Bridging the Gap

Evaluation of DNA methylation in the human genome: Why examine it and what method to use

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Summary

Purpose: Since its discovery 50 years ago, DNA methylation has been found to be an important part of gene regulation. Newer methods of analysis over the last decade have helped further the understanding of this epigenetic phenomenon. The purpose of this article is to describe current methods of analysis and discuss advantages and disadvantages of each and their possible roles in gynecologic malignancies.

Results: The methods for analysis of DNA methylation are divided into two major categories: 1) methods which utilize chemical methods or restriction enzymes to differentially cleave at cytosine versus 5-methylcytosine sites, 2) methods which utilize sodium bisulfite ($NaHSO_3$) to specifically convert unmethylated cytosines to uracil (thymine after PCR). This recently developed method appears to be more sensitive and allows the investigator to specifically delineate the study site(s).

Conclusion: DNA methylation is important in the human genome. Its role in tumorigenesis is just beginning to be understood. While relying upon newly designed methods of analysis, further understanding of this epigenetic phenomenon and its role in gene expression and tumorigenesis will be forthcoming.

Key words: Methylation; Tumor suppressor genes; CpG; Mutation.

Introduction

DNA methylation is a well-known epigenetic phenomenon that occurs in higher order eukaryotes. It was first discovered almost 50 years ago, and its importance has been slowly uncovered over time [1, 2]. Approximately 5% of cytosines in the mammalian genome are modified to 5-methylcytosine [3]. The majority of 5-methylcytosines within the human genome are found in CpG islands which cluster in promoter regions and the 5' ends of genes [4]. Post-transcriptional modification of cytosine to methylcytosine plays an essential role in the regulation of gene expression [5, 6]. X-chromosome inactivation and imprinting gene expression are both controlled by DNA methylation [6-8]. This epigenetic methylation may play a role in linking aging to the development of cancer [9]. This appears to occur in two different ways. First, within the human genome, methylcytosine appears to be inherently more mutable than cytosine [10]. These mutation are most commonly missense mutations (specifically transitions) and are manifested as changes from cytosine to thymine or guanine to adenine (Figure 1). Second, gene expression can be altered, either upregulated or more commonly silenced by promoter methylation. These epigenetic phenomena occur in a gene's promoter in the CpG island region (Figures 2 and 3) [11-16].

Silencing tumor suppressor genes by this mechanism may serve as an alternative to gene mutation. As with the two-hit hypothesis of Knudson, one wild-type gene could be silenced by promoter methylation and the other gene could be lost or mutated resulting in two hits and, thus, a tumorigenic phenotype. Alternatively, methylation of both alleles could result in silencing [14].

Multiple methods exist for studying this genomic phenomenon. The first method utilizes chemical methods or restriction enzymes to differentially cleave at cytosine versus 5-methylcytosine sites. The second method utilizes sodium bisulfite ($NaHSO_3$) conversion of unmethylated cytosine to uracil (thymine after PCR) (Figure 4). Each of these techniques has many variations. These variations, as well as advantages and disadvantages of the techniques, will be discussed.

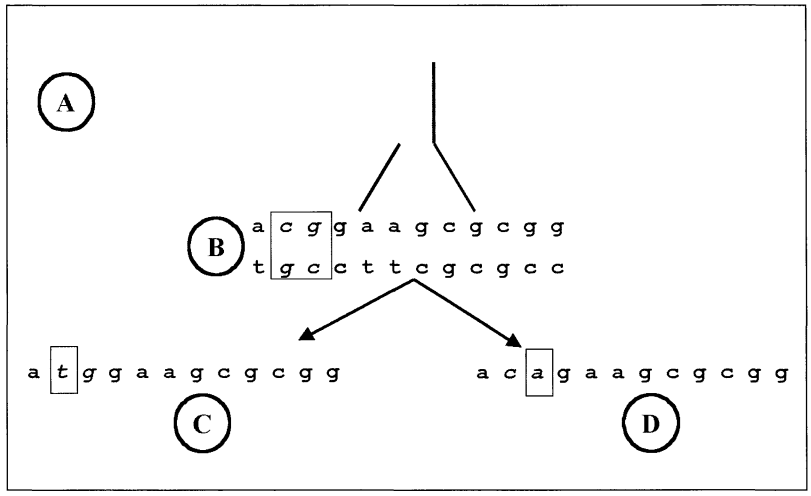

Figure 1. — A) Diagram of DNA double helix. B) Close-up diagram of a short segment of DNA. Top strand is the sense (5'→3') strand. Bottom strand is the anti-sense strand. Italicized bases in the box represent a CpG site on both the sense and anti-sense strand. C) Sense strand with mutated CpG site (C→T) boxed in. D) Sense strand demonstrating mutated CpG site. The base change is from (G→A) on the sense strand because there was a C→T mutation on the anti-sense strand which in turn becomes a G→A mutation on the sense strand instead of the classic CpG→TpG.

Methods for studying DNA hypermethylation

Differential Chemical Cleavage

Specific chemicals can be used to differentially cleave between unmethylated and 5-methylated cytosines. Saluz and Jost analyzed the different interaction of hydrazine with cytosine versus 5-methylcytosine [17]. The cytosine ring is affected by hydrazine treatment resulting in piperidine cleavage except in the presence of a 5-methyl modification. Therefore, linear fragments can be amplified and missing bands will designate the presence of DNA methylation [17, 18]. In contrast, potassium permanganate gives a positive display of the 5-methyl modified base. However, it is less complete than that given by hydrazine [18].

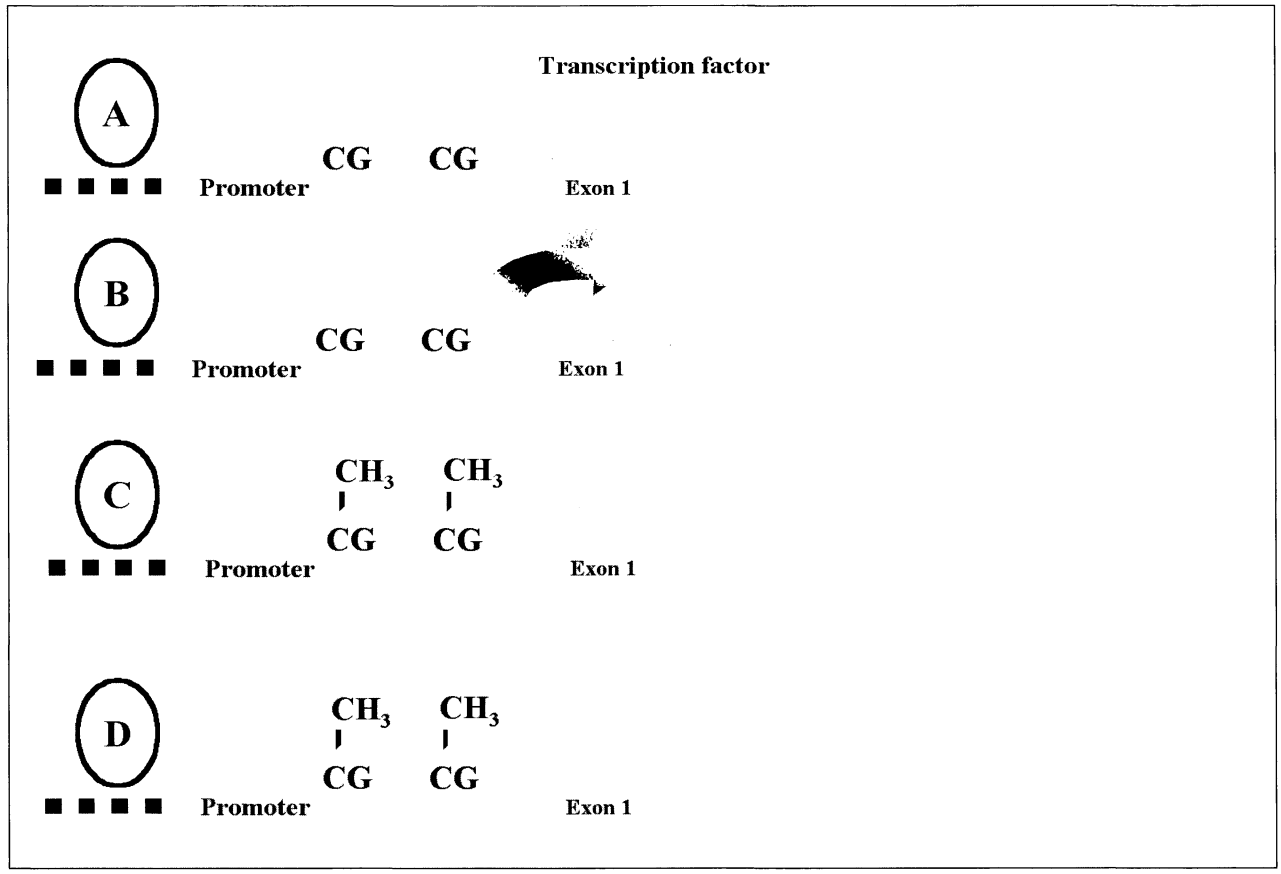

Figure 2. — A) Diagram of a theoretical promoter with CpG sites. DNA is represented by (---). The transcription factor is also shown. B) Transcription factor is shown bound to the promoter with transcription beginning (the arrow). C) The promoter's CpG sites are methylated changing the promoters configuration. D) Since the configuration is changed by the methylation, the transcription factor cannot bind and transcription cannot begin.

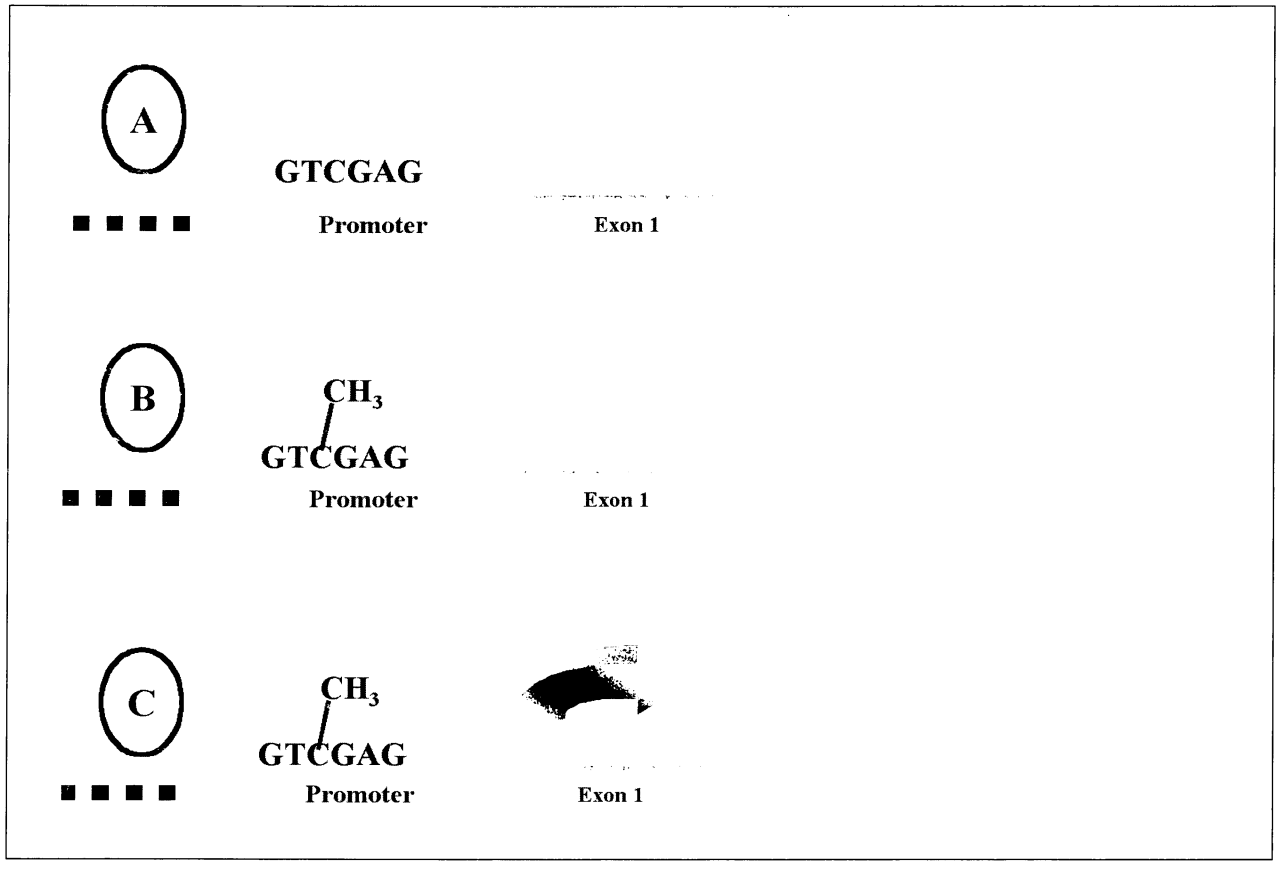

Figure 3. — A) Diagram of a theoretical promoter. B) Methylation of a CpG site changes the promoter's configuration. C) The changed configuration allows inappropriate binding of a transcription factor that does not normally bind to this promoter. Thus, inappropriate upregulation of the gene product can occur.

The advantages and disadvantages are fully explained later. However, one unique disadvantage of this method is the "shotgun" cleavage of unmethylated cytosines. Narrowing the aim of chemical cleavage is not possible. Therefore, this method wastes a large amount of DNA (5 □ g or more), which is then not usable for other experiments.

Methylation specific restriction enzyme digest

Methylation specific restriction (MSR) enzymes (i.e., *Msp*I, *Hpa*II, *Bst*BI, *Bst*U1, *Hha*I, *Sac*II, *Sma*I) are able to differentially restrict DNA at specific sites of cytosine methylation. *Msp*I and *Hpa*II are MSR enzymes

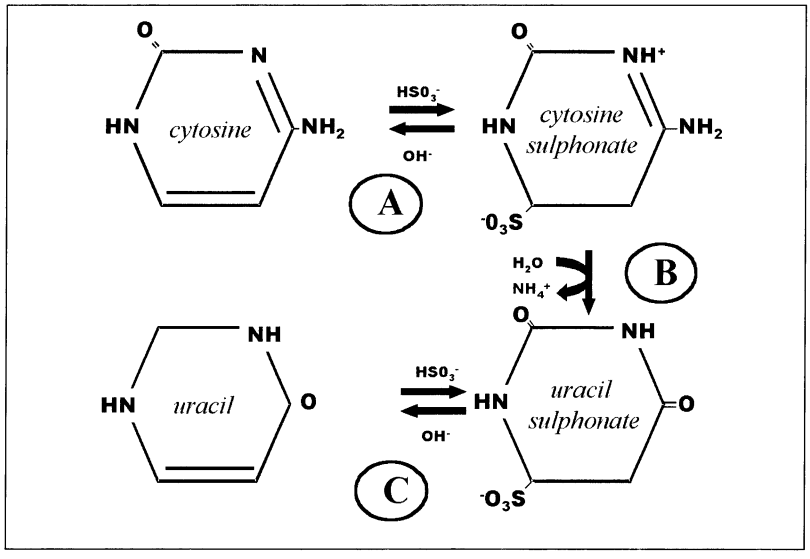

Figure 4. — This diagram demonstrates the sodium bisulfite conversion of cytosine to uracil (thymine after PCR). A) Reversible sulfonation of cytosine to cytosine sulphate (methylation blocks step). B) Hydrolytic deamination (non-reversible). C) Desulfonation (reversible).

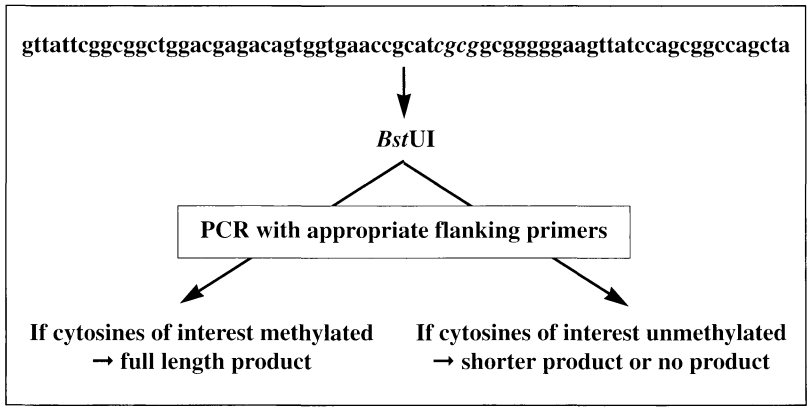

Figure 5. — An example of a potential sequence containing a BstUI restriction site (italicized) is shown. If the cytosines in the restriction site are methylated, full length products will be seen on a gel after amplification with appropriate primers.

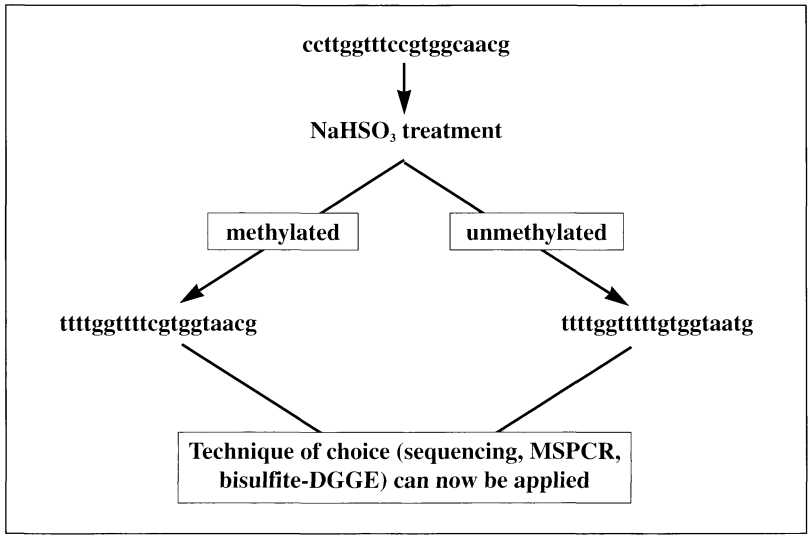

Figure 6. — Unmodified DNA (sense strand only) demonstrated at top of figure. The sequence changes based on the presence/absence of methylated cytosines. Primers must be designed to account for this sequence change. The original sense/anti-sense strands are no longer complementary, so it may be easier to design primers paying particular attention to the modified sense strand only.

that are isoschizomers of each other. In certain situations, both enzymes restrict DNA between the cytosines in the sequence *'CCGG'*. However, *Hpa*II will not restrict DNA if either cytosine is methylated. In contrast, *Msp*I can cleave the DNA appropriately if the internal cytosine is unmethylated. Southern hybridization can be used to ascertain the length of the restricted fragments and determine methylation status.

A more advanced version of this technique involves the combination of MSR enzymes and PCR. Primers are designed to flank both sides of an MSR enzyme restriction site. An enzymatic digestion is carried out using the MSR enzyme of interest (Figure 5). *Bst*UI, which cleaves DNA at unmethylated *'CGCG'* sites, is used in this example. If the cytosines are methylated, a PCR reaction using appropriately designed primers will result in a full-length product on agarose gel. If the cytosines are unmethylated, a PCR reaction using appropriately designed primers will give a shortened product or be absent.

There are distinct disadvantages with these methods. First, a large amount of DNA is required (5 □ g or more) [19]. Second, if there is incomplete cleavage of the unmethylated site, it will result in a methylated product (a false positive) and appear as a full length band on Southern blot analysis and/or be amplified by the PCR primers. Also, paraffin samples are unsuitable for methylation specific restriction enzyme digest [19]. Another problem is that a small number of methylated alleles will not be discovered by these methods. Finally, the MSR enzymes can only be used at designated sites. There is slight variation between different MSR enzymes. On the other hand, one advantage of this method is its simplicity in studying samples with high levels of methylated alleles (imprinting genes and X-chromosome inactivation) [20, 21].

Sodium Bisulfite Chemical Modification Methods

Since its description by Clark *et al.* in 1994, chemical modification of unmethylated cytosine to uracil by $NaHSO_3$, followed by subsequent change from uracil to thymine by PCR, has become the method of choice for analyzing the epigenetic phenomenon of methylation in human DNA [2]. At least three modifications of this method are now in use: bisulfite sequencing, methylation specific PCR (MSPCR), and bisulfite denaturing-gradient gel electrophoresis (bisulfite DGGE) [2, 14, 19, 22, 23]. Each of these are described with apparent advantages, disadvantages, and potential artifacts. In general, these methods require adaptation of typical primer design to account for the change by the bisulfite of both unmethylated and methylated single

strands of DNA. Figure 6 describes this interesting problem. The modified base, 5-methylcytosine, is resistant to bisulfite treatment, but overheating the DNA may lead to modification of this base rather than modifying cytosine without methylation [2].

Bisulfite Sequencing

Bisulfite sequencing has been described by multiple authors. DNA is treated with bisulfite, amplified by PCR (using appropriately designed primers), and sequenced to analyze the area of interest [2, 14, 23]. One advantage of this method is that it gives a positive view of changes on a sequencing gel. There are several disadvantages. First, this method can be labor intensive since cloning may be required to obtain accurate sequencing [19]. Low numbers of methylated alleles (less than 25%) may be missed [19].

Aggerholm and colleagues have described another modification of this method called bisulfite-DGGE [22, 24]. This method uses the bisulfite technique and follows it with non-discriminatory PCR amplification of methylated and unmethylated alleles. Allele resolution is accomplished with denaturing gradient gel electrophoresis. These authors found that different results were obtained with this method compared to the MSPCR method (described later) when studying the death associated kinase promoter in acute myelogenous leukemia [22, 24, 25]. This difference may be due to the fact that MSPCR appears to be a more sensitive method to amplify a low incidence of methylation [26]. Boyes and Bird found that the density of promoter methylation is important [27]. A weak promoter may need only partial or incomplete CpG island methylation to silence a gene while a strong promoter may require that dense methylation is present [27].

First described by Herman and colleagues, the MSPCR method is a modification of the bisulfite treatment of DNA [19]. After treating DNA with $NaHSO_3$, specific primers that account for potential sequence changes caused by modification are used to examine a designated area of interest. Selection criteria for primers should include the analysis of CpG cytosines along with other cytosines to detect methylation. This technique has been used to study multiple tumor suppressor genes and other genes of interest in human disease and aging [13, 19, 25, 28-30]. Recently, the MSPCR has been used to study the role of CpG promoter methylation in ovarian cancer [31-33]. Specifically, methylation, as demonstrated by MSPCR, was shown to be of importance in BRCA1, hMSH2, hMLH1 but not BRCA2 [31-33].

One advantage of this technique is the simplicity of laboratory methods. It does not require labor intensive methods such as cloning. Another advantage may actually be a disadvantage to some. It is the ability to determine areas devoid of overwhelming methylation [22, 26]. A final advantage is the ability to examine very specific areas of interest, rather than relying upon the presence of an MSR site.

Disadvantages are also present with this technique. As with any primer, non-specific priming may occur. Due to the modification of DNA with the bisulfite technique, the chance for false priming may be difficult to predict. This technique is also dependent upon the number of PCR cycles. The use of more than 35 cycles is discouraged by developers of the technique due to over-amplification of a small number of alleles [19, 26].

Conclusion

All of the methods discussed have several advantages and disadvantages. While advocates for each method support each technique to the exclusion of others, many scientists agree that this epigenetic phenomenon should be extensively studied. One area of continued controversy is determining which came first, methylation or gene silencing [34, 35]. In other words, does gene silencing lead to a phenotype that allows increased CpG methylation, or does increased CpG methylation lead to gene silencing? The question has been controversial and difficult to answer. However, the issue may, in point, be mute clinically if not biologically. Since clinical correlation exists between methylation and disease specific characteristics, it may not matter if the chicken or egg came first [9, 12, 13, 14, 15, 19, 36].

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