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Methods for genome-wide analysis of DNA methylation in intestinal tumors

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Abstract

Recent studies show that colorectal cancer is strongly associated with aberrant DNA methylation, which has been linked to the origin and progression of the disease. This fact indicates a need for deep analysis of DNA methylation alterations during colorectal carcinogenesis. The knowledge obtained from such studies will elucidate the mechanisms of epigenetic changes and, through the identification and characterization of DNA methylation markers and disease-specific methylation patterns, will help improve the diagnosis and treatment options for patients. The introduction of new methods for genome-wide analysis of DNA methylation has been an important step towards achieving these goals. In this review, we discuss the role of DNA methylation in intestinal carcinogenesis as well as the different methodological approaches that are currently being used for methylation analysis on a genome-wide scale.

Keywords

DNA methylation; epigenetics; colon cancer; methylated-CpG island recovery assay; MIRA

1. DNA methylation patterns

Carcinogenesis is commonly linked to aberrant DNA methylation patterns. In mammals, DNA methylation occurs at cytosines in CpG dinucleotide sequences. Since methylated cytosine is prone to deamination and conversion to thymine, most of the CpG dinucleotides have been lost over evolutionary time while others were retained in specific areas of the genome and have formed CpG rich regions, the so-called CpG islands. Approximately 60% of all human promoters are associated with CpG islands. In the genome of untransformed cells, ~90% of all promoters are unmethylated [1]. In cancer, promoters of tumor suppressor genes frequently become silenced, and this process is often associated with aberrant DNA hypermethylation of CpG islands overlapping the promoters. In addition to tumor suppressors, a variety of other genes acquire the aberrant DNA methylation mark in promoter regions during carcinogenesis. To this group belong genes regulating cell cycle progression, apoptosis, response to growth factors, DNA repair, invasiveness and

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transcription factors functioning during development, such as homeobox genes [2–4]. It was shown that DNA methylation frequently occurs in tissue-specific silent genes [1,5,6]. This fact raises the question if DNA methylation follows gene inactivation, or if in some way DNA methylation can directly induce gene silencing. For example, comparison of DNA methylation in normal and malignant prostate cells indicated a group of genes, which become silent and methylated during malignant transformation [6]. Whereas in normal cells most of the CpG islands are unmethylated, CpG dinucleotides located outside of CpG islands are usually methylated. In addition to CpG island hypermethylation, loss of genome-wide DNA methylation frequently takes place in cancer. These epigenetic changes leading to loss of methylation occur most significantly in repetitive elements and may result in genome instability, in an increased number of mutations and in promotion of tumor formation [7–11].

2. Genetic defects in colorectal cancer

Every year, approximately one million people are diagnosed with colon cancer and around 500,000 patients die from this disease according to the WHO [12]. In 88–94% of colon cancer patients, colon cancer develops sporadically due to different risk factors such as sex, age, lifestyle, diseases, diet, environment, hormonal factors and personal history of sporadic tumors. Only 5–10% of colon cancer cases are associated with inheritance and are predisposed by syndromes like hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). Chronic inflammation caused by ulcerative colitis and Crohn's disease is responsible only for 1–2% of colon cancers. It is assumed that the adenoma-carcinoma sequence is linked to accumulation of genetic and epigenetic alterations important during development of colorectal cancer. Genomic instability observed in colon cancer divides cancer development mechanisms into two commonly suggested pathways: chromosomal instability (CIN) and microsatellite instability (MSI) [13]. Carcinogenesis in familial adenomatous polyposis and in 85% of sporadic cancer cases is associated with CIN caused by diverse mutations or epigenetic changes of tumor suppressors and oncogenes [12,14–17]. In contrast, MSI originating from inactivation of mismatch repair genes is responsible for hereditary nonpolyposis colorectal cancer and for 15% of sporadic cancers. Germline mutations of mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*, or *MSH6*) cause HNPCC [18–23]. In contrast, in sporadic colon cancer, MSI is frequently associated with epigenetic silencing of *MLH1*, which is an early event in adenoma formation [24–29]. DNA hypomethylation is also involved in colon cancer and is associated with genomic instability. Colorectal cancer is linked to LINE-1 hypomethylation, which is linearly associated with a decreased survival rate of patients [30–32]. Analysis of genome-wide DNA methylation in colorectal cancer indicated that DNA hypomethylation is associated with CIN [33]. Later, it was observed that hypomethylation of LINE-1 is inversely associated with MSI [34].

3. DNA methylation in colon cancer

During the past decade, aberrant DNA methylation has become established as a marker for cancer initiation and progression. Hundreds of CpG islands become specifically methylated in most types of human cancer and in particular stages of cancer progression. The search for such DNA methylation markers and their characterization may have great importance for cancer diagnosis and for the identification of potential tumor suppressors.

Aberrant DNA hypermethylation plays an important role in colorectal cancer and is responsible for silencing of a number of key genes including p16, p14 and *APC* [35–37]. In addition to cancer-specific DNA methylation, age-dependent DNA methylation is frequently observed in colon tumors [38]. During aging, genes like *ERα*, *MYOD* and *IGF2* accumulate

aberrant DNA methylation in colonic epithelium, which is also found in neoplasia [39–42]. Additionally, cancer-related changes, such as promoter methylation of *MLH1*, and p14 can be detected in aged tissues, which indicate a role of aging in colonic neoplasia [43,44]. Epigenetic changes divide colon cancer into two phenotypes, the so-called CpG island methylator phenotypes (CIMP), CIMP+ and CIMP– [42].

CIMP+ cancers are characterized by aberrant DNA methylation of a large number of loci, which are characterized by cancer-specific DNA methylation. CIMP– is associated with very low numbers of these loci affected by aberrant DNA methylation. CIMP+ is correlated with the methylation status of p14, p16, *THBS1* and *MLH1* [42,44]. It was shown that CIMP+ is frequently present in older patients with poorly differentiated malignancies [45–47]. CIMP+ is associated with MSI and low CIN abnormalities suggesting that CIN and CIMP+ are established by different mechanisms [48,49]. Development of the CIMP+ phenotype takes place in very early stages of cancer development and is detected in adenomatous polyps and aberrant crypt foci [42,50,51]. As mentioned earlier, colon cancer can be caused either by CIN or by MSI [13]. However, colon tumors without CIN and MSI were also observed [49,52]. Analysis of CIMP in these tumors revealed CIMP+, which indicates an alternative pathway of colon cancer development [49].

Based on these facts, three main pathways of development of sporadic colon cancer were postulated by Issa [53]. The first pathway is responsible for development of serrated adenoma in the proximal colon and is associated with CIMP+ and *BRAF* mutation. Further, MSI caused by *MLH1* silencing results in colon cancer with good prognosis. The second pathway avoids genomic instability and is initiated with CIMP+ establishment and with mutations of *KRAS* and *APC* causing precursor lesions including villous adenoma. In this pathway, further establishment of aberrant DNA methylation due to CIMP results in colon cancer with poor prognosis. The third pathway of colon cancer development is caused by *APC* mutation, which leads to tubular adenoma formation and *TP53* mutation with CIN. This pathway is associated with typical colon cancer in the distal colon. Therefore, two (first and second) of the three pathways leading to sporadic colon cancer are associated with aberrant DNA hypermethylation and the third pathway is characterized by CIN, which is linked to DNA hypomethylation of the genome [33,53]. These correlations suggest that aberrant DNA methylation plays a central role in colorectal cancer. Further investigations must elucidate the mechanism of establishment of these aberrant methylation changes in colon cancer and how they promote the malignant phenotype.

Since DNA methylation patterns in colon tumors with diverse origin are different, establishment of subtype-specific DNA methylation markers can help in diagnosis and in stratification of treatment. Discovery and establishment of these markers can be accomplished by genome- wide analysis of DNA methylation patterns in patients. The main approaches for this analysis are discussed in the following sections.

4. Overview of genome-scale analysis of DNA methylation

Malignant transformation is associated with extensive changes in DNA methylation patterns of the genome. Identification of these changes plays a crucial role in elucidation of mechanisms responsible for carcinogenesis and will be important for developing new diagnostic tools. There are four major groups of methods which have been applied for detection of these epigenetic changes at the genome-scale level: (1) methods based on reactivation of methylated promoters after 5-aza-2'-deoxycytidine (5-aza-dC) treatment, (2) methods based on the ability of restriction enzymes to cut or not cut methylated DNA, (3) methods based on the affinity of certain antibodies or proteins for 5-methylcytosine, and (4)

methods based on conversion of unmethylated cytosine into uracil after bisulfite treatment of genomic DNA [54–63]. These four principles are outlined in Figure 1.

5. Reactivation of genes with 5-azacytidine

The first approach is based on inhibition of DNA methyltransferase activity due to formation of a stable complex and trapping of DNA methyltransferase by 5-aza-dC incorporated into DNA [64]. Since incorporation of 5-aza-dC into DNA results in DNA demethylation followed by reactivation of demethylated promoters, genes silenced by DNA methylation can be detected by the identification of gene transcripts reactivated by 5-aza-dC treatment (Figure 1A) [63,65,66]. This approach is in fact one of the first methods that has been employed for detection of aberrant DNA methylation and was used to demonstrate a role of DNA methylation in silencing of the X-chromosome [65,66]. Today, the usage of expression arrays allows one to analyze gene reactivation after 5-aza-dC treatment genome-wide [63,67]. However, this approach has limitations since only a limited number of hypermethylated genes undergo reactivation after 5-aza-dC treatment [68]. Also this method can reasonably be applied only for cell culture. 5-Aza-dC is a toxic agent with a short life span in buffered solutions [69,70].

6. Methods based on methylation-sensitive restriction enzymes

Over several decades, scientists have tried to analyze changes in DNA methylation patterns by using methylation-sensitive restriction enzymes. Restriction Landmark Genomic Scanning (RLGS) is one of the earliest approaches used for identification of multiple CpG islands undergoing DNA methylation changes [61,71]. Radioactively labeled genomic DNA is separated by 2D gel electrophoresis after digestion with methylation-sensitive restriction enzymes, such as NotI. The radiograms displaying radioactively labeled spots on the gel are compared to control radiograms and changes in patterns of spot intensity indicate DNA methylation differences between the analyzed sample pairs, e.g. tumor versus normal tissue. DNA spots of interest can be isolated and sequenced.

One other method for DNA methylation profiling, which uses methylation-sensitive restriction enzymes and gel electrophoresis, is Amplification of Inter-Methylated Sites (AIMS) [59]. In this approach, genomic DNA is digested with SmaI, a methylation-sensitive restriction enzyme, which generates blunt ends after restriction. The next step is a second restriction with the methylation-insensitive isoschizomer of SmaI, XmaI, which generates sticky ends. These sticky ends are ligated with linkers and amplified by ligation-mediated PCR. Therefore only DNA fragments located between two methylated XmaI sites can be amplified (as shown in Figure 1B). PCR products from different samples are resolved by denaturation gel electrophoresis and compared to each other. Since ladders with different patterns in different samples indicate DNA methylation differences between samples, these ladders can be studied in detail by band isolation and sequencing.

The advantage of SmaI and its isoschizomer XmaI for enrichment of DNA fractions with methylated DNA was employed in another technique, Methylated CpG island Amplification (MCA) introduced earlier by Issa and colleagues [72]. MCA is based on amplification of genomic DNA after SmaI-XmaI digestion followed by linker ligation to XmaI sites. In this approach, detection of methylated CpG islands is performed by combination of MCA with a subtractive technique, representational difference analysis (RDA) using the tester and driver principle [72,73]. Later, the combination of the MCA technique with microarrays simplified this approach for DNA methylation analysis [74].

With the introduction of microarrays, several techniques have been developed for genome-wide DNA methylation analysis based on methylation sensitive enzymes. Examples include

HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) and Differential Methylation Hybridization (DMH) [56,57,60]. The HELP method is based on differential microarray hybridization of genomic DNA after either HpaII digestion or MspI digestion followed by ligation-mediated PCR amplification [57]. DNA regions characterized by differential hybridization on arrays are differentially methylated. HELP has the advantage of avoiding amplification of repetitive DNA sequences.

In contrast to HELP, DMH is based on enrichment of only the hypermethylated DNA fraction and comparison of two different samples on the array [56]. For DMH, sonicated DNA after linker ligation is digested with two DNA methylation sensitive enzymes, HpaII and HinP1I and amplified using linker primers. In this case, only DNA fragments with methylated HpaII and HinP1I sites will be amplified, while unmethylated DNA will be digested and will lack one of two or both linkers for PCR amplification. Therefore, enrichment of the DNA fraction after ligation-mediated PCR will reflect the methylation state of restriction sites of these enzymes and differential hybridization of different samples on two-dye arrays will reflect differences in DNA methylation patterns.

Most methods for DNA methylation analysis based on methylation-sensitive restriction enzymes have limitations due to possibly incomplete DNA digestion and due to the location of the restriction sites. For example, only 3.9% of CpGs located in nonrepeat sequences can be recognized by HpaII [75]. Therefore, information about some DNA regions will be missed if these regions have no particular restriction sites or have too big a distance between the restriction sites to be amplified by PCR.

To overcome this problem, several new approaches have been developed recently. Introduction of McrBC, a methylation-specific restriction enzyme system for DNA methylation analysis, has eliminated many of the limitations associated with methods based on methylation-sensitive DNA restriction, since McrBC has the ability to digest methylated DNA, generates overlapping DNA fragments and has no requirement for a highly specific recognition site. This endonuclease system cleaves DNA containing two recognition sites (G/A)C^m within distances between 22 bp and 3 kb with optimal separation between the two sites of 55–103 base pairs [76,77]. However, the exact restriction sites of McrBC are unknown, which reflects a potential weakness of the methods using this enzymatic system. In the different approaches for genome-scale DNA methylation analysis, McrBC can be used alone or in combination with other endonucleases followed by microarray hybridization [78–81]. For example, in a method developed by Lippman and colleagues, McrBC is used alone for exclusion of the methylated DNA fraction from genomic DNA and is further compared to undigested DNA [80].

7. Methods based on sodium bisulfite conversion of cytosine

For analysis of genomic DNA methylation patterns, an alternative for methylation-dependent restriction is the usage of the differences between methylated and unmethylated cytosine after bisulfite conversion (Figure 1C).

For example, the GoldenGate® assay for DNA methylation profiling is based on a single nucleotide polymorphism (SNP) genotyping system for C/T polymorphisms, which occur after bisulfite treatment of CpGs and depends on the methylation status of these dinucleotides [62,82]. The GoldenGate® assay approach provides information about the methylation status of up to 1536 targeted CpG sites from 371 genes [82,83]. For each analyzed CpG island, the methylation status of only a few CpGs is examined by using this technology. The approach is based on the assumption that the methylation status of the analyzed CpGs reflects the methylation status of adjacent CpG sites, which is often the case

[84,85]. In this application, bisulfite-treated DNA is amplified with primers specific to unmethylated or methylated alleles, respectively. PCR products of methylated and unmethylated alleles are differentially labeled and hybridized to a universal IlluminaCode array. The methylation level of the analyzed locus is determined by comparison of signals from methylated and unmethylated alleles in the analyzed sample.

An alternative for the GoldenGate® assay methodology is a highly informative newly developed method for whole genome sequencing of bisulfite-converted DNA [86–88]. In contrast to GoldenGate® technology, this approach provides information about the DNA methylation status of each CpG. However, this methodology has limitations since after bisulfite treatment unmethylated DNA consists chiefly of three DNA bases (A, T and G). This fact is responsible for an enormous ‘bioinformatics’ challenge related to the analysis of bisulfite treated DNA sequencing data. A similar problem is the main technical hurdle for development of microarray-based approaches for analysis of DNA methylation genome-wide after sodium bisulfite conversion. Reduced representation bisulfite analysis or potential target enrichment approaches for bisulfite-converted DNA will provide single base resolution methylation data for specific genomic loci, e.g. CpG islands, but will not readily allow analysis of the entire mammalian genome [87].

8. Methods based on antibodies or proteins that bind to methylated DNA

The last group of methods for detection of DNA methylation changes on a genome-wide scale is based on the high affinity of antibodies or proteins specifically binding to methylated DNA (Figure 1D).

Methylated DNA immunoprecipitation (MeDIP) is a new method, which was developed based on the affinity of specific antibodies to methylated cytosine [55]. In this approach, sheared genomic DNA is heat-denatured and incubated with monoclonal antibodies specific to 5-methylcytosine. The methylated DNA-antibody complex is captured by magnetic bead separation. Purified methylated DNA can be used for analysis of the methylation status of specific DNA regions by PCR reactions or for analysis of genome-wide DNA methylation patterns by microarray approaches or high-throughput sequencing. The MeDIP approach has been applied for DNA methylation profiling in human tissues and in *Arabidopsis thaliana*, and for identification of cancer-specific DNA methylation changes [55,89,90].

In contrast to MeDIP, the Methylated-CpG Island Recovery Assay (MIRA) uses a protein complex rather than antibodies for purification of methylated DNA. This method is based on the high affinity of the complex of two proteins, MBD2B and MBD3L1, to methylated DNA. In this complex, MBD2B binds to methylated DNA and MBD3L1 serves as an enhancer of MBD2B binding [2,91]. Among methyl-CpG-binding proteins, MBD2B has the highest affinity to methylated DNA and the greatest capacity to differentiate (30–130 times) a methylated and unmethylated DNA sequence [92]. Affinity of MBD2B to methylated DNA is increasing with the density of methylated CpGs in the DNA sequence. As a heterodimerization partner of MBD2B, MBD3L1 lacks the methyl-CpG binding domain and has no ability to bind methylated DNA by itself [91,93]. However, in complex with MBD2B, MBD3L1 strongly increases the affinity of MBD2B to methylated DNA [58].

In the MIRA procedure, the methylated fraction of genomic DNA is isolated by incubation of sonicated genomic DNA with MBD3L1-His- and MBD2B-GST-tagged proteins and further isolation of the DNA-MBD3L1-MBD2B-GST complex by usage of glutathione-coated magnetic beads (Figure 1D). This method has high sensitivity and has no need of large amounts of starting material in contrast to most other methods for genome-scale DNA methylation analysis. Presence of MBD3L1 in the complex allows detection of methylated

DNA by PCR after MIRA by using only 1 ng of input genomic DNA. This complex lacks a defined sequence specificity, which was confirmed by cloning and random sequencing of MIRA-enriched DNA fractions in our laboratory and is able to enrich DNA sequences with two or more methylated CpGs effectively [2]. Analogous to MeDIP, MIRA is independent of locations of restriction sites, which gives MIRA and MeDIP an advantage in comparison with the techniques using restriction enzymes. However, in contrast to the MeDIP method, which uses single-stranded DNA, the MIRA assay requires double-stranded DNA for MDB2B-MBD3L1 binding [58,94]. In contrast to the bisulfite sequencing approaches, both methods provide information about DNA methylation for specific DNA loci at a resolution of approximately 50–100 bp and not for each individual CpG, but both methods can readily be applied for genome-scale analysis.

The combination of the MIRA method with microarrays has opened new opportunities to study DNA methylation genome-wide [1–3,5,95]. Using this approach, the analysis of the whole human genome DNA methylation pattern in CD19+ B cells was performed at a resolution of 100 bp [1]. It was found that ~10% of all promoters are methylated in B cells and an important correlation between DNA methylation in gene bodies and gene expression, and between DNA methylation and Giemsa staining of chromosomes, was uncovered [1]. Using the MIRA-assisted microarray method for analysis of lung cancer specimens, 20 lung cancer biomarkers were identified, which are specifically methylated at high frequency in lung cancer and unmethylated in matching normal tissue [3]. The same approach revealed hypomethylation of repetitive elements and frequent methylation of homeobox genes in lung cancer [2,3,95].

9. DNA methylation in intestinal inflammation and cancer

In our laboratory, MIRA assisted microarray analysis was used to identify a role of inflammation and aging in intestinal tumorigenesis of glutathione peroxidase, Gpx1 and Gpx2, double knockout mice (Gpx-1/2-KO mice) [5]. These mice are model animals for inflammatory bowel disease [96–98]. In Gpx1/2-KO mice, ileocolitis, which begins around weaning and is caused by a non-germ-free environment, leads to chronic intestinal inflammation and later on to tumor development [96,98]. Analysis of DNA methylation in Gpx1/2-KO mice revealed hundreds of genes, which become methylated during aging and inflammation.

Analysis of the methylated targets showed distinct differences between the methylation patterns established during aging and inflammation. Approximately 60% of the genes methylated in intestinal tumors were methylated in inflamed tissues in contrast to only 10% of tumor-associated DNA methylation, which correlated with age-dependent DNA methylation. This fact indicates a role of inflammation in establishment of aberrant DNA methylation patterns, which is observed during intestinal tumorigenesis. Comparison of age-dependent and inflammation-dependent DNA methylation also showed differences in the role of Polycomb proteins and localization of methylated CpG islands relative to transcription start sites in establishment of DNA methylation during these processes. This fact was indicated by more frequent recruitment of Polycomb proteins during embryonic development to CpG islands, which become methylated later during inflammation (~70%) rather than during aging (~50%). Also in our animal model, inflammation-dependent DNA methylation occurred more frequently in promoter regions than age-dependent DNA methylation.

The comparison of inflammation-dependent DNA methylation patterns and the histone H3 lysine trimethylation (H3K27me3) binding pattern after chromatin immunoprecipitation and microarray revealed that the Polycomb repressive mark, H3K27me3, is present in ileum and

predisposes to aberrant DNA methylation during inflammation. Previously, the same mechanism was proposed for aberrant DNA methylation in cancer where Polycomb targets in embryonic stem cells were frequently observed methylated in cancer [2,95,99–101]. Our analysis of the H3K27me3 changes during inflammation revealed that an increase of DNA methylation is often accompanied by loss of the Polycomb repressive mark, H3K27me3 during inflammation. A similar phenomenon of exchange of the unstable repressive mark, H3K27me3, for the more stable mark, DNA methylation, was reported in the prostate cell line PC3 [6]. These data suggest that inflammation-dependent DNA methylation and tumor-associated DNA methylation are driven by the same epigenetic mechanism in the intestine.

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Figure 1. Methods representing four different principles of genome-wide DNA methylation analysis

A. Detection of gene promoters silenced by DNA methylation by using 5-Aza-dC for gene reactivation. **B.** Analysis of DNA methylation by use of restriction enzymes differentially digesting methylated and unmethylated DNA. A combination of the MCA method with a microarray approach is shown. **C.** Analysis of DNA methylation by bisulfite conversion. **D.** Analysis of DNA methylation changes by employing the high affinity of specific antibodies or proteins, which bind to CpG-methylated DNA. The Figure represents a chart-flow for the MIRA approach. MeDIP is based on a similar principle and uses antibodies specific to single-stranded methylated DNA.