Restriction Site–Specific Methylation Studies of Imprinted Genes with Quantitative Real-Time PCR

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BACKGROUND: Epigenetic studies, such as the measurement of DNA methylation, are important in the investigation of syndromes influenced by imprinted genes. Quick and accurate quantification of methylation at such genes can be of appreciable diagnostic aid.

METHODS: We first digested genomic DNA with methylation-sensitive restriction enzymes and used DNA without digestion as a control and nonmethylated lambda DNA as an internal control for digestion efficiency. We then performed quantitative real-time PCR analyses with 6 unique PCR assays to investigate 4 imprinting control regions on chromosomes 7 and 11 in individuals with uniparental disomy of chromosome 7 (UPD7) and in control individuals.

RESULTS: Our validation of the method demonstrated both quantitative recovery and low methodologic imprecision. The imprinted loci on chromosome 7 behaved as expected in maternal UPD7 (100% methylation) and paternal UPD7 (<10% methylation). In controls, the mean (SD) for percent methylation at 2 previously well-studied restriction sites were 46% (6%) for both H19 and KCNQ1OT1, a result consistent with the previously observed methylation rate of approximately 50%. The methylation percentages of all investigated imprinted loci were normally distributed, implying that the mean and SD can be used as a reference for screening methylation loss or gain.

CONCLUSION: The investigated loci are of particular importance for investigating the congenital Silver–Russell and Beckwith–Wiedemann syndromes; however, the method can also be applied to other imprinted regions. This method is easy to set up, has no PCR bias, requires small amounts of DNA, and can easily be applied to large patient populations for screening the loss or gain of methylation.

Given the notion that DNA methylation and histone modifications can cause heritable characteristics that occur without changing the DNA sequence, epigenetic studies have become increasingly important. In mammals, a methyl group can be attached to a cytosine base when it is located 5′ to a guanosine, a process referred to as CpG methylation. This type of methylation is of interest in the contexts of various cancers, imprinting, and regulatory effects involving the promoters of many genes (1).

Imprinting refers to gene expression specific to the parent of origin and is maintained through mitosis and reset in the germ line. Imprinting control regions (ICRs), which are typically located in CpG islands in proximity to imprinted genes, govern the nonexpression of one allele, which is often accompanied by parent-specific methylation patterns (2). The importance of these genes for human development and growth has been demonstrated in various syndromes, such as the Angelman, Prader–Willi, Beckwith–Wiedemann, and Silver–Russell syndromes (2). Genetic irregularities that influence the dosage of imprinted genes, such as uniparental disomy (UPD), duplications/deletions, and loss of the parent-specific methylation markers (loss/gain of methylation) are frequently found in these syndromes (3, 4). Methylation studies can be used to identify such genetic aberrations.

DNA methylation can be studied in site-specific, large-scale, and global manners. When the loss or gain of methylation at imprinted genes is investigated, the
study of DNA methylation at specific sites is often sufficient and is the focus of the present study. One can distinguish 2 main types of approaches, those based on digestion with methylation-sensitive restriction enzymes and those based on bisulfite treatment of DNA (5). Digestion with methylation-sensitive restriction enzymes is typically followed by Southern blotting, which is cumbersome and has limitations. It requires several micrograms of DNA and allows the study of only 1 restriction site in each experiment. The use of hot-stop PCR after digestion with methylation-sensitive restriction enzymes has also been described (6). Bisulfite treatment of DNA is typically followed by sequencing or methylation-specific PCR assays (both quantitative and conventional) to monitor methylation at specific sites (7–10). Bisulfite-based methods often enable a detailed study of all CpGs in a region of interest and are thus important for exploratory studies in regions where ICRs have yet to be defined.

The study of the loss or gain of methylation at specific sites should be quantitative, because cell populations may be a mosaic with respect to the methylation status at a given site. For the study of the loss or gain of methylation at imprinted genes, investigators have used Southern blotting, different PCR-based methods, and a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) approach with bisulfite-treated DNA (6, 7, 11–14).

We evaluated a site-specific methylation analytical approach that combines simple digestion with methylation-sensitive restriction enzymes and subsequent quantitative real-time PCR (qRT-PCR) analysis of previously characterized ICRs (15–18). This approach offers a number of practical advantages in the study of imprinted genes in that all steps can be performed in a microtiter plate format, several loci can be studied from the same digest, small amounts of DNA are needed, and the qRT-PCR methodology is widely available.

Materials and Methods

STUDY PARTICIPANTS
Forty Finnish individuals (21 females, 19 males) of typical height [mean (SD), 161 (6.7) cm and 175 (6.1) cm, respectively] were used as controls. Included in the study were 7 patients with maternal UPD for chromosome 7 (matUPD7) (1 patient with segmental matUPD7q31–qter and 6 patients with whole-chromosome matUPD7) and 1 patient with whole-chromosome paternal UPD for chromosome 7 (patUPD7), all of whom have previously been described (19–23). For analyses of H19 (H19, imprinted maternally expressed transcript) methylation, we included 20 patients with Silver–Russell syndrome (11 males and 9 females), all of whom had been diagnosed as previously described (22). The study was approved by the appropriate ethics review boards at the University of Helsinki, Finland, and the Karolinska Institutet, Sweden.

SssI METHYLATION OF A DNA
We methylated 1 µg genomic λ DNA with 4 U of the CpG methyltransferase SssI in 20–µL reaction volumes with 1× NEBuffer 2 and 160 µmol/L S-adenosylmethionine (all reagents from New England Biolabs) for 2 h at 37 °C and subsequently inactivated the enzyme at 65 °C for 20 min (for the formulation of NEBuffer 2, see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3). Four reactions were run in parallel to ensure optimal methylation efficiency and were subsequently pooled. Methylated λ DNA (100%) was diluted with nonmethylated (0%) λ DNA to create a dilution series of 75%, 50%, and 25% methylation. We then digested these DNA dilutions with HpaII (New England Biolabs) and measured the methylation percentage as described below.

MEASUREMENT OF DNA METHYLATION
DNA methylation was assayed in genomic DNA extracted from whole blood. We digested 130 ng of DNA with 2 U of the methylation-sensitive restriction enzyme HpaII (New England Biolabs) in a 50-µL volume and incubated another 130 ng of DNA with 1× NEBuffer 1 only (for the formulation of NEBuffer 1, see the online Data Supplement). We included an aliquot of an amplified λ DNA fragment with an HpaII restriction site in both reactions as an internal control for digestion efficiency. To reduce the variance, we first prepared the genomic DNA, NEBuffer 1, and the internal control together and then divided it before adding the HpaII enzyme in 1× NEBuffer 1. We incubated DNA (with or without enzyme) overnight at 37 °C in a PCR machine (Mastercycler; Eppendorf) to ensure complete digestion and then inactivated the enzyme at 62 °C for 20 min. To test for methylation at additional imprinted sites, we digested all samples similarly with the methylation-sensitive restriction enzyme NotI.

The digested DNA and corresponding undigested DNA were then assayed in parallel with 7 different assays using the SYBR Green dye. Primers were designed with Primer3 software (24) and tested by electronic PCR for specificity (http://genome.ucsc.edu/cgi-bin/hgPcr). Table 1 presents the primer sequences and their annealing temperatures used for the assays. We carried out qRT-PCR analyses in 20-µL volumes with 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 5 ng DNA, and 200 nmol/L of each primer. Each sample was quantified in duplicate or triplicate. Typical cycling conditions were as follows: 95 °C for 10 min...
and 40 cycles [95 °C for 30 s, the annealing temperature (see Table 1) for 30 s, and 72 °C for 45 s], followed by 72 °C for 10 min and dissociation analysis. SYBR Green fluorescence was measured during the extension step. If we observed a primer peak in the dissociation curves, which occurred for some amplicons, we confirmed the specificity by electrophoresing the product on a 1.5% agarose gel. The primers in the MEST [mesoderm specific transcript homolog (mouse)] ICR required a touch-down PCR profile consisting of 3 cycles each at annealing temperatures 65 °C, 63 °C, and 61 °C, followed by 31 cycles with annealing at 58 °C. All PCR runs were manually inspected with 7500 Fast System SDS software, version 1.3.1 (Applied Biosystems).

SEQUENCI NG REACTIONS
PCR reactions were carried out in 25-μL reactions containing 0.5 ng/μL of genomic DNA, 2.5 mmol/L MgCl₂, 400 μmol/L of each deoxynucleoside triphosphate, 800 nmol/L of each primer, and 0.03 U/μL of HotStarTaq DNA polymerase (Qiagen). The PCR was performed with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C, with a final extension of 10 min at 72 °C. PCR products were dephosphorylated with 400 U/L shrimp alkaline phosphatase (Amersham Biosciences/GE Healthcare) and visualized with Sequencer version 3.0 software (Amersham Biosciences/GE Healthcare). Sequences were assembled and analyzed with Pregap and Gap4 software (www.cbi.pku.edu.cn/tools/staden).

STATISTICAL ANALYSIS
The relative degree of methylation at the investigated loci was calculated by subtracting the mean of the undigested DNA threshold cycle (Ct) values (controls for the starting DNA amount) from the mean of the digested DNA threshold cycle (Ct) values. We then calculated the amount of undigested DNA relative to the amount of digested DNA, PM, by exponentiating the obtained difference with a base of 2: PM = 2x, where x = (mean Ctdigest) – (mean Ctnondigest). The methylation percentage was calculated as 1/PM. In cases in which a nonmethylated restriction site was assayed, digestion efficiency was calculated as: 1 − 1/PM. The SE of the methylation percentage was calculated as previously described (25).

We used the Kolmogorov–Smirnov test to evaluate whether relative amounts of methylation at the investigated loci were normally distributed in our sample by comparing the observed data to a randomized normal distribution with a mean and SD derived from the observed data. We used 2-sided Student t-tests to analyze differences between means. All statistical analyses were performed in the R statistical environment (26).

Results
QUALITY ASSESSMENTS AND VALIDATION
We used a combination of digestion with methylation-sensitive restriction enzymes and subsequent qRT-PCR analysis to measure methylation (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3). To assess the quantitative recovery and
imprecision of methylation measurements, we digested a dilution series of SssI-methylated DNA samples with HpaII and measured the degree of methylation in quadruplicate. A linear regression analysis of the data revealed quantitative recovery across the entire methylation range (0%–100%), with a slope of 0.983 and an adjusted $R^2$ of 0.989 in the dilution series (Fig. 1). We repeated the methylation measurement in an identical manner and obtained similar results (slope, 0.987; adjusted $R^2$, 0.970). The individual SDs for the Ct values of replicates were low (mean, 0.09; median, 0.08). Because the observed SDs were well below the difference expected for an imprinted locus (Ct/H110051), we proceeded to measure methylation effects.

We studied a total of 6 loci in the 4 ICRs of GRB10 (growth factor receptor-bound protein 10) (7p14), MEST (7q32), H19, and KCNKQOT1 (11p15.5) via digestion with the methylation-sensitive 4-base cutter HpaII and the methylation-sensitive rare-cutter NotI (Table 1). We measured mean and median digestion efficiencies by cutting nonmethylated DNA, and they were both 96%. The success rate of the PCR assays was 99.7%. To assess the imprecision of the reactions, we measured the SDs of replicate Ct values. The mean (SD) values were 0.14 (0.13) for GRB10-NotI, 0.08 (0.07) for MEST-HpaII, 0.11 (0.09) for H19-HpaII, 0.15 (0.13) for KCNKQOT1-HpaII-1, 0.14 (0.13) for KCNKQOT1-NotI, and 0.10 (0.08) for KCNKQOT1-HpaII-2. The distribution of the SDs for the replicates is shown for each assay in Fig. 2.

**IMPRINTED LOCI ON CHROMOSOME 7 BEHAVE AS EXPECTED IN matUPD7 AND patUPD7**

We analyzed restriction sites in the ICRs of GRB10 and MEST in 7 patients with matUPD7, in 1 patient with patUPD7, and in the 40 control individuals. We found the ICR restriction sites of GRB10 and MEST to be hypermethylated (approximately 100% methylation) in matUPD7 and hypomethylated in the patUPD7 patient (<10% methylation). The controls showed intermediate but distinct methylation patterns (Table 2). A patient with segmental matUPD7 covering 7q31-qter showed normal methylation for GRB10-NotI and hypermethylation at MEST-HpaII (7q32), results that are consistent with the localization of the genes with respect to the UPD segment (Table 2). In the controls, we observed a lower mean methylation percentage and SD for GRB10-NotI and a higher methylation percentage for MEST-HpaII. We also assayed the patUPD7 and matUPD7s for the imprinted loci on chromosome 11 (see below), and the results revealed the typical methylation patterns (Table 2), confirming the normality of these data apart from chromosome 7.

**COMPARISON OF METHYLATION PERCENTAGE AND SOUTHERN BLOTTING RESULTS**

We then studied 2 ICR restriction sites that had previously been studied with Southern blotting: HpaII site 25 in the H19 ICR (H19-HpaII) and the NotI site in the KCNKQOT1 ICR (KCNQ1OT1-NotI). In the 40 controls, the mean (SD) methylation percentage was 46% (6%) for both H19-HpaII and KCNKQ1OT1-NotI (Table 2). These methylation percentages are similar to those of previous observations and are consistent with the 50% methylation level expected at an ICR (11, 13, 27, 28).
showed the most similar methylation percentage. In our analysis, one control individual appeared to be hypermethylated at the KCNQ1OT1-HpaII-2 site but not at the adjacent sites. Sequencing of this individual revealed a heterozygous C-to-T transition at the restriction site (ss74800507, to appear in Build 128 of the dbSNP database; see http://www.ncbi.nlm.nih.gov/projects/SNP/). Although the parental origin of the transition could not be determined, our failure to digest DNA at this site suggests that it originated from the nonmethylated paternal allele.

**DISTRIBUTION OF METHYLATION PERCENTAGE AT IMPRINTED LOCI**

We tested whether the observed data followed a normal distribution to ascertain how methylation percentage varied among individuals. A normal approximation seemed to describe the data well for all loci, and the null distribution of the SDs of replicate measurements for all 6 PCR assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Chromosome</th>
<th>Restriction site previously studied?</th>
<th>Controls (n = 40), %</th>
<th>matUPD7 (n = 7), %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>GRB10-NotI</td>
<td>7p14</td>
<td>No</td>
<td>24.1</td>
<td>6.4</td>
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<tr>
<td>MEST-Hpall</td>
<td>7q32</td>
<td>No</td>
<td>60.5</td>
<td>6.7</td>
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<tr>
<td>H19-Hpall</td>
<td>11p15.5</td>
<td>Yes</td>
<td>45.7</td>
<td>6.1</td>
</tr>
<tr>
<td>KCNQ1OT1-Hpall-1</td>
<td>11p15.5</td>
<td>No</td>
<td>37.6</td>
<td>6.5</td>
</tr>
<tr>
<td>KCNQ1OT1-Noli</td>
<td>11p15.5</td>
<td>Yes</td>
<td>45.6</td>
<td>6.4</td>
</tr>
<tr>
<td>KCNQ1OT1-Hpall-2</td>
<td>11p15.5</td>
<td>No</td>
<td>44.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* For GRB10-NotI, n = 6; data for segmental matUPD7q31.33-qter are not included.

* Testing the null hypothesis that the data are normally distributed in a Kolmogorov–Smirnov test.
hypothesis of a normal distribution could not be rejected in a Kolmogorov–Smirnov test (Fig. 3, Table 2), implying that the mean and SD are meaningful as cutoffs for defining abnormal methylation. To evaluate this supposition, we tested 20 patients with Silver–Russell syndrome for methylation at the \( H19 \) ICR (\( H19-Hpall \)), a locus that has previously been shown to be hypomethylated in a substantial proportion of such patients \( (13, 28) \). The results in Fig. 4 show that hypomethylation at this locus is readily detected in a subset of Silver–Russell syndrome patients at a methylation percentage cutoff of \( -2 \) SDs (\(<33.5\%\)). The wide range of methylation percentage values in the hypomethylated patients (3%–20%) also demonstrates the different extents of mosaicism for the methylation aberration.

**Discussion**

We have shown the feasibility of digestion with methylation-sensitive restriction enzymes followed by qRT-PCR analysis for quantifying methylation at imprinted loci. This approach offers an advantage over currently used methods in that it requires only 2 laboratory steps, the digestion and the quantification, both of which can
be performed in a microtiter plate format (see Fig. 1 in the online Data Supplement).

qRT-PCR is a frequently used method with a wide linear range, and we demonstrated quantitative proportionality across the entire methylation range with linear regression analysis. We ensured digestion efficiency by using excess amounts of enzyme and digesting overnight and monitored efficiency further by adding nonmethylated λ DNA in the digestion and control reactions to measure dosage differences. To demonstrate how the extremes of the methylation range behaved for imprinted loci, we studied matUPD7 and patUPD7 individuals at imprinted loci, which were distinct from the controls and consistent with the expected methylation patterns. These results suggest that the method can also be used to quickly screen for matUPD7 in patients with Silver–Russell syndrome and be easily confirmed with microsatellite markers.

Studies of site-specific loss or gain of methylation have generally focused on imprinting disorders. Such investigations have been based on detailed studies of ICRs, which have shown all sites in defined regions in genomic DNA extracted from blood samples to be stably imprinted and to be representative of the methylation status of the entire region (16). Imprinted regions are unique in this respect, whereas other phenomena, such as cancers, often require a more general investigative approach, such as large-scale or global methylation studies (5). Such studies are further complicated by the presence of cell population–specific methylation profiles (8); however, in instances of the influence of imprinted genes on some cancers, such as IGF2 [insulin-like growth factor 2 (somatomedin A)] and colon cancer, the study of a single restriction site could suffice as a diagnostic marker (29).

To assess the utility of our method and compare it with conventional Southern blotting (the most commonly used method for studying loss or gain of methylation at imprinted regions), we investigated 2 extensively studied restriction sites in ICRs of the chromosome 11p15.5 imprinted clusters (the H19 ICR and the KCNQ1OT1 ICR). The methylation percentages for all controls, matUPD7, and patUPD7 were similar to those reported in previous studies (11, 13, 28). The Southern-blotting approach is limited to a single locus at a time and requires a relatively large amount of DNA, whereas the qRT-PCR approach, which permits the study of several loci with the same digest, is more efficient, both in terms of laboratory work and the amounts of DNA required. Some of the shortcomings of our method are the frequency of restriction sites and the GC-rich nature of the DNA of ICRs, which limit the number of possible PCRs that can be designed. The latter limitation is also problematic for methylation-specific PCR, which is discussed below. Furthermore, polymorphisms and mutations can unexpectedly influence the results for the studied site. This consideration means that if the proposed method is to be used to screen for the loss or gain of methylation, the preferred approach is to use a restriction site that has previously been well studied with Southern blotting, bisulfite sequencing, or another method, to ensure that the mean methylation percentage is relatively invariable among individuals and that the restriction site is in the critical region of the ICR.

Several good methods that are based on bisulfite treatment of DNA are available for studying DNA methylation. A general drawback with bisulfite treatment is the risk of degrading the DNA during the conversion step, which includes NaOH treatment and incubation at high temperatures (5). Degradation is less of a problem with restriction enzyme digestion. A general advantage of bisulfite-based methods is that they are not limited to the evaluation of methylation at restriction sites only; all types of CpG methylation can be studied. Numerous methylation-specific PCR methods are in use (5). These methods require careful design of primers and probes and extensive optimization of efficiency, because such methods are based on different assays that are designed to distinguish between bisulfite-converted and nonconverted cytosines (5).

With our approach, methylation percentage is measured with the same PCR assay, and thus the same efficiency. Combined bisulfite restriction analysis (COBRA) and MALDI-TOF mass spectrometry are 2 other bisulfite-based methods; these are more labor intensive than our approach (see Fig. 1 in the online Data Supplement); furthermore, the MALDI-TOF method requires specialized equipment (12, 14).

We found methylation percentage to be normally distributed at imprinted loci in the control individuals. The quantitative nature of the mean methylation percentage has been reported previously (30). The normal distribution of the data and the magnitudes of the SDs show that methylation can vary between individuals, but only to a certain extent, as is suggested by the range of the measurements. Individuals with a degree of methylation that deviates greatly from the mean are likely to exhibit some phenotypic consequence, such as in the Beckwith–Wiedemann and Silver–Russell syndromes for 11p15.5 (4). Such a finding was exemplified in our screening for the H19 ICR in a small set of patients with Silver–Russell syndromes, in which we demonstrated that a substantial proportion of the patients showed methylation well below −2 SDs of the mean. Even if our method is highly quantitative, the mosaicism that we observed between individuals for the methylation aberration suggests that some individuals may be hard to define as normally methylated or as hypomethylated. In such cases, additional tests at prox-
inal loci or the evaluation of DNA extracted from other tissues might be of aid. Because the mean methylation percentage obtained differs somewhat across techniques, it is advisable to use empirically defined cutoffs for abnormality.

Interestingly, we observed differences in mean methylation percentage both within and between ICRs. In the KCNQ1OT1 ICR, all 3 studied sites showed slightly different mean methylation percentages. The previously studied Norl site had a mean methylation of 46% and was the most centrally located site in the ICR. The other sites, located about 1 kb upstream and about 0.4 kb downstream of the Norl site, showed lower degrees of methylation. Our observation is also supported by the recent report of 30% mean methylation at a distinct locus in the H19 ICR (30), whereas the mean methylation percentage for the well-studied HpaII site 25 (also studied in the present work) has been repeatedly reported to be about 50% (13, 28). We further observed that the restriction sites in the ICRs of GRB10 and MEST had mean methylation percentages that were different from the expected 50%, even if they clearly behaved as imprinted in UPDs of chromosome 7. These results emphasize the difficulty in defining imprinting, because the sites would be acceptable for UPD screening whereas the unexpected mean methylation in controls remains to be explained.

In summary, we have shown that our method of digestion with methylation-sensitive restriction enzymes followed by qRT-PCR analysis is a simple, efficient, and quantitative method for studying methylation, with the potential for parallel investigations of several imprinted loci. The small amounts of DNA required allow extensive analysis of precious sample collections; consequently, this approach may become an attractive alternative to Southern blotting in the future. Furthermore, this approach can easily be established in most genetic laboratories with commonly available instruments. Finally, we have reported the range of methylation percentages for control individuals for 6 restriction sites in 4 ICRs, results that could easily be applied to large patient collections for studies of the loss or gain of methylation.

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