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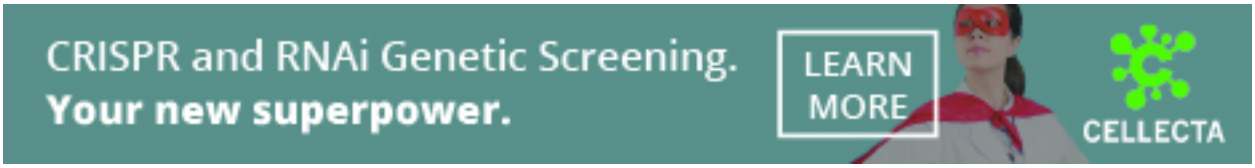

A Novel Method to Quantitate Methylation of Specific Genomic Regions

Mervi Heiskanen,[1] Ann-Christine Syvänen,[2] Harri Siitari,[3] Sinikka Laine,[1] and Aarno Palotie[1]

[1]Department of Clinical Chemistry, University of Helsinki and the Laboratory Department of Helsinki University Central Hospital, 00290 Helsinki, Finland; [2]National Public Health Institute, Department of Human Molecular Genetics, 00300 Helsinki, Finland; [3]Wallac Oy, Analytical Systems Division, 20101 Turku, Finland

A new solid-phase primer extension method has been developed for the quantitation of methylation differences and is described here. The method is less cumbersome than Southern blot analysis, expresses the results in a numerical format, can be adapted to a microtitration well format, and thus allows the analysis of a large series of samples. The model gene analyzed here is the calcitonin gene, but the method can be adapted to the analysis of methylation alterations in any area of the genome. The primer extension method clearly differentiated hypermethylated samples from normally methylated samples and a range for normal values could be determined. In quantitation experiments the method showed linearity in a range from 2% to 100% malignant blasts diluted with normal leukocytes.

Although DNA methylation is known to be essential for mammalian development it also contributes to malignant transformation. In addition to the increased probability of point mutations in methyl cytosine residues, cytosine methylation also affects gene expression, DNA replication, and repair. Generally, hypomethylation is associated with gene activity and hypermethylation with partial or total inactivity.[1,2] Detection of methylation alterations of specific genes is usually based on digestion with isoschizomeric restriction enzymes after which the cleavage of DNA is detected by Southern blot hybridization.

Also, PCR-based methods for detecting methylation alterations have been reported. One of the PCR-based methods takes advantage of the fact that PCR amplification occurs only if the DNA between the two primer sites remains uncleaved by the methylation-sensitive restriction enzyme *Hpa*II.[3,4] In two other approaches, genomic sequencing methods combined with PCR are used to identify methylation cytosine residues.[5–7] Unmethylated cytosine residues of genomic DNA can be discriminated from methylated ones by bisulfite modification, which specifically converts unmethylated cytosines to uracil. The uracil residues are identified after strand-specific PCR amplification by nucleotide sequencing. Base-specific cleavage of genomic DNA followed by ligation-mediated PCR amplification has been used to generate methylation-specific footprints on sequencing gels.[7]

A typical feature of DNA methylation is the variation of a specific methylation state among individual cells in a sample. Most samples available for analysis contain both cell types where a specific DNA site is methylated and cells where the same sequence is unmethylated. Thus, simple methods that can reliably demonstrate quantitative differences in methylation state among samples and can be adapted for diagnostic analyses would be beneficial.

In human neoplasia, both hypo- and hypermethylation have been reported and the short arm of chromosome 11 seems to be a methylation "hot spot" in various malignancies.[8] Because of the numerous *Hpa*II restriction sites in the 5′ region of the calcitonin gene it has served as a marker gene for the detection of methylation alterations in this chromosomal area.[8–12] Of special interest has been the association of calcitonin gene methylation alterations with hematological malignancies, in which connection it has been studied because of its diagnostic and prognostic implications.[10,13–16]

A technique to quantitate the relative amount of *Hpa*II-digested template for monitoring methylation alterations in a specific gene has been developed and is described here. This technique is an improvement of the procedure described by Singer-Sam et al.[3,4]

MATERIALS AND METHODS

Preparation of Samples

Bone marrow aspirates from four patients with acute myeloid leukemia (AML), three with chronic myeloid leukemia (CML), one with acute lymphatic leukemia (ALL), and four with chronic lymphocytic leukemia (CLL) were examined. All patients were treated at the University Central Hospital of Helsinki.

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Bone marrow cells from 10 healthy bone marrow donors served as controls. All analyzed samples were left over from diagnostic bone marrow aspirates and were analyzed after informed consent was given.

The DNA was extracted from the mononuclear cell fraction of the bone marrow aspirates as described earlier.[13,17] DNA (200 ng) was digested with 25 units of restriction endonuclease *Hpa*II for at least 16 hr according to the instructions of the supplier, and an additional 25 units was added and incubated for 2 hr more (Boehringer Mannheim).

PCR was performed in an automated thermocycler (Pharmacia, Uppsala, Sweden), and the reaction conditions (100 µl) were as described earlier.[18] The sequences of the primers are shown in Table 1. After initial denaturation at 100°C for 5 min, the samples were subjected to 30 PCR cycles (1 min at 96°C, 1 min at 61°C, 1 min at 72°C). The PCR products were analyzed by electrophoresis in a 2% agarose gel, and the amplified DNA fragments were visualized under UV irradiation.

Solid-phase primer extension

The principle of the method is summarized in Figure 1. For this detection method PCR primers 4 and 5 had been biotinylated during their synthesis[19] and 50 ng of *Hpa*II-digested DNA was amplified in a 50-µl PCR reaction for 25 and 27 cycles under the conditions described above. The primer concentrations used were adapted for the biotin-binding capacity of the microtitration plate well. In all assays, 5 pmoles of biotinylated 5′ primer and 20 pmoles of 3′ primer were used. An aliquot of 10 µl of the PCR product and 90 µl of PBS–Tween was applied to a streptavidin-coated microtitration plate well (Wallac Oy, Turku, Finland) and the plates were incubated for 1.5 hr at 37°C. The wells were washed five times with 100 µl of buffer [40 mM Tris-HCl (pH 8.5), 1 mM EDTA, 50 mM NaCl, 0.1% Tween 20] using an automated washer (Delfia PlateWash, Wallac Oy). The captured DNA was denatured by incubation in 50 mM NaOH for 5 min at 20°C and followed by washing as described above. Thereafter, a primer extension reaction was performed with 0.5 units of *Taq* DNA polymerase in a 100-µl reaction mixture containing 20 pmoles of the 3′ unbiotinylated PCR primer (~100-fold excess over template), 10 pmoles of [^{3}H]dCTP (TRK 338, Amersham; 64 Ci/mmole), 40 pmoles of dCTP, 50 pmoles of dATP, dGTP, dTTP by incubation for 15 min at 20°C and then for 20 min at 55°C. After the final washing, 180 µl of scintillation fluid (LKB Wallac Oy Optiphase "Hisafe"3) was added to each well and the plate was shaken for 5 min, after which the incorporated radioactivity was measured directly from the plate using a Micro Beta scintillation counter (Wallac Oy Turku, Finland). For controlling the interassay variation, two samples, one hypermethylated and one normaly methylated, were selected and included in each assay.

Southern blot hybridization was performed as described earlier.[13]

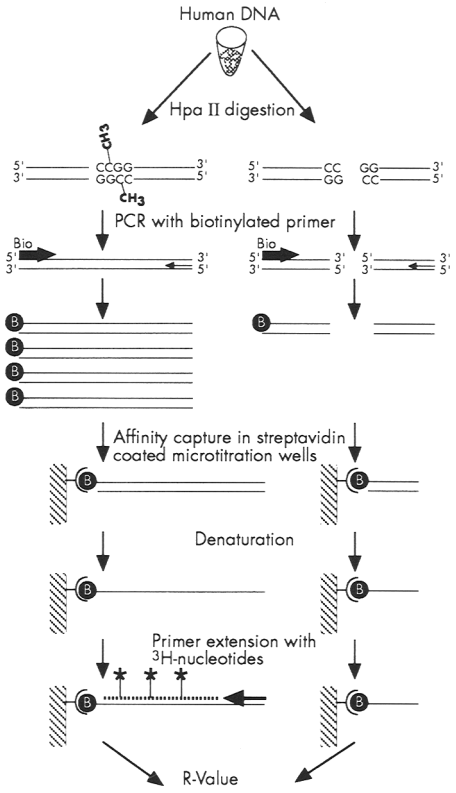

FIGURE 1 Principle and steps of the solid-phase primer extension assay. After *Hpa*II digestion the DNA is amplified with primers flanking the cleavage sites. One of the primers is biotinylated to allow capture of the PCR product on a streptavidin-coated solid phase. The PCR product is quantified by the aid of a primer extension reaction with tritiated nucleoside triphosphates. The amount of incorporated radioactivity is inversely proportional to the quantity of *Hpa*II-digested template DNA. Two assays are performed for each sample, one without digestion and one after *Hpa*II digestion. The result of the assay is given as the ratio (R) of ^{3}H-label incorporated in these two reactions [(R) *Hpa*II digested/nondigested].

Statistical evaluations

The statistical calculations were performed using the Macintosh StatWorks program.

RESULTS

To determine in more detail the methylation status of the calcitonin gene in normal and malignant bone marrow samples, seven potential methylation areas were analyzed (Fig. 2). The DNA sample was digested with the methylation-sensitive restriction enzyme *Hpa*II, and the digested DNA was subsequently amplified using primers flanking the restriction site of interest, run in an agarose gel

TABLE 1 Primer Sequences (5′ → 3′)

Region			Fragment size (bp)
I	1 CTT AGA AGT TAG GCG TTC CCG	1′ GAC CTG CTT GCA TCA MC CTG	547
II	2 TCA GAG GGG GCA CAT GTT GG	2′ TTC CTA CCC TGC CAT CCA TC	262
III	3 GCT TCC GCA TCT GTA CCT TG	3′ CCT CAG ATA GGC AGG GAT CTA	194
IV	4 TCC GTC TCT TGT TTC CCA CG	4′ GCC AGT GCA CAG CM CCA AT	444
V	5 TTC TCA CTC CCT TTC CTC CTC	5′ CGA GAG AGT AAG ACT GGA GTC	175
VI	6 AGA AGG ACA CTG GTA TCA GAC	6′ GGG TGA GM TAG ATC AAC AGG	671
VII	7 TCA CAG CCT GCA CTG AGT TTG	7′ GTT ATG TCT MC CAC GGT ACC	380

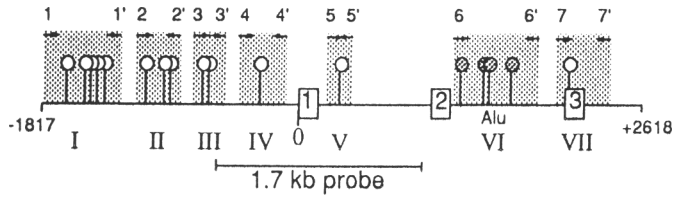

FIGURE 2 A schematic representation of the 5′ area of the calcitonin gene and the flanking sequences. *MspI*/*HpaII* restriction sites in normal lymphocytes are shown as unmethylated (open circles) and methylated (shaded circles). PCR-amplified areas I–VII, the flanking 5′ primers (1–7), and the 3′ primers (1′–7′), are shown. The positions of exons 1–3 and the 1.7-kb probe used in the Southern hybridization are also shown. [Modified from a figure by Broad et al.[12]]

electrophoresis, and visualized with ethidium bromide. If a PCR product was observed in agarose gel electrophoresis, it indicated the existence of uncleaved DNA, and it was interpreted as a sign of methylation in all of the *Hpa*II restriction sites of the corresponding DNA fragment. In agreement with previous results obtained by Southern blot analysis, the clearest differences were observed in the regions IV and V (data not shown). It became evident that the ethidium bromide visualization was not sufficient to quantitate differences among individual samples. Thus, a more accurate solid-phase primer extension procedure was developed (Fig. 1). This method is also based on the methylation sensitivity of the *Hpa*II enzyme and PCR, but it allows a more accurate comparison and thus quantitation of the amount of methylation. The ethidium bromide-stained agarose gels were also run to ensure the exclusive amplification of the specific product.

The results of the assay are calculated as ratios (R-values) between the radioactivity incorporated in a PCR product from an *Hpa*II-digested DNA sample, divided by the radioactivity incorporated in the PCR product from the undigested sample. Thus, a low R-value indicates that a large proportion of the template was digestible by *Hpa*II, which is a sign of a large proportion of unmethylated cells in the sample. A low R-value is to be expected in bone marrow samples of healthy individuals. The mean value for controls was calculated from the results of 10 different samples.

Because the exponential range of PCR amplification may vary among samples, the analysis was done at two different PCR cycles (25 and 27 cycles). The R-values and standard errors obtained from the gene regions IV and V are shown in Table 2. The normal upper limit for the R-value was set to be two times the standard deviation above the mean value in the 10 control samples. The quantitative character of the method was demonstrated by the range of R-values among control samples. In normal bone marrow the Southern blot hybridization always reveals distinct restriction fragments indicating a normal state of methylation.[8,10,11,13,14] Surprisingly, a wide range of values among controls was observed. In region IV using 25 PCR cycles, values from as low as 0.063 up to 0.200 were observed. The quantitative nature of the assay was assessed further by diluting AML blasts with peripheral blood leukocytes from a healthy individual and performing the assay on region IV after 25 PCR cycles. The method is relatively linear in a range from 2% to 100% of malignant cells (Fig. 3).

TABLE 2 Ratio of Methylated DNA Obtained by the Solid-phase Primer Extension Method

Sample no.	R-values[a] IV[b] 25	S.E.	IV[b] 27	S.E.	V[b] 25	S.E.	V[b] 27	S.E.	Southern analysis
AML 101	0.54	0.02	0.52	0.03	0.34	0.02	0.21	0.01	normal
AML 102	0.85	0.05	0.67	0.01	0.56	0.07	0.58	0.04	abnormal
AML 104	0.55	0.01	0.61	0.01	0.96	0.03	0.95	0.07	abnormal
AML 105	0.42	0.03	0.47	0.01	0.33	0.01	0.38	0.04	abnormal
CML 201	0.70	0.08	0.59	0.03	0.18	0.01	0.47	0.10	abnormal
CML 202	0.35	0.03	0.35	0.02	0.70	0.02	0.95	0.02	abnormal
CML 203	0.66	0.06	0.48	0.05	0.21	0.03	0.27	0.03	abnormal
ALL 301	0.23	0.01	0.37	0.01	0.23	0.01	0.43	0.01	abnormal
CLL 401	0.57	0.03	0.56	0.03	0.51	0.02	0.52	0.01	abnormal
CLL 402	0.54	0.02	0.51	0.03	0.79	0.01	0.71	0.06	abnormal
CLL 403	0.16	0.01	0.28	0.05	0.48	0.01	0.57	0.06	abnormal
CLL 404	0.50	0.03	0.66	0.01	0.34	0.02	0.40	0.01	abnormal
Mean of all controls[c]	0.15	0.01	0.15	0.01	0.16	0.01	0.18	0.01	
Upper limit of normal[d] values	0.20		0.20		0.25		0.28		

[a]The R-value is the ratio between radioactivity incorporated into *Hpa*II-cleaved and uncleaved samples (see Materials and Methods and Fig. 1 for details).

[b](IV and V) The *Hpa*II cleavage regions given in Fig. 2; (25 and 27) the number of PCR cycles employed. (S.E.) Standard error. The given values are means of four determinations.

[c]The mean R-value of 10 control bone marrow samples, from a total of 12 to 40 determinations. The mean of all patients was compared with the mean of controls in each column (patients in IV 25 vs. controls of IV 25, patients of IV 27 vs. control of IV 27, etc.) by the *t*-test. In all comparisons $P < 0.0001$.

[d]The upper limit of the normal R-value was set at +2 S.D. of the mean of all controls.

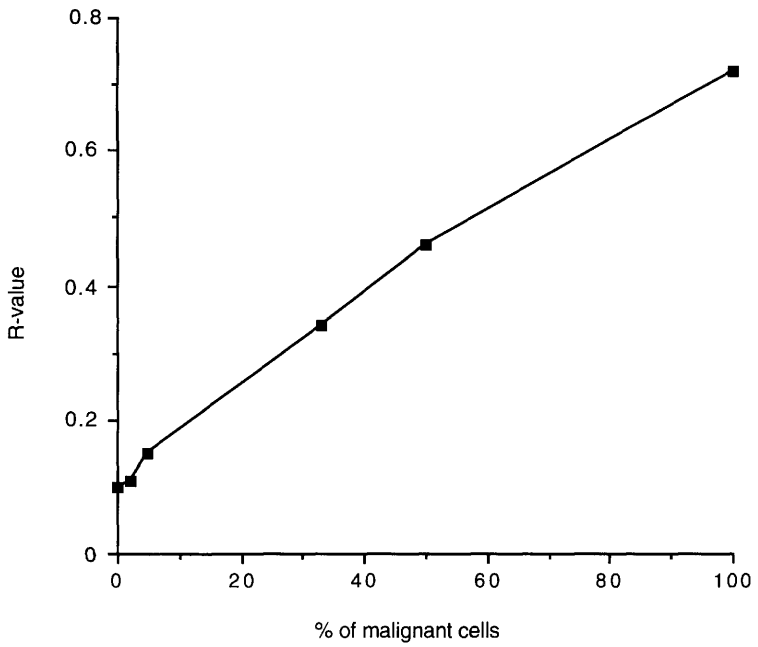

FIGURE 3 Dose–response curve obtained by the primer extension reaction. DNA extracted from AML blasts was diluted with leukocyte DNA from a healthy control. The *x*-axis indicates the proportion of DNA from AML blasts; the *y*-axis indicates the R-value obtained in the primer extension reaction.

The primer extension assay was applied further to study the methylation state of regions IV and V of the calcitonin gene in various hematological malignancies. The numerical results of the test were compared with the results obtained previously by Southern blot analysis (M. Heiskanen et al. unpubl.). Twelve patients with either myeloid (AML, CML) or lymphoid (ALL, CLL) malignancies were studied. Of these patients, a hypermethylated calcitonin gene was detected by Southern blot in all but one patient (AML 101). Of the 11 positive samples, as determned by Southern blot analysis, the mean R-values obtained in the primer extension assay are 0.50 (area IV, 25 PCR cycles) and 0.48 (area V, 25 PCR cycles). The corresponding R-values after 27 PCR cycles are 0.50 and 0.57, respectively. When the R-values from patients and controls are compared, there is a statistically significant difference between the two groups (*t*-test, $P<0.0001$). A discrepancy between the results obtained with Southern blot analysis and the solid-phase primer extension assay was seen in AML patient 101, who had a normal pattern in Southern blot hybridization but distinctly low R-values in the analysis of region IV. This discrepant result is probably caused by the higher sensitivity of the primer extension assay compared with Southern blot analysis.

DISCUSSION

In this study, a solid-phase primer extension reaction to quantitate methylation alterations in a specific gene area was developed. The method provides an additional, more quantitative alternative to the PCR-based procedure described by Singer-Sam et al.[3,4] To obtain an overall picture of the methylation status of a gene, ethidium bromide visualization following agarose gel electrophoresis of the PCR products produced from the regions containing *Hpa*II restriction sites is sufficient. However, when an observation of low background methylation is to be related to an altered methylation state, visual judgement after ethidium bromide staining is not accurate enough and a more quantitative method is needed. Thus, a method that expresses the differences between normal and abnormal methylation states in a numerical format was developed. Our results from the primer extension assay show that the normal and hypermethylated samples could be clearly distinguished from each other.

Based on the dilution experiments, it is possible to quantitate the proportion of methylation among samples. The results are expressed as ratios of incorporated tritiated nucleotides, because a precise expression, such as percent of methylated alleles, is not possible. This is because of the methylation phenomenon that makes the generation of unequivocal controls, where a site is either methylated or unmethylated in all template molecules, impossible. Because of the lack of such standards the absolute sensitivity of the solid-phase primer extension protocol is difficult to estimate. However, the method is well suited for comparing samples, for example, from different patients or follow up of methylation alterations during transformation of cultured cells. More generally, the solid-phase primer extension reaction developed here is a valuable approach to quantitate specific PCR products. A similar approach, which allows accurate and highly sensitive quantitative PCR analysis, is the solid-phase minisequencing assay designed to detect point mutations.[18,20]

In addition to the numeric format of the results obtained, another advantage of our method is the relative simplicity of the analysis. The solid-phase primer extension method combined with modern laboratory equipment is easier to perform than Southern blot hybridization, and is well suited to an analysis of a large number of samples in a clinical laboratory. Here, the method was set up using a microtitration plate that can be directly measured in a liquid scintillation counter. Obviously, the detection system does not have to rely on radioactivity, but both colorimetric and chemiluminescence methods can be adapted to the solid-phase primer extension protocol.

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