Sensitive and quantitative universal Pyrosequencing™ methylation analysis of CpG sites

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DNA methylation is implicated in multiple normal cellular processes, including the regulation of tissue- and development-specific gene expression, imprinting, X-chromosome inactivation, DNA repair, and the suppression of parasitic DNA sequences. Aberrant gene promoter methylation and resulting altered gene expression have been associated with cancers (1,2). Altered methylation patterns have been described in human sporadic cancers: genome-wide hypomethylation and localized hypermethylation have been observed, and both alterations can be present in the same tumor (3). Together with loss-of-heterozygosity and gene mutations, transcriptional silencing by methylation has been shown to be a major inactivating event of tumor suppressor genes in accordance with Knudson's two-hit hypothesis (4). Gene inactivation by promoter hypermethylation and resulting loss of function have been shown for several cancer genes involved in DNA repair, cell cycle control, apoptosis, angiogenesis, differentiation, metastasis/invasion, transcription, and signal transduction (1).

A wide range of methods exists to detect genomic DNA methylation, including approaches to detect genome-wide and gene-specific methylation levels (5). Most methods used to analyze the methylation status of a specific sequence are based on bisulfite modification of the DNA. Following the treatment, the methylation status can be assessed as a sequence difference by sequencing, methylation-specific PCR, methylation-sensitive single-stranded conformational polymorphism, methylation-sensitive single nucleotide primer extension (Ms-SNuPE), or restriction enzyme digestion. Recently, high-throughput methods to detect site-specific methylation have been developed using the TaqMan® technology (6,7)

We developed a highly quantitative method to assess DNA methylation levels at specific sites using the Pyrosequencing™ technology (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing methylation analysis (PyroMethA) is a modification of the combined bisulfite restriction analysis (COBRA), where the restriction analysis is substituted with the highly quantitative Pyrosequencing reaction. Recently, Uhlmann et al. (8) reported the independent development of a Pyrosequencing-based method to detect methylation. Our assay designed for a different gene further validates the use of the Pyrosequencing technology to detect methylation at specific CpG sites. In addition, we developed and validated universal PyroMethA as an approach for high-throughput methylation detection. Standard PyroMethA and universal PyroMethA were equally robust and quantitative.

Figure 1A compares the experimental approaches for PyroMethA and COBRA for the *CDKN2A* (p16) promoter region tested. Although methylation per se does not affect the primary genomic DNA sequence, after bisulfite treatment all unmethylated cytosines are converted into uracil, while the methylated cytosines remain unchanged. Thus, the presence of methylation in a CpG island can be detected and quantified as a chemically induced C-to-T transition. Pyrosequencing is a real-time sequencing technique based on the detection of the release of inorganic pyrophosphate during nucleotide incorporation (9). The pyrophosphate released in the DNA synthesis reaction is quantified by monitoring a luciferase reaction. The luciferase reaction produces a signal proportional to the number of pyrophosphate molecules released (i.e., to the nucleotides incorporated in the DNA). Using the Pyrosequencing allele quantification software (Pyrosequencing AB), the sequence and allelic contribution is depicted as a quantitative Pyrogram™. This allows highly accurate determination of the frequency of polymorphic sites. The technology has been used successfully to determine mutant to wild-type allele ratios (10), to quantitatively determine allelic states of polyploid organisms (11), and to analyze DNA pools for the determination of allele frequencies in population-based studies (12,13). Here we show that Pyrosequencing can be used to successfully quantify the methylation status of specific CpG sites. In addition, to facilitate high-throughput determination of site-specific methylation levels for multiple genes, we developed a PCR amplification strategy using a tailed reverse primer in combination with a biotin-labeled universal primer in the same reaction (Figure 1B).

Briefly, *CDKN2A* promoter PCR amplification was performed in a 50-µL reaction containing HotStarTaq™ Master Mix (Qiagen, Valencia, CA, USA), 0.1 µM forward primer CDKN2A-F, 5′-GGTTGTTTTYGGTTGGTGTTTT-3′, and biotinylated reverse primer CDKN2A-R, 5′-ACCCTATCCCTCAAATCCTCTAAAA-3′. The amplification was carried out according to the general guidelines suggested by Pyrosequencing: denaturation at 95°C for 5 min, followed by 50 cycles at 95°C for 30 s, 56°C for 1 min, 72°C for 45 s, and a final extension at 72°C for 7 min. To reduce the cost/assay, we developed an amplification protocol using a universal primer approach (Figure 1B). Briefly, the biotinylated reverse primer was substituted with a 5′ tailed (5′-gacgggacaccgctgatcgttta-3′) unlabeled reverse primer, CDKN2A-UNIVR, 5′-gacgggacaccgctgatcgtttaACCCTATCCCTCAAATCCTCTAAAA-3′, and a biotinylated

universal primer, UNIV, 5′-gggacaccgctgatcgtttta-3′ at a ratio of 1:9 [uppercase, nucleotides specific for the target gene; lowercase, nucleotides specific for the universal primer tail; lowercase underlined, universal primer (UNIV) sequence]. PCR conditions were as described before, except that the annealing temperature was lowered to 54°C. Pyrosequencing reactions were performed according to the manufacturer's specifications with a *CDKN2A*-specific pyrosequencing primer (CDKN2A-PS, 5′-TTTTTGTTTGGAAAGAT-3′) and run on the PSQMA system (Pyrosequencing AB). Bisulfite treatment and COBRA were performed as described previously (14).

To determine the sensitivity and specificity of PyroMethA and universal PyroMethA, we performed a dilution series experiment on DNA extracted from RKO, a cell line with known *CDKN2A* promoter methylation levels of approximately 90%, as detected by COBRA. We prepared samples containing different fractions of methylated and unmethylated genomic DNA by mixing RKO methylated DNA with OVCAR-3 unmethylated DNA in the *CDKN2A* promoter (100%, 70%, 50%, 30%, 20%, 10%, 5%, and 0% RKO). We performed duplicate experiments for PyroMethA and universal PyroMethA. For one series, one-half of the same PCR product for each sample was analyzed by COBRA and universal PyroMethA. All three methods provided comparable results (Figure 2A). Overall, the methylation levels detected by all three methods were slightly lower than the predicted ratio of methylated/unmethylated DNA. This lower than expected ratio could be due to preferential PCR amplification of the normal unmethylated allele and/or the nature of the dilution/mixing experiment. In spite of these quantitative differences between predicted and observed results, there was complete concordance among the three assays. COBRA and PyroMethA showed a high degree of correlation ($r > 0.98$), and the correlation between regular PyroMethA and universal PyroMethA was even higher ($r > 0.99$). Neither method showed statistically significant differences.

We repeated COBRA and PyroMethA multiple times for the *CDKN2A* promoter region on two cell lines, HCT116 and RKO. To compare the variance of the two methods, we used a standard F-test and determined that PyroMethA is overall less variable than COBRA (Figure 2B). In addition, we measured *CDKN2A* promoter methylation in nine cell lines using COBRA and multiple replicates (2–4) using PyroMethA (Figure 2C). For 10 matched pairs of tumor and normal samples, we determined methylation levels by COBRA once and by PyroMethA and universal PyroMethA twice (Figure 2D). ANOVA testing of the results for the 20 normal/tumor samples showed no statistically significant differences between regular PyroMethA and universal PyroMethA. Results obtained by PyroMethA for the *CDKN2A* promoter region were overall comparable to those obtained by COBRA. The observed variation could be due to biological variability of methylation levels in different cell culture preparations and/or experimental variability unrelated to

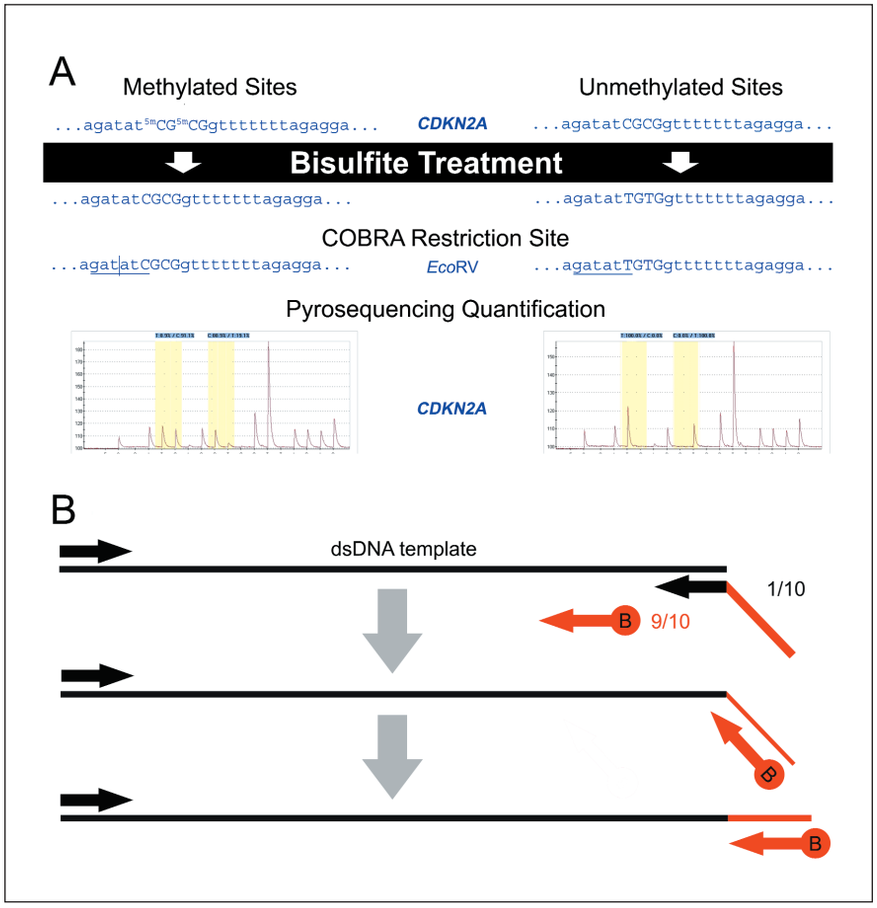

Figure 1. Schematic outline of PyroMethA and universal PyroMethA. (A) Comparison of the experimental approaches for COBRA and PyroMethA/universal PyroMethA to determine *CDKN2A* promoter methylation levels for specific CpG sites within their respective CpG islands. Bisulfite treatment converts unmethylated cytosines in genomic DNA to uracil, and methylated cytosines remain unaffected, resulting in a chemically induced methylation-dependent C-to-T polymorphic site, which can be detected by COBRA or PyroMethA. COBRA is based on the presence/absence of an informative restriction recognition sites (underlined sequence) for *Eco*RV in *CDKN2A*. PyroMethA is independent of the presence of an informative restriction site and quantitatively measures the relative levels of both nucleotides for the methylation-dependent single nucleotide polymorphism, which is represented in a Pyrogram. It is noteworthy that PyroMethA allows the simultaneous determination of methylation levels for multiple cytosines (capital letters) in a given CpG island. Methylation levels in neighboring CpG sites can be variable as shown here for *CDKN2A* (Figure 2). In contrast, using COBRA, methylation levels are determined at essentially a single CpG site. (B) Schematic of the universal PyroMethA amplification strategy. The biotinylated reverse primer is substituted with a 5′ tailed unlabeled reverse primer and a biotinylated universal primer mixed at a ratio of 1:9.

the method of quantification. These data indicate that PyroMethA/universal PyroMethA and COBRA always give concordant calls for the methylation status when a 10% threshold to declare methylation is used. (Figure 2, A–D).

Several techniques have been developed to assess gene-specific methylation events. However, most of them do not lend themselves to high-throughput screening. COBRA has been used extensively in methylation studies, but it is especially laborious. Thus, the development of a reliable quantitative method with high reproducibility, ease of use, and potential for high-throughput analysis is highly desirable. Here we developed and validated PyroMethA as a new method to quantitatively determine methylation levels. PyroMethA is similar to the Ms-SNuPE technique (15), except that Pyrosequencing allows the interrogation of multiple consecutive sites and does not require radioactivity. Like COBRA (14) and Ms-SNuPE (15), PyroMethA is a PCR-based method. It works better on small PCR products (100–150 bp), which is advantageous when working with DNA from clinical or archival samples, since partial DNA degradation can be a major problem for some of the other techniques. Like other site-specific methylation meth-

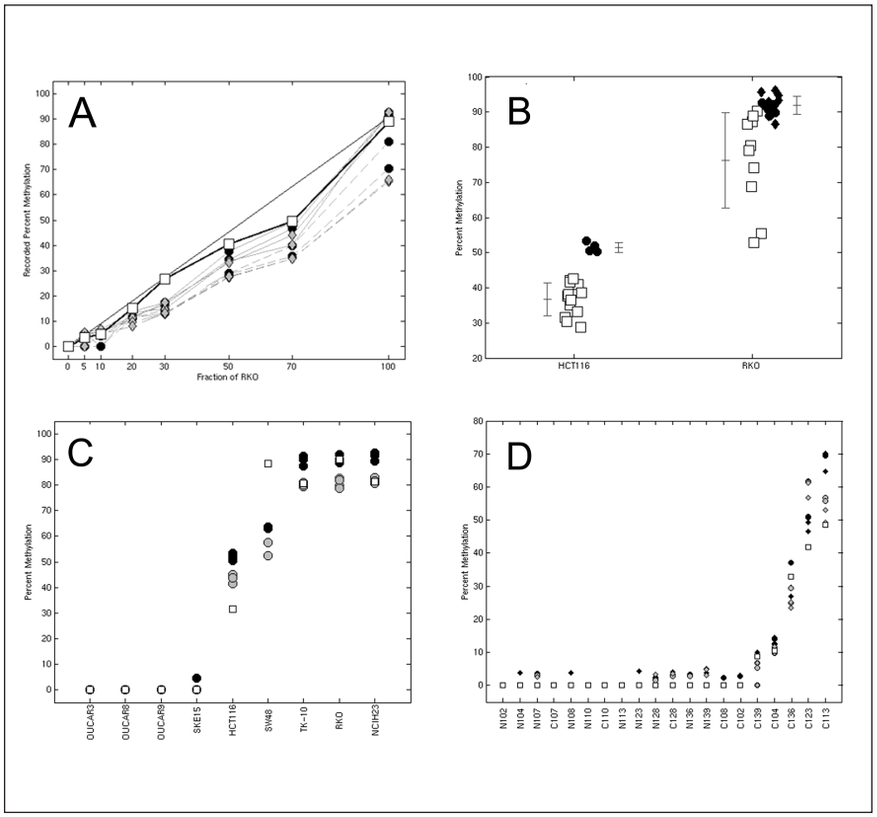

Figure 2. Methylation levels for the gene promoter regions of *CDKN2A*. (A) Comparison of COBRA (open squares) with PyroMethA (filled circles) and universal PyroMethA (shaded diamonds) in a dilution series of RKO genomic DNA with specific levels of *CDKN2A* promoter methylation. The straight line through the origin represents the expected results for the dilution series at the first CpG site based on RKO cell line methylation levels previously determined by COBRA. Symbols connected by straight lines represent observed data at the first CpG site; symbols connected by stippled lines represent data observed at the second CpG site. (B) Comparison of COBRA (open squares), PyroMethA (filled circles) for cell lines HCT116 and COBRA (open squares), PyroMethA (filled circles), universal PyroMethA (filled diamonds) for cell line RKO, to determine reproducibility. $\bar{x} \pm SD$ levels are indicated. (C) COBRA (open squares) and PyroMethA results (site 1, filled circle; site 2, shaded circle) for a panel of nine cancer cell lines in ascending order of methylation levels, as determined by PyroMethA. (D) COBRA (open squares), PyroMethA (circles; site 1, filled; site 2, shaded), and universal PyroMethA (diamonds; site 1, filled; site 2, shaded) results for a panel of 10 pairs of matched normal/colorectal cancer samples in ascending order of methylation levels, as determined by COBRA.

ods, PyroMethA can be applied only to known sequences. Although the design of the assay for the CpG sites we studied was relatively straightforward, assay design and quantification can be affected by sequence context.

Our data indicate that both PyroMethA and universal PyroMethA compare favorably to COBRA in terms of sensitivity, specificity, and robustness. Moreover, validating and implementing the universal-primer strategy in the initial PCR, we substantially reduced the cost/assay. In addition, with the assay we developed for *CDKN2A*, we demonstrated that PyroMethA provides a more comprehensive tool to determine methylation levels at multiple CpG sites throughout a given CpG island. Since our method is not dependent on informative restriction sites, the spectrum of target sequences that can be assayed is broader than that for COBRA. Our data indicated that methylation of neighboring CpG sites within the same CpG island can be variable. Differential levels of methylation at different CpG sites in a given promoter could underlie differential expression of affected genes and may thus be of biological significance.

With the completion of the sequence of the human genome, gene promoter sequences and regulatory CpG islands can be readily identified in silico (16). The development of new methods, such as PyroMethA/universal PyroMethA, will enable the more detailed study of candidate genes affected by methylation. Large-scale screening for promoter methylation at specific sites will provide a better understanding of the epigenetic and epigenomic contributions to the molecular processes of development and disease. For example, high-throughput studies targeted at promoter regions of candidate tumor suppressor genes subject to epigenetic inactivation by methylation can be easily designed and carried out. To determine the efficacy of de-methylating agents in clinical trials, PyroMethA would also constitute a suitable high-throughput platform. In conclusion, PyroMethA/universal PyroMethA is a new, highly sensitive method to quantify CpG site methylation, which can be easily adopted for high-throughput studies of gene-specific methylation events.

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