

Main Principles and Outcomes of DNA Methylation Analysis

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Abstract

Epigenetic modifications, including DNA methylation, are critically important mediators of normal cell function over the course of our lives. These modifications therefore also can play prominent roles in the development of disorders and diseases, including ovarian cancer. Genome-wide studies are now beginning to comprehensively decipher the methylome in normal and diseased tissues and cells, providing new insights into the distribution, specificity, and magnitude of modifications that occur and raising questions about these changes at specific loci. Further study of these alterations in specific tissues usually involves targeted approaches, of which there are a number available, all with distinct advantages and disadvantages. Here we provide a brief overview of DNA methylation and some of the methylation alterations that have been identified in ovarian cancer, as well as some of the technical approaches used to study these modifications.

Key words DNA methylation, Ovarian cancer, Bisulfite conversion, Methylation-specific PCR, Bisulfite sequencing, Pyrosequencing

1 DNA Methylation as a Biological Phenomenon

There are several major forms of epigenetic gene regulation that can become deregulated and contribute to the initiation and progression of a number of human diseases, including noncoding RNAs, histone modifications, and DNA methylation, which together interact to remodel chromatin. DNA methylation is a powerful form of gene regulation that plays a crucial role in normal mammalian development [1, 2]. The ability to methylate DNA extends to bacteria and plants, and this modification is involved in many gene regulatory systems. Cross-species comparative epigenomic analyses have revealed intriguing trends for both conserved and divergent features of DNA methylation in eukaryotic evolution. In mammals, DNA methylation is critical for the proper function of the genome, including genomic imprinting, X-chromosome inactivation, and transposon silencing and for controlling the expression of endogenous genes.

Patterns of methylation are stably maintained through somatic cell division and may even be inherited across generations. These patterns are sometimes perturbed in important human diseases, such as those associated with imprinting disorders and cancer [3–5].

Genome-wide studies are beginning to elucidate complex methylation profiles present within the human genome, and how changes in these profiles are associated with disease. DNA methylation occurs primarily at cytosine-guanine (CG) dinucleotide pairs, at the 5-carbon position of the cytosine base. About 70–80 % of the CG dinucleotides throughout the genome are methylated. DNA methyltransferase enzymes are responsible for the covalent transfer of a methyl group to cytosine using S-adenosylmethionine as the methyl group donor. CG dinucleotides are overall depleted throughout the genome, thought to be due to endogenous deamination of methylated cytosines to thymines over the course of evolution [6]. In spite of this relative depletion of CG dinucleotides, there are regions of the genome where dense clusters of CGs exist; these are often unmethylated and associated with the transcription start sites of genes [7]. Within gene bodies, CG methylation appears to be favored in exons over introns. Although the biological function of gene body methylation or mechanisms by which gene bodies are targeted by the methylation machinery are not well-understood, the preferential methylation of exons in plant and animal species appears to be an evolutionarily conserved phenomenon [8].

2 Alteration of DNA Methylation and Ovarian Carcinogenesis

In many types of cancer, DNA methylation is altered such that regions of the genome that normally exhibit methylation, such as repetitive elements, become hypomethylated [9] while other regions of the genome that are normally unmethylated, including promoter regions of tumor suppressor genes, acquire methylation [10]. The stability and somatic heritability of DNA methylation make it an attractive molecular biomarker for risk assessment, early disease detection, prognosis, and prediction of response to therapy. DNA methylation may also be useful for disease monitoring and for the identification of new therapeutic targets in cancer.

A preliminary study of DNA methylation in specimens from 50 women with ovarian cancer analyzed matched preoperative serum, tumor tissue, and ascites/peritoneal washings and reported that one or more of the tumor suppressor genes analyzed (breast cancer 1, early onset (*BRCA1*), RAS association domain family member 1, isoform A (*RASSF1A*), adenomatous polyposis coli (*APC*), *p14^{ARF}*, *p16^{INK4a}*, and death associated protein-kinase (*DAPK*)) showed methylation in all 50 of the tumors, regardless of histology, stage, or grade of the tumor [11]. Serum from 41 of

the patients showed an identical pattern of hypermethylation as that detected in the tumors. The peritoneal fluid from 27 of 29 women also showed the same methylation profiles as the matched tumor. These findings suggest the ability to noninvasively detect alterations in methylation that are associated with the disease. Furthermore, the serum methylation profiles from all eight women with stage I disease matched that of the tumor. None of the tissues examined from 40 control women (serum, non-neoplastic tissues, or peritoneal fluid) showed hypermethylation of these genes. This study therefore indicates that, at least for this subset of genes, methylation changes occur early in the disease process. This study supports the utility of using DNA methylation profiles for early detection of epithelial ovarian cancer using specimens obtained through relatively noninvasive means.

3 Assessment of DNA Methylation Status in Ovarian Cancer Diagnostics and Therapy

Discovery and quantitation of methylation changes offer great potential for disease detection and prognosis and may also impact individualization of therapies used to treat the disease [12]. The fast-paced development of technologies for assessing DNA methylation has provided powerful means to also monitor changes in these profiles. Abnormal DNA methylation of CpG islands in the promoter region of tumor suppressor genes in ovarian cancer is well established as a common mechanism of gene silencing and serves as an alternative to genetic mutation to abrogate gene function. Classic tumor suppressor genes, including *BRCA1*, *p16^{INK4a}*, *mutL*, *E. coli* homolog of 1 (*MLH1*), and *RASSF1A*, and many more have been identified as hypermethylated with associated loss of expression in ovarian cancers [13–18]. Thus far, only limited numbers of genes have been shown as specifically aberrantly methylated in tumor cells based on histologic origin or other common features. For example, *BRCA1* methylation is believed to be restricted to ovarian and breast cancers [19]. However, DNA methylation biomarkers that are specific to epithelial ovarian cancer remain to be discovered.

The methylation status of individual genes has been investigated for potential prognostic use in ovarian cancer. Studies have shown an association between DNA methylation of insulin-like growth factor-binding protein 3 (*IGFBP-3*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *BRCA1*, and *MLH1* and increased risk of ovarian cancer progression [20–23]. Determining a methylation signature that can predict time to relapse and/or overall survival would greatly impact individualized care regimens. Understanding and overcoming resistance to chemotherapy is central to improving survival of ovarian cancer patients.

Several studies in breast and ovarian cancers have shown that *BRCAl* is hypermethylated in 10–15 % of sporadic ovarian tumors and that hypermethylation is associated with the loss of RNA and protein expression. Ovarian cancer patients with *BRCAl* hypermethylation have better survival [24], which may be due to reduced ability to repair DNA damage induced by chemotherapy, thereby improving clinical response to treatment [25]. However, the low frequency of *BRCAl* methylation in epithelial ovarian cancers suggests that genome-wide screening for additional informative DNA methylation targets will be required.

4 Bisulfite Modification as an Essential Step for DNA Methylation Analysis

Sodium bisulfite has differential effects on unmethylated and methylated cytosines that lead to deamination of unmethylated cytosines with conversion to uracils under acidic conditions. Methylated cytosines are not affected, enabling discrimination of methylated from unmethylated cytosines throughout the converted DNA. Bisulfite treatment has played a pivotal role in the analysis of DNA methylation. Successful bisulfite modification is essential for ensuring accuracy in assessing DNA methylation. Incomplete bisulfite conversion is the single largest confounder of bisulfite-based methods of DNA methylation analysis [26]. To achieve high bisulfite conversion, it is important that the DNA is of good purity and that appropriate reagents and procedures are used for bisulfite modification. We have found excellent performance, ease of use, and consistently high conversion rates using commercially available kits, such as those offered by Zymo Research or Qiagen. We typically treat 500–800 ng of DNA with sodium bisulfite, which can provide sufficient template for up to 40 PCR reactions (25 μ l reaction volume) in our hands.

There are several important considerations regarding the treatment of genomic DNA with sodium bisulfite. Incubation of genomic DNA with sodium bisulfite is a harsh chemical treatment and leads to DNA fragmentation. Depending on the intended application, this fragmentation may hinder the downstream assays being performed. This may be alleviated in part by adjusting the time of the incubation step of the DNA with the sodium bisulfite. Several companies have recently developed kits for sodium bisulfite modification that substantially reduce this incubation time, which should lead to decreased DNA fragmentation. Use of shorter incubation times should be accompanied by methods to confirm the conversion efficiency. DNA extracted from formalin fixed, paraffin-embedded (FFPE) tissues presents an even greater challenge since the formalin fixation also leads to fragmentation of the DNA before the bisulfite treatment. Reversal of the DNA-protein cross-links followed by ligation of the DNA fragments has been successfully used to generate data from FFPE specimens on the Illumina

Infinium platform [27]. This platform utilizes technology requiring a single-base extension of the bisulfite-modified DNA so the mosaicism generated by performing ligation of DNA fragments from the entirety of the genome is not an issue, but it does require DNA fragments of at least 1 kb to work effectively. Another consideration is that DNA extracted from buccal swabs or saliva is often contaminated with substantial amounts of bacterial DNA. It is therefore important to determine that there is sufficient human DNA present in the specimen to ensure an adequate quantity for bisulfite treatment and your intended application.

5 Methods of DNA Methylation Analysis

Three PCR-based techniques for targeted analysis of DNA methylation that utilize bisulfite-modified DNA are described in the following chapters.

The first technique we describe is methylation-specific (MS) PCR, which is a relatively fast and inexpensive method for generation of qualitative (methylation present or absent) data for the region analyzed. We then describe a protocol for bisulfite sequencing of cloned alleles, which is useful for capturing the methylation status of each individual CG dinucleotide within the region studied. This method can also provide semiquantitative information about the number of alleles showing a given methylation profile and allow for detection of methylation patterns across alleles. Lastly, we describe bisulfite pyrosequencing, a quantitative method of determining the actual percent methylation of each CG within the sequence analyzed for the DNA specimen under study. There are many variations of these protocols that are beyond the scope of this chapter. We describe our preferred methodologies.

In traditional MS-PCR, two sets of primers are designed for the amplification of methylated or unmethylated DNA followed by gel electrophoresis. MS-PCR is a simple, fast, sensitive, and specific method for determining the methylation status of virtually any CG-rich region [28]. The use of PCR as the step to distinguish methylated from unmethylated DNA in MS-PCR allows for a significant increase in the sensitivity of methylation detection and thus can be used with limiting amounts of DNA. In addition, MS-PCR is suitable for the analysis of large numbers of samples as it can be carried out in a 96-well plate format. Although many newer methods have been developed, MS-PCR is still a popular technique used for analysis of DNA methylation. However, disadvantages of MS-PCR include the low quantitative accuracy and the increased risk for false positives. MS-PCR can indeed be performed in a more quantitative fashion by incorporating use of fluorescent hydrolysis probes (MethyLight) or fluorescent dyes (such as SYBR) that enable real-time detection of the MS-PCR amplification products. The tremendous amplification of the starting template during

PCR can also lead to inaccuracies in estimations of the level of methylated or unmethylated DNA present, especially when the reactions are allowed to plateau due to exhaustion of reagents or primers. Use of fewer PCR cycles may help prevent this type of discrepancy but also decreases the analytical sensitivity of the assay. Use of appropriately designed primers and a higher annealing temperature may also help prevent false-positive events.

Bisulfite sequencing of cloned alleles offers a more quantitative approach to methylation analysis as compared to MS-PCR. Sequencing of individual cloned alleles provides the methylation status for individual molecules and provides a measure of the proportion of alleles showing methylation at each CG cytosine when sufficient numbers of clones are sequenced. However, sequencing of single alleles can be time-consuming because it requires the selection and analysis of multiple clones, independent PCR, and sequencing reactions for each and can be costly when used as routine methodology.

Another quantitative approach that is more amenable to high-throughput analysis is bisulfite pyrosequencing, which utilizes pyrophosphate, released after each nucleotide is incorporated, to drive an enzymatic cascade that results in the luciferase-catalyzed conversion of luciferin to oxyluciferin and production of light. The light produced as the end result of this reaction is directly proportional to the number of nucleotides incorporated and hence the number of template molecules present in the reaction mix. Bisulfite pyrosequencing therefore yields quantitative information regarding the methylation status of single CG sites. Pyrosequencing requires careful primer design since the reaction is carried out at relatively low temperature, and the assays should be tested to insure that there is not substantial bias in the amplification efficiency of methylated versus unmethylated template DNA for the primer pair used. As with any bisulfite-based method of DNA methylation analysis, pyrosequencing also requires complete bisulfite conversion, which in part depends on high purity DNA. Unlike the other methods of analysis, bisulfite pyrosequencing incorporates bisulfite conversion controls into every sequence run, thus providing an internal control for each template to check the efficiency of the conversion. This method can be performed using a small amount of DNA (we use 40 ng of template DNA per pyrosequencing reaction, assuming complete recovery of the bisulfite-modified DNA). The main disadvantages of this method are the requirement for specialized instrumentation and the limitation of the DNA length analyzed (~115 bp maximum) so that only a limited number of CG sites are included within the sequence analyzed. However, because of the highly quantitative nature of this method coupled with the precision it affords and the reliability of the data, pyrosequencing is widely used by many research laboratories.

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