Clinical applications of DNA methylation in gastrointestinal cancer

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CHAPTER 1

Synthesis of universal unmethylated control DNA by nested whole genome amplification with Φ29 DNA polymerase

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Abstract

Optimization of highly sensitive methods to detect methylation of CpG islands in gene promoter regions requires adequate methylated and unmethylated control DNA. Whereas universal methylated control DNA is available, universal unmethylated control (UUC) DNA has not been made because demethylase is not available to remove methyl groups from all methylated cytosines. On the basis that DNA synthesized by DNA polymerase does not contain methylated cytosines, we developed a method to create UUC DNA by nested whole genome amplification (WGA) with Φ29 DNA polymerase. Contamination of the template genomic DNA in UUC was only $3.1*10^7$, below the detection limit of sensitive methods used for methylation studies such as methylation-specific PCR. Assessment of microsatellite markers demonstrated that even nested Φ29 WGA achieves highly accurate and homogeneous amplification with very low amounts of genomic DNA as an initial template. The UUC DNA created by nested / 29 WGA is practically very useful for methylation analysis.

Introduction

Cytosines of CpG dinucleotides in DNA of higher order eukaryotes are partially methylated [1], and this modification has important regulatory effects on gene expression, especially when it involves CpG-rich areas (CpG islands) in the promoter region[2,3]. Epigenetic gene silencing by promoter hypermethylation is as significant as deletions or mutations for inactivation of tumor suppressor genes[4-6]. Because these events play a significant role in malignant transformation and immortalization of cells, assessment of gene promoter hypermethylation has become important to understand tumor progression. Among the available methods for detecting specific methylation status of genes, methylation-specific PCR (MSP) and its derivatives are currently the most widely used techniques because they have high sensitivity for virtually any block of CpG sites in CpG islands[7]. Because the MSP results are highly dependent on the specificity of primer annealing, the annealing temperature of thermal cycling and other PCR conditions must be optimized carefully with proper methylated and unmethylated control DNA to avoid nonspecific amplification which causes false-positives or false-negatives. However, universal unmethylated control (UUC) DNA is not available whereas universal methylated control (UMC) DNA can be made from normal genomic DNA with a CpG methylase SssI[8]. Therefore, DNA from peripheral blood leukocytes (PBL), sperm, or other tissues is usually utilized as an unmethylated control, depending on the methylation status of the target site. However, it is labor consuming and sometimes very difficult to verify the absence of CpG methylation at the target site of the template DNA used as an unmethylated control. In addition, it is impossible to find an unmethylated control for global methylation analysis because there is no completely unmethylated genome in humans. Therefore, artificially synthesized UUC DNA would be highly valuable in any methylation analyses such as global methylation analysis or assessment of promoter hypermethylation of tumor-related genes in tumors and serum. On the basis that DNA synthesized by DNA polymerase does not contain methylated cytosines, we aimed to create UUC by whole genome amplification (WGA), but the conventional thermal cycling WGA methods were not adequate because they inefficiently amplified GC-rich areas[9], the targets of methylation studies. Recently, a WGA technique by Φ29 DNA polymerase, which is from the bacteriophage Φ29, has been developed[10,11]. The Φ29 polymerase continuously amplifies single- or double-stranded circular- or linear-DNA by strong strand displacement activity. Therefore, after an initial heat-melting step, the Φ29 polymerase does not require further thermocycling to initiate nascent strand synthesis and can amplify highly GC-rich sequences. In addition, Φ29WGA has been shown to have high fidelity and near complete genome representation[12]. However, because the amplification power of this method is only 10^3–10^4, we designed a protocol using nested Φ29 WGA to make a UUC and confirmed its utility for practical methylation studies.

Materials and Methods

Template genomic DNA

The genomic DNA of PBL obtained from healthy donor volunteers was used as the template DNA for WGA. Peripheral blood was centrifuged and the PBL fraction was isolated. DNA was extracted using DNAzol reagent (Molecular Research Center, Cincinnati, OH) and quantified with an UV absorption spectrophotometer.

Creation of UUC by nested WGA with Φ29 DNA polymerase

GenomiPhi DNA Amplification Kit (Amersham Biosystems, Piscataway, NJ) utilizing Φ29 DNA polymerase was used to create UUC from genomic DNA. For primary WGA, 1.0 ng of genomic DNA prepared in 1 µl was amplified in a total volume of 20 µl following the instruction provided by the kit manufacturer. DNA was diluted with 9 µl sample buffer containing random primers, heat-denatured at 95 °C for 3 min, cooled to 4 °C, and then mixed with 9 µl reaction buffer and 1 µl enzyme mix containing Φ29 DNA polymerase. All the buffers used were provided as premixed in the kit. The mixture was incubated at 30 °C for 18 h, and then the enzyme was deactivated by heating at 65 °C for 10 min. For nested WGA, 0.1 µl of the product of primary WGA was amplified in a total volume of 20 µl with the same protocol as the primary reaction. DNA products synthesized by primary and nested WGA were quantified with UV absorption spectrophotometer after purification by QIAquick PCR purification kit (Qiagen, Valencia, CA). The nested WGA product was electrophoresed on 2% agarose gel, and the DNA length was analyzed.

Creation of UMC by SssI methylase

SssI methylase (New England Biolabs, Beverly, MA), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5'. . .CG. . .3', was used to create UMC in accordance with the manufacturer's protocol[8].

Microsatellite analysis of UUC

To ensure the fidelity and representation of nested WGA, allelic ratios at 34 microsatellite markers (mononucleotide repeat markers BAT25 and BAT26; dinucleotide repeat markers TGFbR2, TP53, D1S228, D2S123, D5S229, D5S346, D6S1678, D6S1700, D6S286, D8S261, D8S262, D8S321, D9S171, D10S197, D10S393, D10S591, D12S1657, D12S1706, D12S327, D12S346, D12S393, D14S51, D14S62, D16S421, D16S422, D17S1832, D17S849, D17S855, D18S61, and D18S70, and tetranucleotide markers D12S1059 and D12S296)[13-15] of the genomic DNA (template) and UUC (nested WGA product) were compared. Primer sequences were obtained from the National Cancer for Biotechnology Information (NCBI) database. Forward primers were labeled with WellRED dye-labeled phosphoramidites (Beckman Coulter, Fullerton, CA). PCR was performed with 10 ng of genomic DNA or nested Φ29 WGA product, 2.5 mM Mg2+, and 0.2 µM of each primer in a 10-µl reaction volume for 36 cycles: 30 s at 94 °C, 30 s at suitable annealing temperature for each primer set, 30 s at 72 °C, and 7-min final extension at 72 °C. The amount and size of the PCR amplicon were determined by capillary array electrophoresis (CAE) with the CEQ 8000XL system (Beckman Coulter). Allelic ratio deviation of nested

WGA products on each heterozygous marker was calculated using the following formula: Max (R_G, R_U)/Min (R_G, R_U) - 1.0, where R_G, R_U are the CAE intensity ratios of two alleles in genomic DNA and in UUC, respectively.

SBM on UUC and UMC

SBM was applied on UUC and UMC as previously described[16]. A 5 µg DNA sample in 46.7 µl was denatured by the addition of 3.3 µl of 3M NaOH and incubated at 37 °C for 15 min. After addition of 520 µl of 2.5M sodium metabisulfite at pH 5.0 and 30 µl of 10 mM hydroquinone, DNA was incubated at 60 °C for 4 h in the dark. The bisulfite-treated DNA was desalted using the Wizard DNA cleanup system (Promega, Madison, WI) and eluted in 50 µl H2O. DNA was then desulfonated by 5.6 µl of 3M NaOH at 37 °C for 15 min and neutralized by 14 µl of 3M sodium acetate. After ethanol precipitation, the DNA pellet was resuspended in 50 µl of 10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0.

Detection of methylation of promoter region of genes

To test the UUC, we demonstrated the methylation status of p16 (INK4a) gene promoter region by SBM direct sequencing and MSP. This gene is one of the most intensely studied tumor suppressor genes in malignant tumors[5,17]. DNA sequence of p16 gene isoform-1 promoter region with indication of the primers is shown in Fig. 1. We also demonstrated the methylation of promoter regions of seven other genes (RASSF1A (ras association domain family protein 1), hMLH1 (mutL homologue 1), TWIST, ID4 (inhibitor of DNA binding 4), ESR1 (estrogen receptor 1), 14-3-3σ, and MGMT (methylguanine-DNA methyltransferase)) by MSP.

acacgcctttgctggcaggcggggggagcgcggctgggagcagggaggccg
gagggcggtgtgggggggcaggtgggggaggagcccagtcctccttccttgc
caacgctggctctggcgagggctgcttccggctggtgccccgggggggaga
CCCAACCTGGGGCGACTTCAGGGGTGCCACATTCGCTAAGTGCTCGGAGT
TAATAGCACCTCCTCCGAGCACTCGCTCACGGCGTCCCCTTGCCTGGAAA
GATACCGCGGTCCCTCCAGAGGATTTGAGGGACAGGGTCGGAGGGGGCTC
TTCCGCCAGCACCGGAGGAAGAAAGAGGAGGGGCTGGCTGGTCACCAGAG
GGTGGGGCGGACCGCGTGCGCTCGGCGGCTGCGGAGAGGGGGAGAGCAGG
CAGCGGGCGGCGGGGAGCAGCATGGAGCCGGCGGCGGGGAGCAGCATGGA
GCCTTCGGCTGACTGGCTGGCCACGGCCGCGGCCCGGGGTCGGGTAGAGG
AGGTGCGGGCGCTGCTGGAGGCGGGGGCGCTGCCCAACGCACCGAATAGT

Fig. 1. *DNA sequence of p16 gene isoform 1 promoter region with indication of the primers for methylation detection analyses. Lowercase: upstream of 50' untranslated region; uppercase: exon 1. Open boxes: SBM sequencing primers; solid underlines: methylated-specific MSP primers; and dotted underlines: unmethylated-specific MSP primers. CpG sites are indicated in bold.*

For SBM direct sequencing, 1 µl of SBM UUC was amplified by PCR with the SBM sequencing primers and 2.5 mM of Mg2+ in a 50-µl reaction volume for 36 cycles: 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a 7 min final extension at 72 °C. Purified PCR products were bidirectionally direct-sequenced by CAE using CEQ DYE Terminator Cycle Sequencing kit (Beckman Coulter). The cycling program included 30 cycles: 20 s at 95 °C, 40 s at 55 °C, and 4 min at 60 °C.

For MSP, each primer was designed to cover two or more CpG sites. Unmethylated-specific and methylated-specific PCR was performed on 1 µl SBM UUC with 0.2 lM of each primer and 2.5 mM Mg2+ in a 10-µl reaction volume for 36 cycles: 30 s at 94 °C, 30 s at optimized annealing temperature for each primer set, and a 7-min final extension at 72 °C. Forward primers were labeled with WellRED dyelabeled phosphoramidites (Beckman Coulter). PCR products were detected and analyzed by CAE.

Results

Nested WGA product

The amount of UUC created by nested WGA in 20 µl of reaction volume was 15.9 ± 1.1 µg (mean ± SEM, n = 4), which included only 5 pg of template genomic DNA (1/200 amount of the initial template DNA). The total amplification ratio was $3.2*10^6$, and the contamination ratio of genomic DNA was $3.1*10^7$. When 100 ng of nested WGA product was used as a template for subsequent analysis, estimated contamination of the genomic DNA was as low as 0.02 copies per reaction, below the minimum detection level of a highly sensitive method such as MSP. When the nested WGA product was electrophoresed on 2% agarose gel, the DNA fragment length was widely distributed but >5 kb fragments were dominant, indicating wide coverage of the genes and their promoter regions (Fig. 2A).

Microsatellite analysis

All 34 microsatellite markers were sufficiently amplified on UUC. When amplicon length and distribution were compared for each marker, all the peaks shown by CAE were equivalent and no aberrant bands were observed. No significant amplification error occurred on the nucleotide repeats, and the amplicon size was completely preserved after nested WGA reactions (Fig. 2B). In 16 heterozygous markers, allelic ratios were highly maintained in all markers; the deviations caused by nested WGA were only 5.9 ± 0.8% (mean ± SEM) with a maximum value of 11% in D12S327 (Figs. 2B and C). Thus, the two alleles were equally amplified by nested WGA, and it was demonstrated that Φ29 WGA achieves highly accurate and homogeneous amplification, even on nested reactions with very low amounts of genomic DNA as a template.

Methylation status of gene promoter regions in UUC

As a demonstration, SBM direct sequencing and MSP at p16 promoter region on the UUC were performed. Sequencing showed that all cytosines of CpG dinucleotides were converted to uracils; thus, there was no methylation on CpG dinucleotides (Fig. 3A). In MSP, only the unmethylated-specific peak was observed (Fig. 3B). Restriction enzymatic digestion

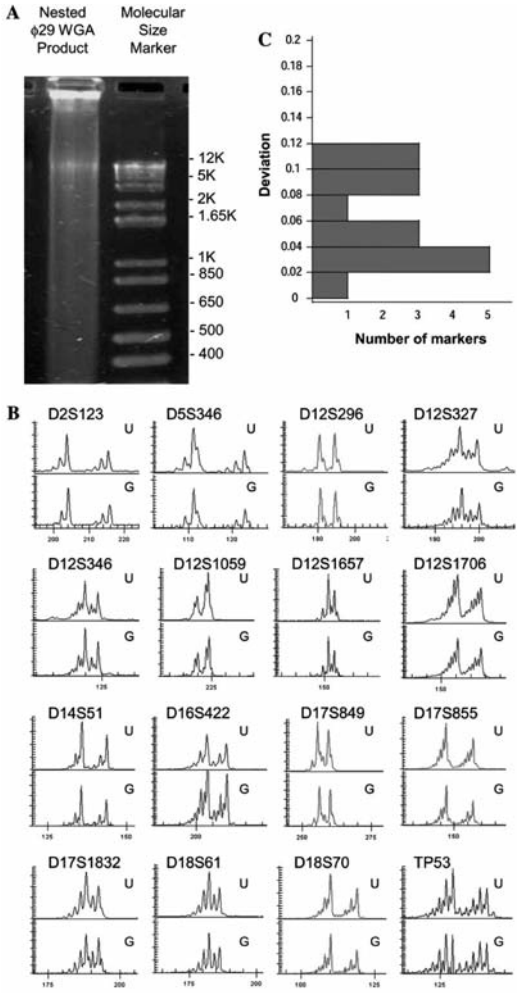

Fig. 2. *(A) Nested /29 WGA product electrophoresed on 2% agarosegel along with molecular size marker. DNA fragment lengths were widely distributed, but >5 kb fragments were dominant. (B) CAE results of 16 microsatellite markers showing heterozygosity on genomic DNA among 34 tested markers. Upper figure (U) in each marker is of UUC and lower figure (G) is of genomic DNA used as a template. The vertical axis represents the fluorescent intensity indicating the amount of PCR amplicon and the horizontal axis represents the PCR amplicon size. All the peaks shown by CAE were equivalent and no aberrant bands were observed. Allelic ratios were highly maintained in all markers. (C) Histogram of allelic ratio deviation of UUC; the allelic ratio deviation was 5.9 ± 0.8% (mean ± SEM).*

study using methylation-specific restriction endonuclease HpaII confirmed that the CpG within the recognition site was not methylated in UUC (data not shown). COBRA analysis using restriction endonuclease HpyCH4 IV further confirmed that specific CpG site in p16 promoter region had no methylation (data not shown). Similarly, MSP analysis of the promoter regions of RASSF1A, hMLH1, TWIST, ID4, ESR, 14-3-3σ, and MGMT showed only unmethylated-specific peaks (Fig. 3B). In all primer sets, the PCR amplicon length and the shape of the peaks were identical for UUC and PBL DNA. Thus, nested Φ29 WGA did not affect promoter sequence.

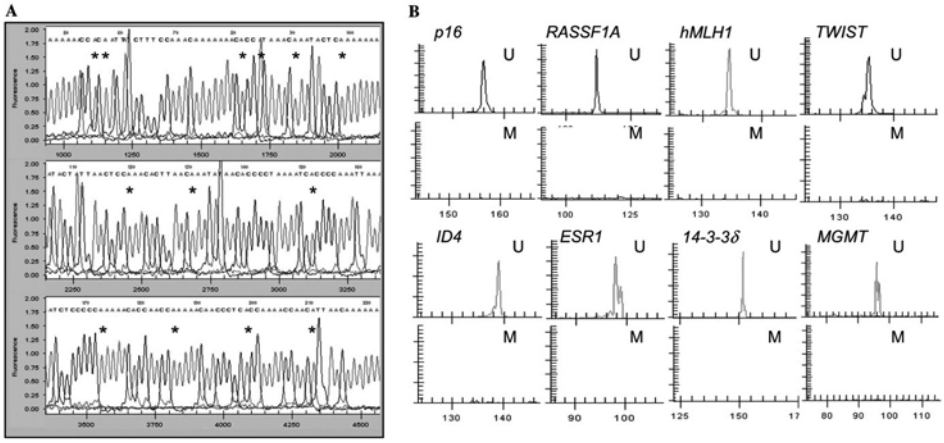

Figure 3. *(A) SBM direct sequencing result at p16 promoter region on UUC in reverse direction. It is shown that all the cytosines of CpG dinucleotides in UUC were converted to uracils (shown as A in reverse sequence with *), representing that there was no methylation on CpG dinucleotides. (B) Methylated- and unmethylated-specific MSP for p16, RASSF1A, hMLH1, TWIST, ID4, ESR1, 14-3-3d, and MGMT on SBM UUC. Unmethylatedspecific peaks were observed in unmethylated-specific MSP in upper figures (U), but no peaks were observed in methylated-specific MSP in lower figures (M). The vertical axis represents the fluorescent intensity indicating the amount of PCR amplicon and the horizontal axis represents the PCR amplicon size.*

Discussion

There are two major types of WGA: thermal cycle amplification using thermostable DNA polymerase and continuous amplification at a stable temperature using DNA polymerase such as Φ29. To make a UUC DNA, the entire genome including GC-rich sequence must be equally amplified. However, thermal cycling methods amplify the genome unequally depending on the distribution of primer annealing sites, and the length of products is relatively short. In addition, because GC-rich sequences are not efficiently amplified[9], thermal cycling methods may not be adequate for the CpG islands, which are the usual targets of interest for methylation studies. In contrast, a continuous amplification method using Φ29 DNA polymerase, which synthesizes the DNA strand displacing the bound complementary DNA, has demonstrated that genome representation was comprehensive and estimated to be 99.8% complete, there was no degradation in the accuracy of single nucleotide polymorphism (SNP) genotyping, and the estimated error rate (9.5 · 10 6) was equivalent to that for unamplified samples[12]. In our results of microsatellite markers, it was demonstrated that Φ29 WGA achieves highly accurate and homogeneous amplification, even on nested reaction with very low amounts of genomic DNA as a template. Contamination of methylated CpG dinucleotides of the template genomic DNA must be minimized in UUC. However, amplification ratio of Φ29 WGA is only 10^3–10^4, and it is insufficient because

the subsequent method such as MSP can detect as low as 10^4 level of contaminated methylated DNA[7]. Nested amplification can reduce the percentage of the contaminating genomic DNA used as the template. In this study, a nested Φ29 WGA amplified the template DNA by a factor of $3.2*10^6$. The final concentration of contaminant was only 0.02 copies for the subsequent reactions, a negligible amount. Nested Φ29 WGA maintained the allelic ratio and the size of microsatellites, representing the high fidelity of the amplification. As shown in gel electrophoresis, the fragment of DNA synthesized by nested Φ29 WGA had sufficient length to cover promoter regions, and the CpG islands in the promoter regions were adequately amplified. In addition, results of MSP and SBM direct sequencing demonstrated that the promoter regions were maintained in the nested Φ29 WGA products. For highly sensitive methods, both negative and positive controls are essential to optimize conditions. UUC is very useful and necessary for methylation studies, especially MSP, which is a sensitive and powerful technique but critical for condition settings. Inappropriate settings easily cause false-positive and/or false-negative results. The designing of MSP primer sets can have difficulties because of restrictions such as limited annealing sites. Optimization of the PCR is essential but can be difficult without adequate controls. In conclusion, the UUC DNA created by nested Φ29 WGA is practically very useful and highly essential for adequate methylation analyses such as evaluations of promoter methylation status of tumor-related genes in tumor tissues. Contamination ratio of the template genomic DNA in UUC was below the detection limit of MSP or other sensitive methods. Because the UUC contains near complete whole genome sequence and does not contain methylated cytosines, it can be utilized as a standard control material for various genomic methylation analyses.

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