Rapid and quantitative method of allele-specific DNA methylation analysis

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Several biological phenomena depend on differential methylation of chromosomal strands. While understanding the role of these processes requires information on allele-specific methylation, the available methodologies are not quantitative or labor-intensive. We describe a novel, rapid method to quantitate allele-specific DNA methylation based on the combination of bisulfite PCR and Pyrosequencing™. In this method, DNA is first treated with sodium bisulfite, which converts cytosine but not 5-methylcytosine to uracil. Genes of interest are subsequently amplified using PCR. Allele-specific methylation can then be determined by pyrosequencing each allele individually using sequencing primers that incorporate single nucleotide polymorphisms (SNPs) that allow differentiation between the two parental alleles. This allele-specific methylation methodology can potentially afford quantitative analyses relevant to the regulation of X chromosome inactivation, allele-specific expression of genes in the immune system, repetitive elements, and genomic imprinting. As an illustration of our new method, we quantitated allele-specific methylation of the differentially methylated region of the H19 gene, which is imprinted. Although we could reliably determine allele-specific methylation with our technique, additional studies will be required to confirm the ability of our assay to measure loss of imprinting.

INTRODUCTION

Background

Variation in allele-specific expression is common in humans (1,2) and underlies normal human variability and predisposition to human diseases (3). Genomic imprinting is an example of allele-specific expression, where only one chromosomal allele is expressed. In imprinting, this monoallelic expression is dependent on the parental origin of the chromosome (4). Since normal genomic imprinting is regulated, in part, by methyl groups on cytosines within cytosine-guanine (CpG) dinucleotides, normal or loss of imprinting (LOI) can be predicted by DNA methylation in regions that are differentially methylated between parental alleles (differentially methylated regions; DMRs) (5,6). As a proxy for parent-specific expression, there are two current methodologies used to analyze DNA methylation. The first is bisulfite genomic sequencing

(7) of multiple alleles, a process that is labor-intensive. The second is bisulfite pyrosequencing, which does not assess allele-specific methylation levels, but instead, averages methylation levels of both alleles in a highly quantitative manner (8). Our new method uses both bisulfite PCR and allele-specific pyrosequencing to measure DNA methylation of a single allele. To illustrate our new methodology, we analyzed allele-specific methylation of the *H19* gene, a gene that is normally imprinted and expressed only from the maternal allele.

Outline of the Allele-Specific Methylation Methodology

To obtain the methylation status of the CpG sites of interest, we adapted a standard method known as bisulfite genomic sequencing. In brief, this method is based on the selective deamination of cytosine to uracil by treating DNA with bisulfite and the sequencing of subsequently generated PCR

products. In contrast to cytosine, 5-methylcytosine does not get converted by bisulfite and remains as cytosine. This inherent selectivity of bisulfite to induce a primary sequence change can be used to distinguish DNA methylation status (7).

DNA methylation at a specific CpG dinucleotide can then be quantitated as a ratio of cytosine to thymine, for which several methods have been developed (8–16). To amplify the *H19* imprinting center, specifically the region harboring 19 CpG sites within and surrounding a CTCF binding site, primers specific to the bisulfite-converted template that are not dependent on the methylation status were used.

To measure DNA methylation of each allele individually, we used Pyrosequencing™, with sequencing primers that were directed to a heterozygous single nucleotide polymorphism (SNP). Thus, a pair of sequencing primers was used to measure methylation of each allele separately. Hence, the sequencing primers incorporate

the polymorphic information of the parent-specific mark (Figure 1). We demonstrate that this methodology correctly identified the percentage of allele-specific methylation within a 3% margin of error.

An immediate health application of this allele-specific methylation methodology is to classify persons as to whether or not they have LOI in prospective epidemiologic studies. As a proof of principle, we present the application of this methodology to study a methylation-dependent imprinting center: the *H19* imprinting center, which regulates the paternally imprinted noncoding RNA H19 (17–19). We can distinguish the methylation levels of each individual allele by exploiting a SNP in the upstream region of the *H19* gene (rs2071094; dbSNP build 124) (20) (Figure 1). By doing so, we can quantitate methylation on each allele at a single base resolution for persons who are heterozygous for the SNP.

MATERIALS AND METHODS

Cell Lines and Human Tissue Samples

The Colo205 colorectal carcinoma cell line was cultured in Dulbecco's modified essential medium at 37°C in a humidified atmosphere with 5% CO₂. Tissue samples were collected through the University of Southern California Pathology Core Laboratory. Informed written consent was obtained, and the collection was approved by the University of Southern California Institutional Review Board. The informative samples for the allele-specific methylation assay are samples heterozygous for a SNP in the *H19* imprinting center. Genomic DNA was extracted using standard phenol-chloroform methodology.

Sodium Bisulfite Modification of Genomic DNA

Sodium bisulfite modification of genomic DNA was performed as described previously by Frommer et al. (7), with minor modifications. In brief, DNA (approximately 2 µg) suspended in 50 µL distilled water was denatured

in 5.5 µL 0.2 M NaOH at 37°C for 10 min. After the addition of 30 µL 10 mmol hydroquinone and 520 µL 3 M sodium bisulfite, pH 5.0, the DNA was incubated at 50°C for 16 h. The bisulfite-modified DNA was purified with Wizard® Plus kits (Promega, Madison, WI, USA) according to manufacturer's instructions. NaOH (5.5 µL 3M) was added and allowed to incubate for 5 min at room temperature. The bisulfite-treated DNA was precipitated with 0.6 volumes 10 M sodium acetate and 3.0 volumes ethanol using glycogen as a carrier. The final precipitate was resuspended in 20 µL water.

H19 Genotyping Assay

Heterozygosity for the G/A SNP within the *H19* imprinting center (rs2071094, dbSNP build 124; GenBank® accession no. AF125183, nucleotide 8008) was determined by Pyrosequencing (Biotage, Uppsala, Sweden). A 135-bp region was amplified on human genomic DNA with the following PCR primers, 5'-GGTCTCACCGCCTGGATC-3' and 5'-BIOTIN-GACCCGGGACGTTTCCAC-3', and genotyped with the sequencing primer 5'-ACAGCCCCGAGCCCCG-3'.

Amplification of Bisulfite-Converted DNA

Bisulfite-modified DNA (2 µL) was amplified in a primary PCR and followed by a second nested PCR using 1 µL primary PCR product. PCR was performed in a 25-µL volume using 10 pmol of each primer and the Eppendorf® HotMaster™ Taq DNA kit (Eppendorf, Westbury, NY, USA) at reagent concentrations per the manufacturer's instructions. The following primers in the primary PCR were used to amplify the 503-bp fragment from bisulfite-treated DNA: 5'-GAGTTGTGTTTGGGATAGA TGT-3' and 5'-CAATAAAATATCCCA ATTCCA-3'. The primers for the

Figure 1. The epigenetic haplotyping assay. Samples heterozygous for a single nucleotide polymorphism (denoted as G/T) in the differentially methylated regions (DMRs) are utilized in this assay. Bisulfite PCR is used to convert unmethylated DNA (white lollipops) to T, while methylated DNA remains as C. A pyrosequencing primer directed at G or T SNP is used for allele pyrosequencing to measure DNA methylation 3 and 7 bp downstream of the polymorphism.

nested PCR that amplified the 223-bp fragment (nucleotide positions 7876–8102; GenBank accession no. AF125183) were: 5'-GTTTTTATGAG TGTTTTATTTTATAGATG-3' (nucleotide positions 8102–8075; GenBank accession no. AF125183, reverse-complement bisulfite-modified strand) and 5'-CCTCCTCAAAAATCTTTA TAAATACAC-3' coupled with biotin (nucleotide positions 7903–7876; GenBank accession no. AF125183, reverse-complement bisulfite-modified strand). PCR conditions included one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 53°C (primary PCR) or 62°C (nested PCR) for 30 s, 72°C for 30 s, and followed by one cycle of 72°C for 5 min.

Quantification of Allele-Specific Methylation

The allele-specific methylation was quantitated using Pyrosequencing with allele-specific sequencing primers and the Pyro Gold Reagent kit (Biotage) on a Pyrosequencing 96 HS system (PSQ 96 HS; Biotage) according to the manufacturer's protocol. In brief, 10 µL PCR products for each sequencing reaction were immobilized onto streptavidin-coated beads (Streptavidin Sepharose™ HP; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) in binding buffer (10 mM Tris-HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween® 20) for 10 min. The

biotinylated template was purified with the Pyrosequencing vacuum prep tool (Biotage) and incubated with 10 pmol/reaction individually with each sequencing primer in annealing buffer (20 mM Tris-acetate, pH 7.6, and 2 mM MgAc₂). The biotinylated single DNA strand represents the reverse-complement bisulfite-modified strand (GenBank accession no. AF125183; nucleotide positions 7903–7876). The DNA strands were denatured at 80°C for 2 min and reannealed at room temperature for 10 min. Sequencing was performed according to the manufacturer's instructions. The allele-specific sequencing primers were (i) G allele, 5'-GAATTTTAGTTG-3' and (ii) T allele, 5'-GAATTTTAGTTT-3'. In addition, we used sequencing primers that did not recognize either allele (A allele, 5'-GAATTTTAGTTA-3' and C allele, 5'-GAATTTTAGTTC-

3') as a negative control. The allele frequency (% cytosine or % thymidine) was calculated from the peak height analyzed with the allele quantification module in the PSQ 96 HS software (Biotage). Percentage of methylation was determined by the percentage of cytosine to thymidine conversion [methylation = % cytosine/(% cytosine + % thymidine)].

Statistical Methods

To assess the extent that allele-specific methylation assay measures methylation, linear regression analysis was conducted, regressing measured percentage methylation on predicted ("true") percentage methylation, with three replicates at each true value (0%, 25%, 50%, 75%, 100%). Ninety-five percent confidence bands were calculated around the regression line.

RESULTS

Allele-Specific DNA Methylation in the Region that Regulates *H19* Genomic Imprinting

The working model of our assay is an imprinting regulatory region that has been a paradigm for genomic imprinting: the *H19* imprinting center. We quantitated the methylation levels at CpG dinucleotides within the *H19* imprinting center in the colorectal cancer cell line, Colo205 (Figure 2), which is heterozygous for a SNP near the *H19* imprinting center. The *H19* imprinting center is normally methylated on the paternal allele and unmethylated on the maternal allele at binding sites for the CTCF binding factor (CTCF) protein. CTCF functions as a chromatin insulator and binds to the *H19* imprinting center in a methylation-dependent manner (21). The *H19* imprinting center region in Colo205 is heavily methylated (80.4% and 92.4%, respectively, on the two interrogated CpG sites) on the G allele but not on the T allele (0% and 0%, respectively, on the two CpG sites). Therefore, we infer that the G allele is of paternal origin. This allele-specific methylation data was confirmed by traditional genomic bisulfite sequencing of individual clones. While genomic bisulfite sequencing provides information on the methylation status of all CpG dinucleotides across the chromosomal region of interest, this methodology is cumbersome and may be susceptible to preferential amplification or cloning of one of the parental alleles and selection bias (22). To confirm that our pyrosequencing results were accurate, we used combined bisulfite restriction analysis (COBRA). The COBRA assay quantitated average methylation of both alleles to be 44%, which is in agreement with the 45% average of combining both G and T alleles from our pyrosequencing analyses (see Supplementary Figure S1 available online at www.BioTechniques.com).

We next confirmed our allele-specific pyrosequencing results by performing traditional bisulfite genomic sequencing of the 14 CpG sites in the same region of the *H19* allele. Direct bisulfite sequencing showed 100%

Figure 2. Representation of the light profile of CpG sites generated by sequential nucleotide dispensation (Pyrogram™; Biotage). The emitted light pulse is in proportion to the nucleotides present in the sample. Pyrosequencing of the *H19* imprinted region of the colorectal cell line Colo205. (A) Pyrogram using a sequencing primer that anneals to both the G and T allele. (B) Allele-specific pyrosequencing of the G allele. (C) Allele-specific pyrosequencing of the T allele. Shaded areas highlight the two CpG sites of interest. The percentage of cytosine depicts the percentage of methylation on PCR-amplified bisulfite-modified DNA. The sequence of nucleotides dispensed into the pyrosequencing reactions are listed below each panel, A, adenosine; C, cytosine; T, thymine; G, guanine. Exp. Seq., expected sequences of the *H19* imprinting center surrounding the two CpG sites of interest. Inclusion of a cytosine (fourth nucleotide) and an adenosine (eighth nucleotide) in the dispensation order function as internal controls for accurate quantification of nucleotides in the template.

methylation (154/154 sites) on the G allele and 2% methylation (2/84 sites) on the T allele, which confirms our allele-specific pyrosequencing results (see Supplementary Figure S2).

Allele-Specific Methylation Assay Is Highly Sensitive, Specific, and Reproducible

To test if the allele-specific methylation assay can accurately quantitate allele-specific methylation, we simulated bisulfite-treated DNA populations with varying proportions of methylated DNA by mixing known plasmid DNA. The plasmid DNA contained a bisulfite PCR-amplified *H19* imprinting center, which had been previously sequenced. The plasmid DNA represented the DNA that depicted the four possible allele-specific methylation patterns determined by two states: (i) the SNP adjacent to the CpG site of interest (G/T) and (ii) the methylation status (methylated/unmethylated). Combinations of the plasmids (G-methylated, G-unmethylated, T-methylated, and T-unmethylated) were quantitatively mixed prior to PCR amplification. Specifically, combinations of G-methylated and G-unmethylated were created to simulate 0%, 25%, 50%, 75%, and 100% methylation for the G allele (Figure 3A); likewise for the T allele (Figure 3B). The average methylation at each CpG site of each parental allele is represented on the scale of 0%–100%.

Consistent with previous reports demonstrating that the pyrosequencing technology accurately quantitates methylation (15,23,24), Figure 3 shows that the allele-specific methylation assay is accurate and reproducible across the entire range of possible methylation levels (0%–100%). The allele-specific methylation methodology was highly reproducible on a linear scale (Figure 3), and at low levels of methylation (<50%), this methodology correctly identifies the percentage of allele-specific methylation within 3%.

In contrast to the previous reports that interrogated the average methylation of both parental alleles, Figure 3 presents allele-specific methylation

for the two parental alleles, respectively, on the upper panel (marked by the G allele of the rs2071094 SNP) and middle panel (marked by the T allele). The specificity of our assay was tested in two ways. First, we tested our primers against G and T populations with known methylation status (Figure 3, A and B). We used individual G and T sequencing primers that correctly matched the target SNP against the cloned fragments of G (Figure 3A) and T (Figure 3B) populations, respectively. The pyrosequencing profiles from the sequencing primers on the individual parental alleles (marked as G or T allele) closely matched to the methylation percentages in the corresponding cloned fragments. When the sequencing primers were switched, methylation profiles were not observed for the now mismatched primers (Figure 3, A and B, dashed lines).

Next, we interrogated plasmid mixtures of both the parental alleles. We titrated one of the parental allele against the other in three mixtures: (i) unmethylated G/fully methylated T (G0/T100); (ii) 50% methylated G/50% methylated (G50/T50); and (iii) fully methylated G/unmethylated T (G100/T0). We used individual sequencing primers designed against all four possible nucleotide bases: A, C, G, and T. Methylation profiles were only observed for the sequencing primers specific to the cloned parental fragments. No pyrosequencing profile of any other primer pair apart from the sequencing primer designed for the template was observed (Figure 3C). In summary, the individual sequencing

Figure 3. Allele-specific pyrosequencing of previously quantitated plasmid controls. (A) Cloned PCR products with known percentage of methylation (0%, 25%, 50%, 75%, 100%) of G allele probed with sequencing primers specific for the G allele. (B) T alleles. The dashed lines evaluate any mispriming of the sequencing allele by interrogating the plasmid mixture with the respective mismatched sequencing primer (primer T in the G allele plasmid mixture and vice versa). The concordance of the measured percentage of methylation and the expected percentage of methylation is represented as the solid regression line, and the corresponding standard errors are represented as capped lines. Three separate measurements were performed for each mixture. (C) Vertical axis depicts titration of the G allele against the T allele. Horizontal axis corresponds to the measured methylation status by the allele-specific pyrosequencing.

primers were specific for their respective targeted SNP sites and were able to quantitatively measure allele specific DNA methylation (Figure 3).

Figure 4. Allele-specific methylation analysis in human bladder tissue. (Left panels) Normal margins of bladder tissue that show normally imprinted *H19* expression (monoallelic expression; data not shown). (Right panels) Bladder tumor tissue that shows loss of imprinting (LOI; biallelic expression; data not shown). (Top panels) Methylation on the parental allele marked by the G allele of a G/T single nucleotide polymorphism (SNP) within the *H19* imprinting center. (Bottom panels) Methylation on the parental allele marked by the T allele. The letters below each pyrogram indicate the dispensed nucleotides injected into the real-time nucleotide synthesis to interrogate the expected sequence on the PCR-amplified fragment of the *H19* imprinting center.

Loss of Allele-Specific Methylation in the *H19* Imprinting Center Predicts Loss of *H19* Imprinting

Allele-specific methylation in the *H19* imprinting center regulates the parent-specific expression of the imprinted gene, *H19*. Using a previously described reverse transcription PCR assay (25), we found a bladder tumor that showed biallelic expression of *H19* and therefore LOI. Normal urothelium from this same patient showed monoallelic expression of *H19* and therefore normal imprinting. We analyzed DNA from this tumor using the allele-specific methylation analysis and were able to correlate the loss of allele-specific expression with a decrease in methylation of the G allele (Figure 4). The allele-specific methylation in the *H19* imprinting center is retained in the normal urothelium where the allele marked by the G SNP is heavily methylated (Figure 4: 88.6%, first CpG site; 100%, second CpG site) and the allele marked by the T SNP is unmethylated (Figure 4: 0%, first CpG

site; 6.0%, second CpG site). However, the bladder tumor allele-specific methylation decreased to 46.4% (first CpG site) in the G allele, approximately half of the normal bladder tissue. In our analysis of 41 bladder cancer samples and matched normal urothelium, only four were heterozygous for the SNPs in both our pyrosequencing assay and the previously reported RNA-based LOI assay (26). Of these four informative paired bladder carcinoma and paired normal margin samples, only one showed loss of *H19* imprinting as depicted in Figure 4. Thus, correlation of LOI in bladder cancer and allele-specific methylation will require larger studies.

DISCUSSION

We have developed a rapid method of determining allele-specific methylation at a single base resolution. Bisulfite genomic sequencing is the only method that determines methylation at a similar resolution and represents the gold standard in the field.

However, bisulfite genomic sequencing requires the cloning of PCR-amplified products and direct sequencing of multiple individual clones that each represents one parental allele from a single cell. Recent high-throughput and quantitative methylation methodologies determine average methylation for either a few CpG sites [denaturing high-performance liquid chromatography (13)] or at each individual CpG site [hairpin bisulfite PCR (27,28), pyrosequencing (8,16), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (15,29), methylation-specific PCR followed by denaturing high-performance liquid chromatography (MSP-DHPLC) (30), and single nucleotide primer extension denaturing high-performance liquid chromatography (SNUPE-DHPLC) (14)]. None, however, approaches the ease and allele-specific resolution of our allele-specific methylation measurement method.

This allele-specific methylation methodology possibly affords a more precise tool to dissect the complex methylation changes that occur in the LOI observed in tumors. However, our assay only interrogates 2 of the 41 CpG sites within the sixth CTCF region that are 4 and 10 bp downstream of a SNP. Our bisulfite sequencing data does show that the methylation of our two CpG sites is reflective of the flanking 14 sites (Supplementary Figure S2). To further develop this assay as a biomarker for LOI in epidemiological studies, transitional studies to estimate its sensitivity (true positive rate) and specificity (false positive rate) in discriminating between persons with LOI from persons with normal imprinting should be conducted. This methodology can be extended and used to assess differential gene expression patterns that are due to regulation by allele-specific methylation. This includes allele-specific Alu methylation (31), X chromosome inactivation, differential promoter and insulator activity, noncoding RNA, and monoallelic expression of cytokine genes (32). We applied the allele-specific methylation strategy toward developing a marker for LOI based on DNA methylation. In a limited analysis of bladder cancer samples, we were

unable to conclusively correlate DNA methylation changes to *H19* imprinting status. Our results underscore the need to further develop this assay to identify new biomarkers for LOI that can be used in large epidemiological studies.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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