DHPLC-Based Method for DNA Methylation Analysis of Differential Methylated Regions from Imprinted Genes

BioTechniques 34:356-362 (February 2003)

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ABSTRACT

The bisulfite genomic sequencing method is one of the most widely used techniques for methylation analysis in heterogeneous unbiased PCR, amplifying for both methylated and unmethylated alleles simultaneously. However, it requires labor-intensive and time-consuming cloning and sequencing steps. In the current study, we used a denaturing high-performance liquid chromatography (DHPLC) procedure in a complementary way with the bisulfite genomic sequencing to analyze the methylation of differentially methylated regions (DMRs) of imprinted genes. We showed reliable and reproducible results in distinguishing overall methylation profiles of DMRs regions of human SNRPN, H19, MEST/PEG1, LIT1, IGF2, TSSC5, WT1 antisense, and mouse H19, Mest/Peg1, Igf2R imprinted genes. These DHPLC profiles were in accordance with bisulfite genomic sequencing data and may serve as a type of "fingerprint," revealing the overall methylation status of DMRs associated with sample heterogeneity. We conclude that DHPLC analysis could be used to increase the throughput efficiency of methylation pattern analysis of imprinted genes after the bisulfite conversion of genomic DNA and unbiased PCR amplification.

INTRODUCTION

The detection of 5-methylcytosine of differential methylated regions (DMRs) from imprinted genes has recently found important applications in development, differentiation, aging, and cancer (16,22,23). Many methods have been developed for investigating patterns of DNA methylation (11,12). Early methods that allow for the assessment of CpG dinucleotide methylation status all employ the digestion of genomic DNA with methylation-sensitive enzymes, followed by Southern blot hybridization. However, the main disadvantages are the limitation to available sites for sequence-specific methylation-sensitive enzymes, the occurrence of false positives due to incomplete digestion, and the abundant starting DNA required. Alternatively to conventional methods, gene-specific approaches are now primarily based on the sodium bisulfite treatment of the DNA, which converts all unmethylated cytosines to uracil, leaving 5-methylcytosine unaltered. Several methods have been developed that utilize bisulfite treatment of DNA as a starting point for methylation analysis. Methylation-specific PCR (MSP) uses an individual primer designed to discriminate between converted and non-converted cytosines (15). MSP is semi-quantitative; a restricted number of cytosines can be analyzed simultaneously, and false positives occur frequently (8). Other approaches are the unbiased amplification using primers that do not contain CpG dinucleotides so that the amplification step does not discriminate between templates according to their original methylation status. After cloning and

sequencing, this genomic bisulfite sequencing analysis has the advantage of allowing for the quantification of the cytosine methylation pattern (13,21). Therefore, these labor-intensive and time-consuming steps of cloning and sequencing limit the use of this method for high-throughput analyses. Clearly, there is a need for an alternative rapid and sensitive method using unbiased PCR for methylation analysis of DMRs from imprinted genes, in a way that is complementary with the bisulfite genomic sequencing.

The denaturing high-performance liquid chromatography (DHPLC) method potentially meets these criteria (25). On the basis of column-retention times under partially denaturing conditions, DHPLC analysis allows the discrimination between different modifications in the same DNA segments. Differential elution profiles are produced, based on the temperature-dependent resolution of heteroduplexes from homoduplexes.

The aim of this study was to determine whether, for imprinted genes, the DHPLC method can be employed for the methylation analysis of DMRs in heterogeneous unbiased PCR, amplifying both methylated and unmethylated alleles simultaneously after bisulfite modification, compared to bisulfite genomic sequencing procedure.

MATERIALS AND METHODS

Bisulfite Genomic Sequencing

Genomic DNA was extracted from human blood samples and mouse granulosa cells. The sodium bisulfite conver-

Table 1. DMRs of Imprinted Genes Analyzed in This Study

Genes	GenBank® Accession Number	Nucleotid Positions (nt)	Sequence of the Primers (5'→3')
Human			
<i>SNRPN</i>	NT_010178.11	1315869–1316115	TTTGAGAGAAGTTATYGGTATAGTTGAT CAACAATACTATAAAACCCTAAAAATCCA
<i>H19</i>	AF087017	6005–6326	TGTATAGTATATGGGTATTTTGGAGGTTT TCCTATAAATATCCTATTCCTAAATAACC
<i>MEST/PEG1</i>	Y10620	609–898	TYGTTGTTGGTTAGTTTTGTAYGGTT CCCAAAAACAACCCCAACTC
<i>LIT1</i>	U90095	67301–67502	TTAATTTGAGTTYGGGTGGTATTA ACCRCTTCTACCTAAAACTACRACAA
<i>IGF2 exon 8–9</i>	AC005809	43058–43312	GGGAAAGGGGTTTAGGATTTTAT ATAATTTACTCCCCCTTCAACCTC
<i>TSSC5</i>	AC005950	112571–112804	GTTTTGAGAGGAGGGAGGTTTTT CAAATAAAACRCCAAAACAACCAA
<i>WT1 antisense</i>	NT_009237.11	2601633–2601818	TGAAGAGGGTAAATTATAGGGGTTT CTTCAAAACCTAAAATTAACAAAAACA
Mouse			
<i>H19</i>	U19619.1	2975–3204	GTTTATAAGGGTTATGGGGTGGATATAAT TCCCTTTATTAAACCTAAAATACTCAA
<i>Mest/Peg1</i>	AF017994	2239–2556	GAGATTTATAAGGAAAGAGGGGGTAG ACAACAAAAACAACAACAACA
<i>Igf2R</i> imprinting box	L06446	1857–2101	AGTGGGGTATTTAGTGTTGAGTTTTT TCTACAAATAAAAATAAAATTCRRTACAA

Y: C/T; R: A/G.

sion of genomic DNA was performed as previously described (17,18). Briefly, after denaturation in freshly prepared sodium hydroxide solution (0.3 M NaOH for 30 min), bisulfite solution was added (4 M sodium bisulfite, 125 mM hydroquinone, pH 5.0) to each reaction tube. The reaction mixtures were overlaid with mineral oil and incubated overnight at 55°C. DNA was washed using the DNA Clean-Up Wizard® system (Promega, Lyon, France) and resuspended in water. DMRs regions of human *SNRPN* (5), *H19* (10), *MEST/PEG1* (19), *LIT1* (10), *IGF2* (10), *TSSC5* (10), *WT1 antisense* (20), and mouse *H19*, *Mest/Peg1*, *Igf2R* (6) imprinted genes were analyzed by bisulfite genomic sequencing. PCRs were performed in 10 mM PCR buffer, 3 mM MgCl₂, 0.2 mM dNTP, 0.5 µM each primer, and 1.25 U AmpliTaq Gold® DNA polymerase (Perkin Elmer Life Sciences, Gaithersburg, MD, USA) with the following conditions: 94°C for 10 min, followed by 40 cycles of 94°C for 45 s, 61°C

PEG1, 59°C (*WT1-antisense* and *SNRPN*), or 55°C (*H19* mouse and *Mest/Peg1*) for 45 s, 72°C for 1 min, and a final extension step of 10 min at 72°C. Table 1 lists the accession numbers, the nucleotide position, the primers used in the PCR, the number of CpGs analyzed, and the size of the PCR product. The amplified DNA was cloned into the CloneAmp pAMP1® system (Invitrogen, Cergy Pontoise, France) and sequenced using an ABI PRISM® 377 DNA sequencer (Perkin Elmer Life Sciences). After sequencing, fully unmethylated and methylated clones were used as matrices to generate corresponding products for DHPLC analysis.

DHPLC Analysis

The assessment of methylation patterns was performed by DHPLC scanning on an automated DHPLC instrument (WAVE®, Hitachi model D-7000 Chromatography Data Station Software; Transgenomic Ltd., Cheshire, UK), and a DNasep® Cartridge

(Transgenomic, Santa Clara, CA, USA) was used as a column.

Heteroduplex molecules are formed only when a variation at the heterozygous state is present in a DNA fragment after the PCR product has been denatured and gradually reannealed (Figure 1). PCR products were denatured at 95°C for 5 min and cooled to 56°C for 10 min. The samples were kept cool until 10 µL were applied to a preheated C18 reversed-phase column based on non-porous poly(styrene-divinylbenzene) particles (DNasep). DNA was eluted within a linear acetonitrile gradient consisting of buffer A [0.1 M triethylammonium acetate (TEAA)]/buffer B (0.1 M TEAA, 25% acetonitrile). The mobile phase was heated to the respective denaturing temperature by means of a coil contained in a tin-alloy block (Timberline, Boulder, CO, USA). The elution of DNA could be observed on line at 260 nm. Regeneration of the column was achieved by washing with 100% buffer B for 1 min, followed by an equilibration time of 2 min.

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RESULTS

The DHPLC Method Applied to the DMR of the *SNRPN* Gene

To investigate the efficiency of DHPLC in detecting methylation patterns within a specific PCR product (Figure 1), we compared the results of DHPLC analysis with our sequence data from the bisulfite genomic sequencing of the DMR of the human imprinted *SNRPN* gene using the DNA of normal individuals.

The temperature at which homoduplex and heteroduplex detection occurred was evaluated experimentally by analyzing the elution time and the separation of PCR products derived from fully methylated and unmethylated clones, harboring a known sequence variation (Figure 2). PCR products of fully methylated and unmethylated sequences were obtained by the PCR amplification from corresponding clones previously sequenced after bisulfite genomic sequencing. To identify the optimal running temperature, we compared differential elution profiles of fully methylated and unmethylated PCR products in a range from 50°C to 65°C. The running temperature was determined based on the optimum separation of the different alleles and the peak intensity obtained for the methylated and unmethylated alleles (58°C in this case) (Figure 2b).

Once the conditions for the temperature have been determined, the DHPLC curves of the PCR products from blood samples of control individuals were studied, compared to PCR products from methylated and unmethylated sequences. Shown as chromatograms in Figure 3b, single peaks consisting of homoduplexes characterized homogeneous samples with methylated or unmethylated alleles, whereas multiple peaks consisting of homoduplex and complex heteroduplex formations characterized heterogeneous samples. The pattern of the chromatograms obtained by DHPLC showing methylated, unmethylated, and/or both alleles was in accordance with the methylation profiles assessed by bisulfite genomic sequencing (Figure 3, panels a and b). In the mixed sample containing 50% unmethylated and 50% methylated DNA,

heteroduplexes that form in PCR samples display reduced column-retention time relative to their homoduplex counterparts (data not shown). Elution profiles from the DHPLC analysis of PCR from lymphocytes (individuals A and B) indicate that substantial heteroduplex is formed even with various components of unmethylated and methylated alleles, as judged after bisulfite genomic sequencing. Taken together, these results indicated that, when compared to bisulfite sequencing analysis, the use of DHPLC analysis could be extended to complex DMRs methylation analysis.

The DHPLC reproducibility on pe-

ripheral blood lymphocyte specimens was then evaluated on eight different control individuals (Figure 3c). Because of specific retention times, the methylation patterns could be predicted by DHPLC independently of sequencing information by overlaying the DHPLC profiles with those of reference fragments.

Application of the DHPLC Method to DMRs of Nine Imprinted Genes

We then extended the application of DHPLC to other DMRs of human and mouse imprinted genes (Figure 4). As

Figure 1. A schematic representation for the analysis of methylation patterns by DHPLC. Example of one CpG. After the treatment of the genomic DNA by sodium bisulfite, PCR amplification is done by the use of primers that do not cover any CpG dinucleotides sites in the original genomic DNA. Denaturation and cooling slowly hybridize the PCR products and form a mixture of heteroduplexes and homoduplexes. The resolution of the reannealed DNA species depends on the denaturing conditions and the magnitude of the differences in the melting temperature (T_m). The homoduplexes start to denature, with the T-A wild-type homoduplex being more denaturated than the C-G homoduplex. The heteroduplex DNA fragments start to denature in the region either side of the mismatched bases (A-C or T-G > T-A > CG). The denaturation leads to a reduction in the double-stranded portion of the PCR fragment. Partially denatured DNA fragments corresponding to heteroduplexes elute earlier (single or double peaks) than double-stranded fragments at elevated temperature on a DHPLC column. CG^{5m}, methylated CG; (#), mismatch; (*), heteroduplexes.

for *SNRPN*, the optimal DHPLC sampling temperature for analysis was experimentally determined as the temperature that causes the product to be eluted with discriminative peaks for the methylated and unmethylated alleles [58°C for human *H19*; 62°C for

MEST/PEG1; 55°C for *LIT1*; 57°C for *IGF2*; 58°C for *TSSC5*; 55°C for *WT1 antisense*; 55°C for mouse *H19*; 58°C for *Mest/Peg1*; and 57°C for *Igf2R* (data not shown)]. For each gene studied, products of fully methylated and unmethylated sequences were obtained

Figure 2. Determination of the running temperature for the *SNRPN* imprinted gene. Examples of temperature-dependent resolution at 59°C, 58°C, and 57°C (panels a, b, and c, respectively) of two PCR products (one fully methylated and one fully unmethylated) tested in two separate wells by DHPLC. The 58°C running temperature was chosen as the optimal temperature based on the separation of the different allele and the peak intensity obtained for the methylated and unmethylated alleles. The high number of base differences between the two alleles result in a spectrum of temperatures at which the two alleles are distinguishable. Control methylated and unmethylated samples were done on previously sequenced fully methylated and unmethylated clones. (●), *SNRPN* fully methylated allele; (●), *SNRPN* fully unmethylated allele. (*), heteroduplexes.

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by the PCR amplification of clones previously sequenced after bisulfite genomic sequencing. Methylation analysis of samples was done on individuals A and/or B for human genes and on granulosa cells for mouse genes. As previously indicated, homogeneous samples eluted as a single peak, whereas heterogeneous samples were characterized by multiple peaks consisting of homoduplex and heteroduplex formations. These DHPLC profiles were in accordance with bisulfite genomic sequencing results (data not shown).

DISCUSSION

Many studies have shown the accuracy, sensitivity, and sequence independence of the bisulfite genomic sequencing technique to provide methylation analysis at multiple CpGs sites (11,13). However, time-consuming tasks such as

cloning and sequencing limit the use of bisulfite genomic sequencing for large-scale studies. Different methods that bypass the cloning and sequencing steps have been developed to study methylation patterns after the bisulfite treatment of DNA (12). However, most of these current methods favor either the resolution at the level of individual CpG nucleotide or the overall analysis of multiple CpGs within an amplified region, but none of them simultaneously meet both of these requirements. Analyses at a given CpG are done either with combined bisulfite restriction analyses (COBRA) (26), methylation-sensitive single nucleotide primer extension (Ms-SNuPE) (14), or with microarray-based DNA methylation analysis (MDMA) (1). In the latter approach, after bisulfite treatment and unbiased PCR amplification using fluorescently labeled PCR primers, products are array-hybridized to oligonucleotides that can discrimi-

nate between single bisulfite-converted or non-converted CpG sites of multiple candidate genes. These technologies are powerful approaches for semi-quantitative or quantitative analyses of methylation at specific sites but are still restricted to a limited number of CpGs that can be simultaneously analyzed within a specific gene. In contrast, MSP (15), MethyLight (8), or methylation-sensitive single-stranded conformational polymorphism [bisulfite-PCR-SSCP is also called methylation-sensitive single-strand conformation analysis (MS-SSCA) (4,24)] are based on differential methylation states of alleles and give an overall methylation analysis. In these approaches, differences in methylcytosine patterns are displayed at the PCR amplification step by methylation-dependent primer/probe design (e.g., MSP and MethyLight) or after unbiased amplification of variants (MS-SSCA). These techniques yield semi-quantita-

Figure 3. Comparison between DHPLC and bisulfite genomic sequencing for the *SNRPN* gene. (a) Structural characteristics and methylation profile of the human *SNRPN* gene in the analyzed region. The region analyzed in this study extends from position -144 to +102 relative to the *SNRPN* transcriptional start (arrow). Bisulfite-modified DNA from pools of adult lymphocytes from peripheral blood (individuals A and B) was PCR amplified, and individual clones of the resultant PCR product were sequenced. Each row of squares represents a single cloned allele, and each square represents a single CpG site (open square, unmethylated cytosines; filled square, methylated cytosines). (b) The elution profiles of the PCR products from individuals A and B and PCR products from fully methylated and unmethylated *SNRPN* alleles were tested separately by DHPLC. Because of the difference in their retention times, samples with unmethylated and methylated sequences eluted as single and separate peaks, whereas multiple peaks consisting of the homoduplex and heteroduplex formations characterized heterogeneous samples. As exemplified in Figure 1 for one CpG, complex heteroduplex formations between methylated and unmethylated alleles occur after denaturation, resulting in multi-chromatogram peaks. PCR product from: (●), *SNRPN* fully methylated allele; (○), *SNRPN* fully unmethylated allele; (●), individual A; and (●), individual B. (c) DHPLC reproducibility on PCR from the lymphocytes of eight control individuals. The elution profiles of the PCR products from the eight individuals were tested separately by DHPLC. (*), heteroduplexes.

tive data that are particularly useful for determining the relative proportions of methylated and unmethylated alleles in complex PCR products, although the resolution of methylation states at the nucleotide level is not yet possible. Furthermore, the dependence of MSP or MethyLight on specific nucleotide variations located at primer/probe mating regions restricts methylation analysis to a few CpG sites.

In this work, we showed that the DHPLC method gives an overall picture of methylation patterns of multiple CpGs within an unbiased PCR product, amplifying both methylated and unmethylated alleles simultaneously, as compared to bisulfite genomic sequencing. Because of the reproducible elution profiles and shapes of the DNA peaks obtained on references fully methylated and unmethylated PCR samples, the

profile of unknown samples can be compared and analyzed under exactly the same conditions previously optimized for each gene. Overlaying the profiles of heterogeneous PCR amplifications with that of the reference fragments showed this difference best and may serve as a type of fingerprint that allows us to assess methylation patterns. In contrast to the tedious analysis of sequence data after bisulfite modification, the evaluation of results by DHPLC is quite effortless because the interpretation of the results is based on the possibility of discriminating between single and multiple peaks of elution profiles. In addition, this approach is adaptable for large-scale and semi-automated analysis of known DMRs.

Previous reports have shown the value of DHPLC in methylation analysis studies. In particular, Baumer et al. (2,3)

have described a MSP/DHPLC technique. The method offers a rapid and reliable alternative to conventional methods used after MSP to determine parent-of-origin-specific methylation patterns. Baumer et al. (2,3) also described the advantages of MSP/DHPLC to detect cell mosaicism and the effect of variable levels of methylation at CpG sites located between the priming sequences. However, methylation information in this MSP/DHPLC is limited by the sequence discrimination at the PCR amplification level. Furthermore, Deng et al. (7) and El-Maarri et al. (9), respectively, have shown other applications of such an approach, consisting of the simultaneous detection of CpG methylation after unbiased PCR and single-nucleotide polymorphisms and single-nucleotide primer extension (SNUPE) assays for methylation analy-

Figure 4. Application of the DHPLC method to human (a) *H19* and (b) *MEST/PEG1*, (c) *LIT1*, (d) *IGF2*, (e) *TSSC5*, (f) *WT1 antisense*, and mouse (g) *H19*, (h) *Mest/Peg1*, (i) *Igf2R* imprinted genes. Bisulfite-modified DNA from pools of human lymphocytes from peripheral blood (individual A and/or B) and mouse granulosa cells was PCR amplified as previously described (17). The elution profiles of the PCR products from fully methylated and unmethylated clones and the PCR products from individual A and/or B were tested in separate wells by DHPLC. PCR product from: (●), fully methylated allele; (●), fully unmethylated allele; (●), individual A for human imprinted genes; (●), individual B; and (●), granulosa cells for mouse imprinted genes. (*), heteroduplexes.

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sis at specific CpG sites (9). These applications corroborated the utility of DHPLC in analyzing DMRs of imprinted genes. However, these reports had not described the application of DHPLC to the identification of homo/heteroduplex heterogeneity in the methylation analysis of unbiased PCR products with no additional steps (i.e., digestion assay and SNUPE). In this regard, the current study constitutes an advance and provides a complementary approach for methylation analysis.

Another attractive application of this DHPLC-based technique is the quantification of the relative estimate of methylated and unmethylated molecules. Our preliminary results on imprinting defects, such as in Angelman and Prader-Willi patients or in tumor samples, showed that it is possible to relatively quantitate unmethylated/methylated alleles corresponding to complete loss or gain of methylation (data not shown). However, at first sight, it is reasonable to mention that this method does not allow for the quantitation of the number of methylated and unmethylated copies of a DMR at a given CpG site—such information can only be obtained by MSP-DHPLC as described by Baumer et al. (2,3) and SNUPE-DHPLC as described by El-Maarri et al. (9) (with the latter method being the more quantitative one), or sequencing of clones. Hence, the described assay represents the first step in a comprehensive analysis of methylation status using an unbiased PCR/DHPLC-based approach in the large-scale analysis of imprinted DMRs.

ACKNOWLEDGMENTS

We are grateful to Andras Paldi for helpful advice. This study was supported in part by grants from the Fondation Bettencourt Schuller, Fondation EDF, Fondation pour la Recherche Médicale (FRM). P.C. and K.P. contributed equally to this work.

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Received 14 October 2002; accepted 11 December 2002.

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