery-Nagel, Cergy Pontoise, France).

DNA digestion and methylation-sensitive AP-PCR. Restriction enzymes *Rsa*I, *Hpa*II, and *Msp*I were purchased from NEB (Beverly, MA). The *Taq* polymerase was from Appligen (Illkirsh, France). The MS-AP-PCR method was performed as described by Gonzalgo et al., [9]. Briefly, 500 ng of DNA in 10 μl was digested with either 5 U of *Rsa*I, or 5 U of *Rsa*I + 5 U of the methylation sensitive restriction enzyme *Hpa*II, or 5 U of *Rsa*I + 5 U of *Msp*I. Mixtures were incubated at 37 °C for 16 h, followed by incubation at 65 °C for 20 min to inactivate the enzymes. *Hpa*II and *Msp*I are two isoschizomers known to discriminate between methylated and unmethylated CpG sites. Digestion with either *Rsa*I, *Rsa*I + *Hpa*II, or *Rsa*I + *Msp*I were used as a rapid and efficient means of preparing DNA for MS-AP-PCR analysis. The *Rsa*I restriction enzyme in combination with *Hpa*II and *Msp*I generates smaller fragments of DNA prior to AP-PCR amplification and reduces the number of potential artifacts that might be generated [10]. Two microliters of digested DNA was amplified in 50 μl of total volume, with 50 pmol of a single primer (5′-AAC CCT CAC CCT AAC CCC GG) as sense and antisense, 200 μM each of four deoxynucleotide triphosphates, 1.6 U of *Taq* polymerase, 4 mM $MgCl_2$, and 10 mM Tris–HCl buffer (pH 8.0). The AP-PCR program utilized 5 low-stringency cycles (94 °C for 30 s, 40 °C for 60 s, 72 °C for 90 s) followed by 27 increased-stringency cycles (94 °C for 15 s, 55 °C for 15 s, 72 °C for 60 s). Low stringency PCR permits the identification of differentially methylated bands. The random association of primers with genomic DNA at low annealing temperatures produced multiple PCR fragments detected by fluorescence and visible as peak by the bioanalyzer software [11].

Chip preparation and DNA methylation quantitation by Bioanalyzer 2100. Chip-based separations were performed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), using the DNA 1000 LabChip kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. The instrument allows for highly sensitive laser-induced fluorescence detection using an intercalating dye, which is added to the polymer (gel). The bioanalyzer software automatically calculates size and concentration of each separated band and displays the results in real-time [11]. For analysis of AP-PCR products, gel matrix of the DNA 1000 LabChip kit was prepared by adding 25 μl of dye to a gel vial. The gel/dye mixture was filtered through a spin filter. The chip was filled with the gel/dye mixture and 9 μl of gel/dye mixture were added to the buffer wells. Sample and ladder wells were filled with 5 μl of DNA marker solution before adding 1 μl of ladder and sample in the respective wells. The chip was vortexed and placed in the bioanalyzer for analysis. The PCR products were separated, detected and quantified automatically. The intercalating dye within the sieving matrix allows migrating DNA fragments to be detected using fluorescence imaging. It was not necessary to dilute the PCR products before analyses. The chip preparation time was about 5 min including loading of samples. Typically, a chip assay run time was about 30 min.

Determining linear range of AP-PCR for quantification. The linear range for quantitative PCR amplification was determined as follows: 100 μl of PCR master mix was prepared including 4 μl digested DNA with *Rsa*I + *Hpa*II. The mixture was divided into aliquots which were then subjected to AP-PCR. After the 5 low-stringency cycles (94 °C for 30 s, 40 °C for 60 s, 72 °C for 90 s), aliquots were removed from the thermal cycler at 18, 21, 24, 27, 30 and 33 cycles of the increased-stringency conditions (94 °C for 15 s, 55 °C for 15 s, 72 °C for 60 s), and then resolved in LabChips.

Statistical analysis. Statistical analyses were performed using StatView-5 (SAS, North Carolina, USA) on a Macintosh computer. Comparisons of quantitative data were done by analysis of variance (ANOVA), followed by post-ANOVA Bonferroni/Dunn test. Results are presented as means ± SD. A P value less than 0.05 was considered statistically significant.

Results

The mean tHcy level in DS cases and controls with low-tHcy was 4.3 ± 0.3 μmol/l and 3.7 ± 0.6 μmol/l, respectively. The mean tHcy level for cases and controls with high-tHcy was 14.4 ± 0.4 μmol/l and 15.5 ± 2.3 μmol/l, respectively (Table 1). To be able to assess DNA methylation in different samples, we first determined the linear range of AP-PCR using *Rsa* I + *Hpa* II digested DNA. Cycles 24–30 of the increased-stringency condition were in the linear range. Amplification with 27 cycles was chosen for quantification. Fragments were determined to be hypermethylated if present after *Rsa*I + *Hpa*I digestion but absent or decreased significantly after *Rsa*I + *Msp*II digestion. The presence of a fragment indicated that a methylated -CCGG- site was present between the two positions of primer hybridization. We used concentration of fragments (C_{frag}) generated by bioanalyzer software to calculate the percentage of methylation as follows:

$$\text{Mv} = 100 - \left[\frac{C_{\text{frag}}\textit{Rsa}\text{I} + \textit{Msp}\text{I}}{C_{\text{frag}}\textit{Rsa}\text{I} + \textit{Hpa}\text{II}} \times 100\right]$$

$$= \% \text{ of methylation for identified DNA fragment.}$$

C_{frag}*Rsa*I + *Hpa*II: correspond to the concentration of specified fragment after *Rsa*I + *Hpa*II digestion.

C_{frag}*Rsa*I + *Msp*I: correspond to the concentration of specified fragment after *Rsa*I + *Msp*I digestion.