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Emerging technologies for studying DNA methylation for the molecular diagnosis of cancer

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Abstract

DNA methylation is an epigenetic mechanism that plays a key role in regulating gene expression and other functions. Although this modification is seen in different sequence contexts, the most frequently detected DNA methylation in mammals involves cytosine-guanine dinucleotides. Pathological alterations in DNA methylation patterns are described in a variety of human diseases, including cancer. Unlike genetic changes, DNA methylation is heavily influenced by subtle modifications in the cellular microenvironment. In all cancers, aberrant DNA methylation is involved in the alteration of a large number of oncological pathways with relevant theranostic utility. Several technologies for DNA methylation mapping were recently developed and successfully applied in cancer studies. The scope of these technologies varies from assessing a single cytosine-guanine locus to genome-wide distribution of DNA methylation. Here, we review the strengths and weaknesses of these approaches in the context of clinical utility for the molecular diagnosis of human cancers.

Keywords

DNA methylation; cancer diagnosis; epigenomics; sequencing; microarray

DNA methylation and gene expression: A complex relationship that depends on the context

DNA methylation commonly refers to the covalent addition of a methyl ($-CH_3$) group from the s-adenosylmethionine (SAM) to the fifth carbon of the cytosine base (5mC) which is catalyzed by DNA methyltransferases (DNMTs) enzymes (Figure 1A). It is extensively demonstrated that DNA methylation plays a key role in chromosomal stability, gene expression, genome imprinting, and transcriptional silencing of foreign DNA fragments [1–

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3]. The most abundant DNA methylation in mammalian genomes occurs in cytosines that belong to CpG dinucleotides [4]. Nevertheless, non-CpG DNA methylation mainly occurring in CpHpG trinucleotides (where H can be adenine [A], thymine [T], or C) was also reported in healthy and pathological processes [5]. Interestingly, the human genome is particularly poor in CpG dinucleotides. While at least 132 million CpG dinucleotides are expected (the human genome is composed of ~21% C and ~21% G nucleotides; therefore, the probability of a CpG dinucleotide to occurs is 4.41%), only ~28 million (0.93%) are actually observed, of which 60–80% are generally methylated [4,6]. In fact, DNA methylation is considered a mutagenic event. Deamination of 5mC generates T, which in some cases are identified and removed by mismatch repair mechanisms. But, in other cases, these are considered wild type information and maintained in the mammalian genome. In consequence, the content of TpG dinucleotides in the human genome is higher than expected and presents a negative correlation with CpG abundance [7]. In addition to 5mC, other chemical modifications of C residues were recently identified. These modifications include 5-hydroxymethyl-cytocine (5hmC), 5-formyl-cytosine (5fC), and 5-carboxyl-cytosine (5caC) [8]. 5hmC, 5fC, and 5caC are generated by the successive oxidation of 5mC catalyzed by the ten-eleven translocation methylcytosine dioxygenases (TETs) (Figure 1A) [8]. Finally, 5fC and 5caC can be excised and repaired by thymine-DNA glycosylase (TDG) and base excision repair (BER) systems to generate unmethylated Cs (Figure 1A) [8].

The levels of gene expression are influenced by a multitude of factors, including DNA methylation. Initially, 5mC was proposed to interfere with transcription factor binding; however, its influence appears to involve more complex and indirect mechanisms in which methyl-cytosine binding domain (MBD) proteins recruit multi-protein complexes that ultimately induce chromatin conformation changes [9]. In this model, the strength of the repression would directly depend on the CpG concentration. Far from having a definitive answer, the relationship between DNA methylation and gene expression remains a topic of intense debate. Recent advancements in high-throughput DNA methylation mapping provided new insights about the complexity of this regulatory mechanism.

The CpG context

The distribution of CpG dinucleotides defines regions of importance for the epigenetic regulation of gene transcription. Genomic regions of at least 200 base pairs (bp), in which the G and C content accounts for at least 50% of the nucleotides with an observed/expected CpG ratio>0.6, are denominated ‘*CpG islands*’ [10]. Because of the previously mentioned spontaneous or enzymatically-mediated deamination of 5mCs to Ts, from an evolutionary point of view, it has been *hypothesized* that the conservation of CpG islands is due to absent or infrequent DNA methylation on CpG sites located at these regions [10]. In fact, CpG islands are usually unmethylated in normal cells, and its methylation only occurs in specific and well-controlled physiological situations such as development, differentiation, memory acquisition, genomic imprinting, and X chromosome inactivation [3,4,11–13]. DNA methylation at CpG islands, especially when overlapping gene promoters, was traditionally considered synonymous with gene expression silencing. However, in light of current studies, this statement may need to be reevaluated. In addition to CpG islands, the CpG context defines regions of lower CpG density located around the islands called ‘*CpG shores*’ (up to

2 kb away from the CpG island) and '*CpG shelves*' (2 to 4 kb away from the CpG island), and regions distant from CpG islands (>4 kb away from a CpG island), named by some authors as '*open sea*' (Figure 1B) [14,15]. In spite of a lowered CpG density, these regions have demonstrated to be functionally important for gene expression regulation. For example, CpG shores were found to be associated with cancer-, tissue, and reprogramming-specific differentially methylated regions (C-DMRs, T-DMRs, and R-DMRs) with mechanistic relevance for the expression of associated genes [15,16]. Furthermore, in cell differentiation, the methylation of CpG shores is more dynamic than the methylation of CpG islands, indicating a key role of CpG shore methylation on cell fate determination [16]. In addition, differential methylation of CpG shelves as well as open sea regions have been associated with hepatocellular carcinoma [17], and metastatic melanomas [18].

Gene structure and regulatory elements

The influence on gene expression levels also depends on the location of DNA methylation with regard to gene structures, specifically at promoter, intragenic, and intergenic CpG sites [19].

More than half of human genes contain CpG islands located upstream of their transcription start sites (TSSs). These regions, denominated promoter elements, were traditionally considered the regions where DNA methylation has the strongest influence on gene expression [20]. However, recent discoveries describe a more complex picture. Based on CpG ratio, CpG content, and length of CpG-enriched region, three classes of gene promoters have been recognized [21]. High-, intermediate- and low-CpG density promoters (HCPs, ICPs, and LCPs) represent strong, weak, and non-CpG island promoters, respectively. HCPs are usually unmethylated, even when the transcription of the associated gene is inactive. LCPs are usually methylated without having a blocking effect on the promoter activity. Yet, substantial fluctuation occurs in the DNA methylation level on LCPs according to the developmental stage of the cells. Conversely, ICPs are frequently methylated with a repressive effect on gene expression [21]. Interestingly, these ICP promoters were suggested to be targets of *de-novo* DNA methylation by DNMT3A and DNMT3B with a role in the microenvironment-induced fine-tuning of gene expression variations [22].

Novel evidence indicates that intragenic DNA methylation also influences gene transcription. For instance, methylation of CpG sites located in the 5′ untranslated region (5′UTR) inhibits gene transcription initiation [23]. Conversely, methylation of non-CpG sites located in the gene body can stabilize transcription elongation and influence the selection of alternative splicing sites [5,19]. In addition, methylation of 3′UTR regions is significantly associated with an increased gene expression rate [24]. Furthermore, 5hmC found in gene bodies is positively correlated with gene expression levels, and in contrast to 5mC, is also enriched in active enhancers and CpG-rich promoter regions but it is largely absent from intergenic regions [25].

DNA methylation of intergenic genomic intervals can affect the accessibility to *cis*- and *trans*-regulatory elements, such as enhancers and silencers, greatly influencing the cell-type specific gene expression programs [26]. For example, differences in DNA methylation

levels between neuronal and glial cells are concentrated in enhancer elements which contribute to cell type-specific gene expression programs [27]. Additionally, methylation of repetitive sequences such as *LINE-1* and *Alu* plays an important role in chromosomal stability, mitotic segregation, and suppression of transposable element expression [28,29]. As a general finding, in normal cells, promoter CpG islands present low or null DNA methylation levels, while intragenic and intergenic CpG sites are usually methylated (Figure 1C).

In summary, the relationship between DNA methylation and gene expression is highly complex, and in light of current information, the traditional concept of DNA methylation silencing gene expression by affecting CpG island promoters needs to be carefully reevaluated and expanded.

Role of DNA methylation in cancer: A misbalance between methylation and demethylation

As previously reviewed [30–33], aberrant DNA methylation interacts with genomic and other epigenomic alterations, such as histone modification and nucleosome positioning during tumor initiation and progression. The DNA methylation landscape of normal cells is a well-controlled balance between methylation and demethylation [33]. In this regard, we consider aberrant DNA methylation as deficient, excessive or ectopic methylation/demethylation. Aberrant DNA methylation is observed in both sporadic and inherited cancers; however, specific patterns can be identified in each case [34]. Even though several tumor type-specific DNA methylation alterations have been reported, an epigenetic hallmark for all human cancers appears to be global DNA hypomethylation and a loci-specific DNA hypermethylation [31]. Yet, the timing and the pattern of aberrantly methylated regions vary in different stages and cancer types [35]. Unlike the distribution of DNA methylation in normal cells, cancer cells present increased DNA methylation in CpG-rich regions and decreased DNA methylation in CpG-poor regions (Figure 1D). Traditionally, gene promoter CpG islands hypermethylation has been largely studied in cancer, however, new studies show that aberrant methylation in regions other than gene promoter CpG islands may potentially be novel theranostic markers [36].

In addition to gene-specific aberrant methylation, the characterization of tumor type- and stage-specific genome-wide DNA methylation patterns offers an opportunity to identify unexplored molecular biomarkers. Currently, the multi institutional cooperation effort with The Cancer Genome Atlas (TCGA) aims to unravel novel and functional DNA methylation alterations in a wide variety of human cancers [37]. TCGA has already analyzed the genome-wide DNA methylation patterns of >10,000 human specimens from 33 different tumor types and paired normal controls (https://tcga-data.nci.nih.gov/tcga/). This unprecedented large scale genomic, transcriptomic, epigenomic, and proteomic data, in addition to other independent laboratory efforts, represents a milestone in cancer research and a revolution in the translation of the ‘*-omic*’ era into clinical practices.

Clinical translation of DNA methylation research

The list of clinically-relevant DNA methylation alterations in human tumors is extensive. Examples about the role of DNA methylation markers associated with diagnostic, prognostic, and drug response for different cancer-types have been reviewed [31,32,38,39]. Here, we discuss the most relevant advancements on the clinical utility of DNA methylation mapping of specific human cancers.

Glioblastoma

Perhaps one of the most significant clinical translations of DNA methylation research in solid tumors has been studied in patients with glioblastoma (GBM). These malignant gliomas are the most common and deadly type of primary brain tumors. Nevertheless, survival of these patients has improved from 10 to 14 months after diagnosis in the last 5 years due in part to the identification of patients who benefit from specific treatment strategies [40]. For example, it is now well recognized that O-6-methylguanine-DNA methyltransferase (MGMT) silencing by DNA methylation predicts a better tumor response to the combination treatment of temozolomide with radiation [41]. In addition to its predictive value, *MGMT* methylation is associated with better overall survival, even in absence of temozolomide treatment [42]. This observation was recently confirmed by a randomized phase III clinical trial in which GBMs with *MGMT* methylation improved overall and progression free survival [43]. GBMs were one of the first tumor types explored by TCGA. With >500 surgically-resected untreated primary tumors, integrative analysis of TCGA data identified four major molecular subtypes of GBM [44]. One of those subtypes (pro-neural) contains GBMs with frequent mutations in Isocitrate Dehydrogenase 1 (NADP +), Soluble (IDH1) gene [44]. *IDH1* mutations are associated with a Glioma CpG Island Methylator Phenotype (G-CIMP), in which the global methylation level is significantly higher than that observed in non-G-CIMP GBMs [45,46]. Both G-CIMP and *IDH1* gene mutations are favorable prognostic markers for GBM [47]. More recently, a similar molecular classification scheme was developed using the same DNA methylation mapping approach [48]. This study incorporated two hotspot mutations on the H3 Histone, Family A3 (H3F3A) gene to the molecular subtypes [48]. Interestingly, the authors identified a significant mutual exclusivity between mutations in *H3F3A* and *IDH1* genes, defining a new GBM epigenetic subtype characterized by a global hypomethylation, which has been denominated Glioma CpG Hypomethylator Phenotype (G-CHOP) [48]. G-CHOP tumors present frequent H3F3A^{mut}G34 mutations, specific tumor development location in the brain, and a higher incidence of pediatric GBM [48]. Although the prognostic and predictive significance of these new findings have yet to be fully understood and validated, the identification of these novel and well-characterized molecular subtypes may translate into the development of new targeted therapies. The discovery of G-CIMP and G-CHOP is an example of the importance of performing on a genome-wide scale, as opposed to gene-specific DNA methylation analyses.

Colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed and fatal type of cancer in men and women worldwide [49]. CRC progresses slowly through histologically well-defined stages [50]. The progression to invasive and metastatic stages is prompted by the successive accumulation of mutations in genes that greatly contribute to genome instability reflected by microsatellite instability (MSI) [51]. In parallel, this process is accompanied by the accumulation of a multitude of aberrantly methylated gene promoters [52]. In sporadic CRC, ~15% of the cases display MSI caused by MutL Homolog 1 (MLH1) gene aberrant methylation [50]. Interestingly, *MLH1* methylation presents a significant mutual exclusivity with *MGMT* methylation [53]. Similar to GBM, ~40% of CRCs present transcriptional silencing of *MGMT* throughout aberrant DNA methylation [54]. However, unlike GBM, CRC patients with *MGMT* methylation have a lower response rate to temozolomide [55]. In a phase II clinical trial, patients with metastatic CRC and *MGMT* methylation showed favorable clinical response to dacarbizine [54]. Additionally, *MGMT* methylation is associated with G>A transitions, low MSI (L-MSI), and higher frequency of Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations [53,56]. CRCs can be also classified as high-CIMP (H-CIMP), low-CIMP (L-CIMP), and CIMP-negative [57,58]. Patients with H-CIMP present tumors with frequent proximal colon location, high-MSI (H-MSI), frequent v-raf murine sarcoma viral oncogene homolog B (BRAF) V600E mutation, and low frequency of *MGMT* methylation [59,60]. A recent study reveals that *BRAF* (V600E) mutation is responsible for H-CIMP and *MLH1* aberrant methylation through the activation of the transcriptional repressor v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (MAFG) [61]. This study represents an example of the link between ‘*driver*’ genetic alterations and genome-wide DNA methylation patterns. Additionally, our studies indicate that global hypomethylation, assessed by the methylation status of LINE-1, is an early event in the adenomatous change of colorectal cells and the level of LINE-1 hypomethylation increases with the stage of disease [62,63].

During the past decade, a large number of locus-specific DNA methylation alterations were associated with clinical features of CRC in tumor tissue as well as in blood and stool cell-free DNA [62,64–74]. More recently, a new set of genome-wide DNA methylation signatures were identified with potential prognostic value in CRC [75–78]. Integrative TCGA analyses also revealed new DNA methylation markers with clinical utility for aggressive CRC [79]. Advancements in DNA methylation mapping and analysis techniques will continue to expand discoveries for colorectal-specific biomarkers, and hopefully theranostic markers in order to improve disease management.

Breast cancer

Breast cancer (BC), the most frequent tumor diagnosed in women [49], is often considered a single disease, but it is in fact a compilation of different types of tumors affecting the mammary gland. Molecular stratification based on gene expression profiles has improved the treatment strategy of BC patients [80]. However, large clinical heterogeneity is still observed among patients with the same BC subtype. In this regard, DNA methylation analysis appears to have the potential to improve molecular classification. DNA methylation

research in BC pathogenesis has grown exponentially since the early 90's with the seminal description of differential DNA methylation of the estrogen receptor (ER) gene between BCs with and without ER expression [81]. For example, a recent study found that the methylation level of matrix metallopeptidase 7 (MMP7) gene can effectively distinguish basal-like breast cancers (BLBCs) from other types of triple negative breast cancers (TNBCs) [82]. Another recent study suggests that aberrant methylation of breast cancer 1, early onset (BRCA1) gene has prognostic utility in early-stage TNBCs [83]. Our laboratory and others have described a multitude of aberrantly methylated genes that contribute to BC progression [84–96]. In fact, using independent cohorts, our studies demonstrated a strong association of retinoic acid receptor, beta (RARB) gene methylation and macroscopic lymph node metastasis [86,96]. New studies have identified functional DNA methylation alterations outside of the CpG islands. For example, increased DNA methylation of the 5′CpG shore located on caveolin 1, caveolae protein, 22kDa (CAV1) gene correlates with increased caveolin-1 expression levels [97]. Interestingly, genome-wide DNA methylation landscapes of *in-situ* and invasive BC has been recently investigated [98–104]. To date, TCGA has profiled genome-wide methylomes for a large number of BC tumors and normal tissues (n=1,044). The partial analysis of these data (n=802) revealed that the methylation signature using 574 CpG sites identified five DNA methylation BC subgroups [105]. One subgroup presented a CIMP-like phenotype and contained a higher proportion of luminal B subtype and lobular histology tumors, while another subgroup presented a CHOP-like phenotype highly enriched in BLBCs [105,106]. Further classification of BCs through DNA methylation is needed because it may enable the development of more effective therapeutic regimens for better clinical outcomes.

Melanoma

Melanoma is by far considered the most deadly type of human skin cancer. With a steadily rising incidence during the last three decades, this tumor has become a major challenge for clinic management [49]. Melanoma pathogenesis is greatly influenced by genomic mutations induced by ultraviolet light [107]. A consequence of this mutational landscape is the frequent presence of driver mutations in genes such as *BRAF*, *NRAS*, and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), among others [107]. However, once the primary melanoma is established, a rapid progression to regional and distant organ metastasis is frequently observed [108]. This process requires a highly adaptable gene expression program which, in addition to somatic mutations, reflects the influence of epigenomic alterations. During the past decade, the identification of DNA methylation alterations by our laboratory and others has evolved from targeted single loci to genome-wide approaches [109–120]. Such studies have produced a number of complementary prognostic biomarkers, and identified unexplored therapeutic targets [121]. This new approach identified that alterations in DNA methylation patterns appear early in the progression from benign nevus to primary melanoma [122], and are associated with the prognosis of AJCC Stage IIIC patients [123]. We identified that aberrant methylation of absent in melanoma 1 (AIM1) gene and LINE-1 in tumor or serum are related to melanoma progression and outcome [117]. Our integrative genome-wide DNA methylation, copy number variation, and gene expression analysis identified functional DNA methylation

alterations in the progression from primary melanoma to brain metastasis [18,124–126]. In these studies, aberrant methylation of previously unexplored genes in melanoma, such as homeobox (HOX) and spliceosome factor genes were identified to be associated with progression to brain metastasis. Our laboratory has also identified a melanoma CIMP associated with the development of malignancy through the coordinated inactivation of tumor-related genes and methylation of multiple noncoding, methylated-in-tumor (MINT) loci [127]. In addition, similar to CRC, *BRAF* (V600E) mutation, a frequent alteration of cutaneous melanomas, also causes genome-wide DNA methylation changes in the melanoma epigenome [128].

TCGA network has analyzed >450 melanoma specimens, however, this cohort presents an important limitation. Unlike other solid tumors included in TCGA which are predominantly primary tumors, the melanoma cohort is mainly (~80%) composed of metastatic specimens. This is a consequence of the usually limited size and availability of primary melanomas to perform multiplatform assays. To date, there are no reports summarizing the findings in this TCGA melanoma cohort that can provide an integrative view of melanoma alterations.

Non-small cell lung cancer

Recent advancements in non-small cell lung cancer (NSCLC) treatment involve the inhibition of genes harboring driver mutations in epidermal growth factor receptor (*EGFR*), v-akt murine thymoma viral oncogene homolog 1 (*AKT1*), phosphoinositide 3-kinase (*PI3K*) and erb-b2 receptor tyrosine kinase 2 (*ERBB2*), and fusion of the echinoderm microtubule-associated protein-like 4 (*EML4*) and the anaplastic lymphoma receptor tyrosine kinase (*ALK*) genes (EML4-ALK) [129]. However, patients without these targetable mutations (>50%) are generally treated by non-specific systemic chemotherapy [130] and have a median survival <14 months [131]. The characterization of DNA methylation alterations that can serve as theranostic markers appears to be an alternative for these patients. A recent study identified a NSCLC DNA methylation signature associated with shorter disease-free survival [132]. Importantly, this signature, consisting of five genes (*ALX1*, *HOXA9*, *HIST1H4F*, *PCDHGB6*, and *NPBWR1*) identifies NSCLC stage I patients who have a high risk of recurrence and may benefit from adjuvant chemotherapy. Two studies have reported TCGA integrative genomic and epigenomic data analysis of lung cancers [133,134]. One study, focused on lung adenocarcinomas [133], identified a higher frequency of methylation of the gene *CDKN2A* on proximal inflammatory tumors and classified the tumors into H-CIMP, L-CIMP and intermediate CIMP [133]. Tumors with CIMP-H phenotype presented aberrant methylation of *CDKN2A*, *GATA2*, *GATA4*, *GATA5*, *HIC1*, *HOXA9*, *HOXD13*, *RASSF1*, *SFRP1*, *SOX17* and *WIF1* [133]. The other study, focused on lung squamous cell carcinoma (SQCC) [134], identified a higher overall methylation level in the classical subtype. This study identified that 72% of SQCCs harbor *CDKN2A* gene alterations, of which 21% are due to aberrant DNA methylation [134]. These recent integrative analyses support the important role of aberrant DNA methylation on the etiology of lung cancers, in addition to the mutations on driver genes.

Prostate cancer

Prostate cancer (PCa) is the most commonly detected cancer in men and the second highest cause of cancer deaths in this gender [49]. Patients with high prostate-specific antigen (PSA) levels are regularly subject to invasive needle biopsies. The low specificity of the PSA test leads to an overdiagnosis of clinically insignificant tumors, thus resulting in potentially unnecessary clinical interventions [135]. Identifying tumor biomarkers that provide earlier, more sensitive detection and that can assess the potential biological and clinical aggressiveness is urgently needed for PCa. Aberrant DNA methylation of glutathione s-transferase pi 1 (*GSPT1*) gene is the most common (>81%) molecular alteration reported in PCa [136,137]. The evaluation of *GSTP1* promoter methylation in body fluids, including plasma, urine, ejaculate, prostatic secretion, and biopsy washes [138–140] complements PSA testing and increases the specificity compared with PSA testing alone [141]. This exemplifies the importance of combining current diagnostics tests with DNA methylation-based biomarkers in clinical practice.

Emerging technologies for DNA methylation mapping: New insights on cancer diagnosis

Technologies for DNA methylation profiling utilize a great variety of strategies. The diverse approaches employed vary in the size and the scope of data generated, and in the costs. Consideration of these aspects is critical for making the right decision when designing a cancer diagnostic and prognostic algorithm. For didactical purposes, methods to study DNA methylation can be classified in three categories according to the number and the distribution of CpG sites being evaluated.

Global 5meC quantification approaches

Methods within this category focus on determining the total 5mC content of a genome. Initially, 5mC quantification strategies were based on high profile liquid chromatography (HPLC) [142] and the incorporation of radioactive methyl groups [143]. Most recently, these approaches have been updated to capillary hydrophilic-interaction liquid chromatography/quadrupole TOF mass spectrometry [144], and 5mC antibodies for immunohistochemistry [145], and ELISA (methDNA-ELISA) [146] analyses. Overall, these approaches allow evaluating major changes in the global DNA methylation level in a relatively inexpensive way. However, their main limitation is that region-specific DNA methylation cannot be analyzed.

Locus-specific DNA methylation approaches

Methods included in this category involve determination of DNA methylation status of preselected genomic regions. The surveyed regions contain a variable number of CpG sites, but this number is always limited to the extent of the targeted segment. Generally, locus-specific approaches have a relatively low cost and generate data that are easy to interpret. This makes it a cost-effective strategy and is especially useful when the targeted region

contains well-validated biomarkers. Two major strategies are employed to investigate locus-specific DNA methylation:

Sodium bisulfite modification

Sodium bisulfite modification (SBM) converts C residues to uracil, but leaves 5mC residues unaffected. The seminal sodium bisulfite-based methylation-specific PCR (MSP) [147] was improved with real-time PCR-based approaches such as quantitative-MSP (qMSP) [148] and MethyLight [149], and sequencing-based technologies such as Pyrosequencing [150] and direct bisulfite-sequencing [151]. Our laboratory enhanced MSP capabilities to achieve an Absolute Quantitative Assessment of Methylated Alleles (AQAMA) [68]. This strategy allowed the identification of clinically relevant DNA methylation aberrations in breast, colorectal, and squamous-cell esophageal carcinomas [62,68,92–94,152]. Limitations of SBM are discussed later in the section ‘Genome-wide DNA methylation approaches.

Methylation-sensitive restriction enzymes

Methylation-sensitive restriction enzymes (MSREs) recognize and cut genomic sequences containing unmethylated CpG dinucleotides. Thus, the initial MSRE coupled with PCR or Southern Blot techniques [153] were improved into genome-wide DNA methylation techniques such as the *HpaII* tiny fragment Enrichment by Ligation-mediated PCR (HELP) [154] and the Microarray-based Integrated Analysis of Methylation by Isoschizomers (MIAMI) [155]. The combination of SBM and MSRE denominated COmbined Bisulfite Restriction Analysis (COBRA) has also been largely used in cancer research [156]. Limitations of the use of MSREs are discussed later in the section ‘Genome-wide DNA methylation approaches’.

The main disadvantages of locus-specific techniques involve the requirement of knowing the region(s) of interest beforehand, the limited number of interrogated CpG sites, and the challenge of targeting specific CpG sites located in CpG-rich regions.

Genome-wide DNA methylation approaches

Next-generation high-throughput technologies have had a great impact in improving genome-wide DNA methylation mapping. The approaches used for genome-wide DNA methylation analyses can be classified according to the strategy employed to expose the methylation status: 5mC-specific immunoprecipitation (IP), MSRE digestion, SBM, and third-generation sequencing (TGS) technologies. With the exception of TGS technologies, in spite of the initial strategy, each approach can be evaluated by either microarray or next-generation sequencing (NGS) technologies (Figure 2). Here, we summarize the design, advantages/disadvantages, and cost/benefit ratio for the most relevant technologies that are revolutionizing DNA methylation mapping on human cancer.

Methods based on 5mC immunoprecipitation

These methods consist of the enrichment and isolation of fragments of genomic methylated DNA. 5mC, as well as the proteins that specifically bind to 5mC such as MBD proteins, can

be targeted using specific antibodies. The advantage of these approaches is that they are not affected by either incomplete digestion or bisulfite conversion. Furthermore, since there is no SBM, data processing does not require alignment to a reference sodium bisulfite-converted human genome. However, depending on the quality and potential cross-reactivity of 5mC antibodies, incomplete or non-specific IP can be detected. In addition, 5mC IP-based methods tend to overrepresent high-density CpG regions.

Methylated DNA immunoprecipitation

Methylated DNA immunoprecipitation (MeDIP) provides regional DNA methylation levels in a genome-wide manner [157]. This technique is based on the IP of methylated DNA fragments by using 5mC-specific antibodies. Briefly, genomic DNA is either sonicated or fragmented by restriction enzymes to generate fragments between 100 and 400 bp. Then, the fragmented DNA is heat-denatured to single-stranded DNA and immunocaptured by a 5mC-specific antibody. The enriched methylated DNA can be analyzed by DNA microarrays (MeDIP-Chip) or NGS (MeDIP-Seq). Genomic regions significantly overrepresented in the microarray or in the sequencing data are identified as methylated regions (Figure 2A). Thus, MeDIP-Chip and MeDIP-Seq generate a large amount of information and have been used to identify differentially methylated coding, non-coding, and regulatory genomic regions in breast [158,159], cervical [160], endometrial [161], leukemia [162], ovarian [163], renal [164], and testicular [165] cancers.

Methyl-CpG binding domain (MBD) protein capture

Methyl-CpG binding domain (MBD) protein capture (MethylCap) assay is based on the high affinity purification of methylated genomic DNA using a MBD-specific antibody [166,167]. The procedure and generated data are similar to MeDIP. However, MethylCap has demonstrated to be more efficient at testing CpG islands than MeDIP [168]. Methylated DNA enriched by MethylCap can also be analyzed by both microarrays (MethylCap-Chip) and NGS (MethylCap-Seq) (Figure 2B). MethylCap was recently applied to identify genome-wide DNA methylation biomarkers for bladder [169], colorectal [76], leukemia [170], lung [171,172], ovarian [173,174], and renal [175] cancer diagnoses.

However, both MeDIP and MethylCap have important limitations for routine application in clinical diagnoses. The quality of formalin-fixed paraffin embedded (FFPE) DNA is not appropriate for MeDIP or MethylCap. Moreover, the data generated have a low resolution; there is no information about the methylation status of single CpG sites within the ~150 bp size fragments. Additionally, since it has been demonstrated that some isoforms of MBD1 can bind to both, methylated and unmethylated CpGs [176] via different domains [177], selecting the right MBD domain is critical to exclude false positive results on this technique.

Methods based on methylation-sensitive restriction enzymes

These methods are based on the selective restriction of methylated and unmethylated DNA sequences. Different combinations of MSREs have been employed. Like IP-based techniques, these methods do not require alignment to a reference sodium bisulfite-converted human genome.

HpaII tiny fragment enrichment by ligation-mediated PCR

In HpaII tiny fragment enrichment by ligation-mediated PCR (HELP), the genomic DNA is separately digested with MspI (resistant to 5mC) and HpaII (sensitive to 5mC). The restriction fragments are then amplified by ligation-mediated PCR labeled with two specific fluorochromes (one for *MspI* and the other for *HpaII* fragment products) and hybridized to a microarray (HELP-Chip) or directly sequenced (HELP-Seq) (Figure 2C). The advantage of this method is that it can test methylation levels of genomic regions in spite of their CpG density. However, it is limited to regions that present the HpaII/MspI restriction site (5′-C*CGG-3′), which comprises ~7% of all CpG sites. A recent study using the HELP-Chip assay identified a methylation signature involving 15 loci predictive of survival on acute myeloid leukemia patients [178].

Comprehensive high-throughput arrays for relative methylation

This method uses the enzyme McrBC coupled with a customized microarray hybridization [179]. McrBC is a type IV deoxyribonuclease that, unlike methylation-sensitive enzymes, specifically cuts methylated DNA without affecting unmethylated DNA. Its recognition site contains a purine (A or G) base before the methylated cytosine (5′-A/G5mC-3′). The probes contained in the Comprehensive High-throughput Arrays for Relative Methylation (CHARM) microarray are designed based on the CpG location and density [179] (Figure 2D). Compared with MeDIP-Chip and HELP-Chip, CHARM has better resolution, sensitivity, and specificity [179]. Using this approach, novel DMRs were identified in colon cancer and the importance of CpG shore methylation was evidenced [179].

Unlike IP-based approaches, MSREs-based technologies can be applied to FFPE clinical specimens. However, three important aspects should be considered. First, FFPE-derived DNA has a multitude of fragmentation sites which increases with age or time before fixation. Second, incomplete digestion represents an important issue. Third, these analyses are limited to the number and location of restriction sites in the genome. In addition, these approaches lack single CpG resolution.

Methods based on sodium bisulfite conversion

These methods rely on the mutagenic potential of sodium bisulfite. Importantly, this modification enables the generation of genome-wide maps of 5mC at a single-base resolution. However, the conversion leads to a reduced genome complexity (global decrease of C abundance), generating greater sequence redundancy, increasing the complexity of the sequence alignment (for sequencing-based methods), and decreasing the hybridization specificity (for microarray-based methods). Another disadvantage of SBM-based approaches is that it cannot discriminate between 5mC and 5hmC.

Human Methylation 450k BeadChip

Human Methylation 450k BeadChip (HM450K) is an expansion of the previously clinically-validated GoldenGate and Human Methylation 27k BeadChips [14]. This method quantifies methylation levels at single-base resolution in a genome-wide manner. Genomic DNA is

sodium bisulfite modified and whole-genome amplified. The products are then enzymatically fragmented, purified, and hybridized to the BeadChip [14]. The oligonucleotide probes contained in this array survey the methylation level of over 485,000 cytosines (482,169 CpG dinucleotides and 3,343 CpNpG trinucleotides). Even though these CpG sites only represent 1.73% of all the CpGs, the design covers 21,231 (99%) RefSeq annotated genes with an average of 17.2 probes per gene distributed across the promoter, 5′UTR, first exon, gene body, and 3′UTR regions and 26,658 (96%) annotated CpG islands and their CpG context of shores, shelves, and distant regions identified as 'open sea' [14]. We observed that in this array, CpG island and CpG shore probes are predominantly located at gene promoter regions, while CpG shelf and 'open sea' probes are predominantly located at gene body and intergenic regions [18]. HM450K has demonstrated to be one of the most cost-effective practices for surveying genome-wide DNA methylation variations in human cancers [18,100,106,119,125,126,180–184]. Importantly, this is the method of choice by TCGA, where more than 10,000 specimens have been analyzed. However, some technical limitations with the probe design and analysis pipeline were recently described. First, several probes (~30,000) have a high likelihood of cross-hybridizing to non-targeted genomic regions [185]. Second, the CpG recognition site of many probes (~70,000) contains known single nucleotide polymorphisms (SNPs) [185]. As we recently reported, probes with affinity for non-targeted regions, as well as probes overlapping SNPs should be excluded from the analysis [18]. A general disadvantage of microarray-based approaches is that they only interrogate genomic regions previously determined in which specific synthetic probes can be designed.

Whole-genome bisulfite sequencing

Bisulfite conversion of genomic DNA combined with NGS is the most effective approach to evaluate at a single base resolution the methylation status of almost every C on the genome (~28M) (Figure 2E). This technology has provided evidence about non-CpG methylation [5]. More recently, it has been successfully applied to generate entire methylomes of different normal and respective cancer cell types [186–188]. This approach is generating the most comprehensive DNA methylation maps and promise to provide information about unexplored cancer genomic regions to design most informative diagnostic algorithms and potential therapeutic targets. However, despite of whole-genome bisulfite sequencing (WGBS) advantages, it is still extremely expensive and the data generated represent a challenge for bioinformatics approaches. Also, the large amount of DNA required for WGBS is prohibitive for cancer studies. For all these, WGBS is not yet suitable for routine cancer diagnostics. To maintain the advantages of bisulfite sequencing, while dramatically reducing the costs and the amount of input DNA, different adaptations of WGBS have been developed. Targeted bisulfite sequencing methods, such as reduced representation bisulfite sequencing (RRBS) [189] and bisulfite sequencing of padlock probes (BSPP) [190], which dramatically decrease the costs of WGBS and the complexity of the data analysis. By the other hand, the transposase-based in vitro shotgun library construction ('tagmentation') enables a >100-fold reduction in starting material (~20ng) relative to conventional WGBS protocols [191].

Reduced representation bisulfite sequencing

This is the most widely used adaptation of WGBS, in which a smaller fraction of genomic DNA is bisulfite sequenced [189]. Genomic DNA is first digested with a methylation-insensitive restriction enzyme, such as *MspI*. Then, methylated adaptors are linked to the restriction fragments and the fraction of 40–220 bp is purified and sodium bisulfite modified. The converted fragments are amplified by PCR using primers that are complementary to the adaptor, and then the PCR products are finally sequenced (Figure 2F). Reduced representation bisulfite sequencing (RRBS) generates information about the methylation statuses of 4–6 million of CpG sites at single base resolution. Recently, this protocol was optimized to analyze nanograms of genomic DNA. RRBS has been used to generate genome-wide DNA methylation maps of gliomas [192], leukemia [193], and colon cancer [194].

5mC and 5hmC-specific approaches for sodium bisulfite modification

A significant limitation of current bisulfite-based approaches is the inability to distinguish 5mC from 5hmC. In order to solve this issue, two strategies were recently developed. First is the oxidative bisulfite (ox-BS) modification, which is based on the differential oxidation of 5mC and 5hmC by potassium perruthenate ($KruO_4$). Under this condition, 5mC is not affected, while 5hmC is oxidized to 5fC, which is eventually converted to uracil residues during the SBM step. This approach is coupled with NGS [195] and HM450K BeadChips [196]. The other approach is the TET-assisted bisulfite (TAB) modification, where 5hmCs are protected by glucosylation, and then the 5mCs are oxidized to 5caC by the TET enzymes and then finally converted to uracil residues by SBM. This approach is coupled with NGS (TAB-Seq) [197].

Methods based on third-generation sequencing (TGS) technologies

SBM-based approaches cannot distinguish 5mC from 5hmC, which means that previous studies relying on this approach have pooled both modifications as 5mC. Today, we know that due to their contradictory effect on gene expression, grouping them as 5mC is a mistake that needs to be solved. Single-molecule real-time (SMRT) DNA sequencing method is a TGS platform that allows sequencing of the 5mC without SBM. This is based on the fluorescently labeled nucleotide incorporation kinetics [198]. The covalent modifications of C, such as 5mC and 5hmC, differentially affect the kinetics of the DNA polymerase generating a kinetics signature that allows the discrimination between C, 5mC, and 5hmC [198]. This alternative appears to be a solution for the limitation of SBM-based sequencing approaches. However, it still needs to be properly validated in cancer methylome studies and the kinetic detection systems need higher sensitivity.

Genome-wide DNA methylation pattern as a cancer biomarker: strengths, and weaknesses

Compared with other cancer-specific biochemical modifications, 5mC is a cancer-specific stable modification that can be detected in DNA samples obtained from diverse body fluids,

including whole blood [199], plasma [200], circulating tumor cells [201], serum [112], saliva [202], urine [203], bronchoalveolar lavage [204], sputum [205], stool [206], and fine needle aspirate [207,208], as reviewed elsewhere [209]. These features make the 5mC modification a suitable target for alternative non-invasive diagnostic strategies. Additionally, compared to genetic-based approaches, DNA methylation-based diagnostics present important advantages. First, the number of aberrantly methylated CpG sites is significantly larger than the number of genetic mutations [210]. This allows for the design of more flexible diagnostic strategies. Furthermore, this is particularly important for cancer types with low prevalence of somatic mutations [211–213]. Second, DNA methylation-based diagnostic approaches have the potential to identify tumors of unknown origin. In the example presented in figure 3, using TCGA data, we analyzed the top 4,000 most variable CpG sites across four cancer types (breast, colorectal, glioblastoma, and melanoma). As a representative sample, we included 17 primary tumors for each cancer type. Based on the DNA methylation signatures, the samples clustered into four independent clades corresponding to the tumor type (Figure 3A–B).

On the other hand, three important caveats should be considered with regard to the clinical utility of DNA methylation biomarkers:

First, there is a major interference of intratumor DNA methylation heterogeneity. The cell population under study usually comprises a mixture of phenotypically and epigenetically diverse cell types, and therefore the DNA methylation level is an average value determined by the methylation status of the specific locus and the proportion of cell type under study. This issue has been recently addressed in PCa [214]. In consequence, tissue heterogeneity is a significant issue in DNA methylation studies of cancer. This limitation may be reduced by improving the tissue microdissection techniques to guarantee a high percentage of tumor cell populations. Certainly, this drawback is not limited to DNA methylation since it has also been reported in genomic mutations [215].

Second, culture conditions have the potential to induce variation on DNA methylation patterns that are not present in the original tumor. To evaluate this phenomenon, we generated genome-wide DNA methylation landscapes of DNA samples obtained from brain metastases from melanoma patients processed in three different storage conditions: frozen tissues, FFPE sections, and established cultures. We identified a high correlation between FFPE and frozen tissues (Spearman's rho = 0.913, 0.981, and 0.976; Figure 3C) in the three cases. However, the correlation between DNA methylation profiles obtained from established cultures and either frozen or FFPE tissues was significantly lower (Spearman's rho 0.671, 0.688, and 0.813; Figure 3D). We separately evaluated variations on DNA methylation levels of CpG sites located at CpG islands and CpG sites located at 'open sea' regions. We observe an increase in DNA methylation within a subset of CpG sites located at CpG islands and a decrease in methylation with a subset of CpG sites located at 'open sea' regions (Figure 3E). In summary, due to the DNA methylation variations induced by cell culture conditions, DNA methylation biomarkers identified on *in-vitro* models have to be verified and validated in larger clinical specimen cohorts.

Third, as in the detection of genetic variations, the characterization of aberrant DNA methylation patterns depends on having a normal reference DNA methylome. However, since each cell type presents a specific DNA methylation pattern, defining a reference DNA methylome is a major challenge. In consequence, cancer methylome studies require the evaluation of a large number of normal tissues. Today, the Human Epigenomic Project (HEP) consortium, the International Human Epigenomic Consortium (IHEC), the U.S. National Health Institute Roadmap Epigenomics Program, and the ENCyclopedia of DNA Elements (ENCODE) project are delineating reference maps for DNA methylation, and other epigenomic modifications, for a large variety of normal adult, fetal, and embryonic human cell types. Data generated by these projects are currently used as normal references for different cancer epigenomic studies. We expect that upon competition of these key endeavors, the identification of cancer-related methylation alterations will be significantly enhanced.

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Summary

The understanding of epigenomic alterations in cancers has rapidly increased due to the emergence of more sensitive high-throughput technologies. DNA methylation, one of the most widely studied epigenetic mechanisms, represents an attractive target for cancer diagnosis, prognosis, and treatment. Seminal studies in cancer epigenetics focused on candidate genes mainly centered on the gene promoter. Today, with the advent of recently improved technologies, the focus shifted to genome-wide and whole-genome mapping of DNA methylation distribution. These new approaches revolutionized our understanding of epigenomic regulation and began to modify our preconceptions about the role and clinical utility of DNA methylation in cancer patients.

This *Review* explores emerging technologies for the study of DNA methylation as a cancer biomarker for diagnosis and prognosis. First, it provides new insight into the role of DNA methylation in gene expression, with particular focus on the importance of the cytosine-guanine (CpG) dinucleotide context. Second, it summarizes recent clinical applications of DNA methylation as a cancer theranostic biomarker. Finally, emerging technologies for studying DNA methylation are discussed, including considerations about strengths and weaknesses for each situation.

Expert commentary

DNA methylation is an epigenetic modification with high value for cancer diagnostics. There is a large list of clinically-relevant DNA methylation alterations, with some showing utility as diagnostic, prognostic and/or therapeutic markers. During the past few years, the introduction of NGS and high-throughput microarrays to cancer research led to a paradigm shift in the approaches of characterizing aberrant DNA methylomes. Genome-wide mapping as opposed to single CpG assessment is enhancing the potential use of DNA methylation as a cancer biomarker. In addition, our understanding of the role of DNA methylation marks in the genome has increased. The concept that DNA methylation was only restricted to CpG dinucleotides and that its influence on gene expression regulation was centered on gene promoter CpG islands have been recently challenged by improvements in DNA methylation mapping technologies. With the discovery of additional functions of DNA methylation in transcription regulation, another paradigm that has been modified is the concept that DNA methylation always induces gene silencing. It is now clear that under different CpG and gene structure contexts, DNA methylation can also enhance, stabilize, or define alternative splicing sites, among other functions.

Five-year view

As the cost of NGS-based technologies continues to decrease, the clinical utility of DNA methylation patterns on cancer diagnostics will rapidly increase. The introduction of TGS technologies to cancer studies promises to bring a solution to the multiple problems of DNA methylation mapping using bisulfite-based approaches. Defining the reference DNA methylomes will significantly improve the identification of cancer-specific DNA methylation patterns. This will result in a faster and easier process to identify novel and cancer-specific DNA methylation alterations. However, some improvements should be made before there can be a definite translation of DNA methylation to the clinical practice. First, methylation assays should be improved for low quality/amount of DNA samples, especially those obtained from microdissected FFPE tissues. Second, for current and upcoming cancer diagnostic DNA methylation markers, there is an urgent need for validation studies in large multicenter validation cohorts. Third, comparisons between DNA methylation markers with gene expression and mutation markers should be carried out. This comparison should address the question of whether DNA methylation markers can be used as surrogated for gene expression or mutation markers and identify novel diagnostic strategies in which DNA methylation can be integrated to existing gene expression or mutation markers.

Key issues

- Cancer DNA methylation mapping has rapidly increased with the emergence of high-throughput technologies.
- The traditional view that DNA methylation occurs predominantly at CpG dinucleotides has been challenged with new findings based on NGS approaches.
- The preconception that gene expression is mainly regulated by DNA methylation at promoter CpG islands has been expanded to other functional intra- and intergenic regions.
- Non-CpG methylation in gene bodies is positively correlated with gene expression levels.
- DNA methylation of CpGs outside of a CpG island context, such as CpG shores, shelves, and the so called ‘open sea’, demonstrate functionality for gene expression in normal and tumor cells.
- DNA methylation maps are starting to gain importance as current theranostic markers for cancer management.
- Genome-wide and whole-genome DNA methylation mapping approaches are replacing the traditional locus-specific approaches for the discovery of novel cancer biomarkers.
- DNA methylomes generated from FFPE tissues maintain a high correlation with those generated from frozen tissues. However, DNA methylomes generated from cultured cells present significant differences with FFPE and frozen tissues.
- In contrast to genetic profiling, DNA methylation mapping can identify the cancer-type.
- The human reference methylome of normal tissues is needed to accelerate the discovery of cancer-related DNA methylation variations.

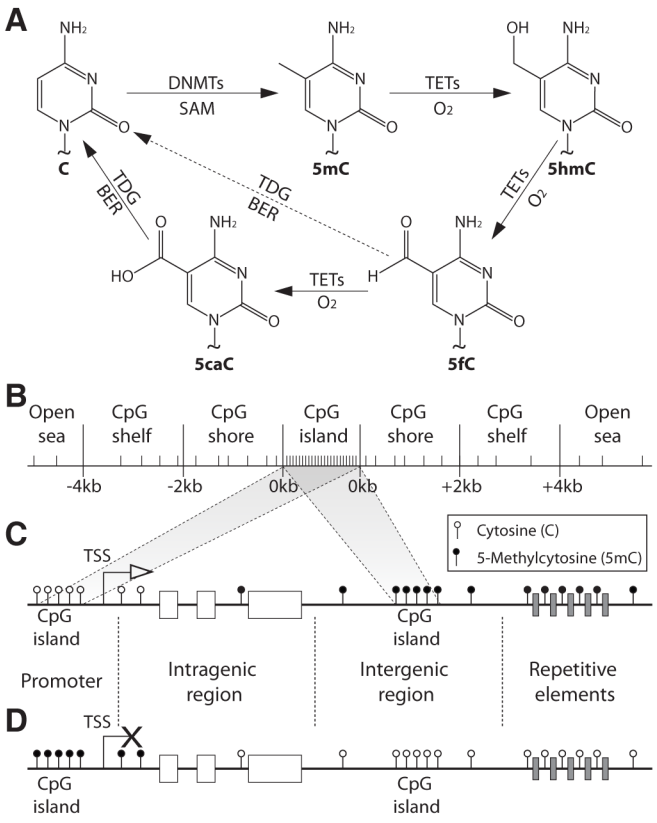

Figure 1. Cytosine modifications and distribution of 5-methylcytosine (5mC in the human genome

A- Chemical structures of the unmodified cytosine (C), 5mC and its oxidation products 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). First, a methyl group is transferred from the s-adenosylmethionine (SAM) to the fifth carbon of cytosine by DNA methyltransferase enzymes (DNMTs). Second, the oxidation of 5mC is carried out by the ten-eleven translocation methylcytosine dioxygenases (TETs) to 5hmC, 5fC, and 5caC. Finally, 5fC and 5caC can be excised and repaired by thymine-DNA glycosylase (TDG) and base excision repair (BER) systems to generate unmethylated Cs. **B-** Representation of the CpG context. **C–D.** Distribution of 5mC in normal (**C**) and cancer (**D**) cells. CpG: cytosine – guanine

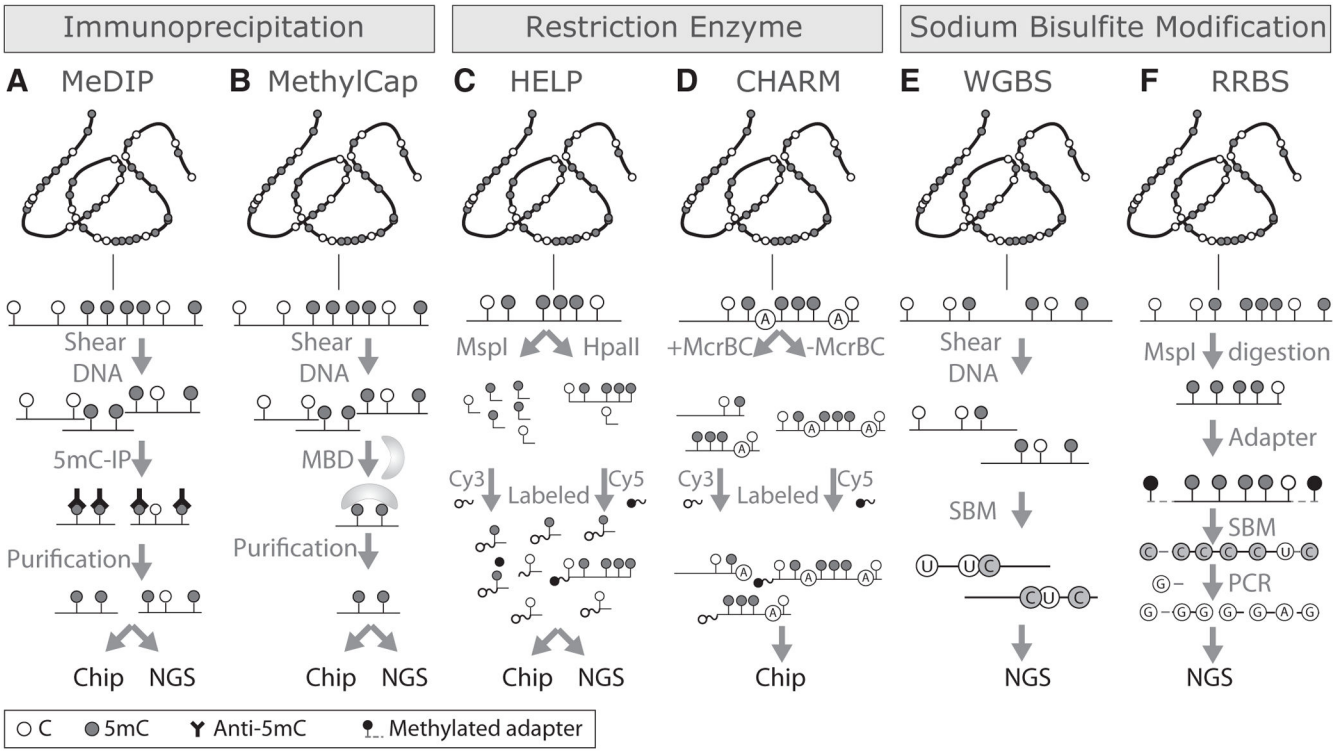

Figure 2. Genome-wide DNA methylation mapping technologies

Technologies based on 5mC immunoprecipitation (IP). **A-** Methylated DNA IP (MeDIP) is based on the use of 5mC-specific antibodies. The product is a fraction of genomic DNA enriched in methylated DNA that can be analyzed by either DNA microarrays (chip; MeDIP-chip) or next-generation sequencing (NSG; MeDIP-Seq). **B-** MethylCap is based on the capture of methylated DNA by methyl-CpG binding domain (MBD) proteins. The fraction of methylated DNA enriched by this approach can also be analyzed by either chip (MethylCap-chip) or NGS (MethylCap-Seq). **C-** HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) is based on the differential labeling of the products of two restriction enzymes, one methylation-resistant (MspI) and other methylation-sensitive (HpaII). **D-** Comprehensive High-throughput Arrays for Relative Methylation (CHARM) uses the restriction enzyme McrBC which cuts 5mC that are preceded by a purine (A or G) base. The restricted (+McrBC) DNA is differentially labeled and compared with the non-McrBC restricted (-McrBC) DNA fragments in a DNA microarray. **E-** Whole genome bisulfite sequencing (WGBS) involves the fragmentation and sodium bisulfite modification (SBM) of genomic DNA followed by NGS. **F-** Reduced Representation Bisulfite Sequencing (RRBS) involves the digestion of genomic DNA by the methylation-resistant restriction enzyme *MspI*. Each fragment is coupled to a methylated adapter and SBM. Finally, only the converted fragments are amplified by PCR using primers that are complementary to the adaptor, and the products are analyzed by NGS.

5mC: 5-Methylcytosine; CpG: Cytosine – guanine.

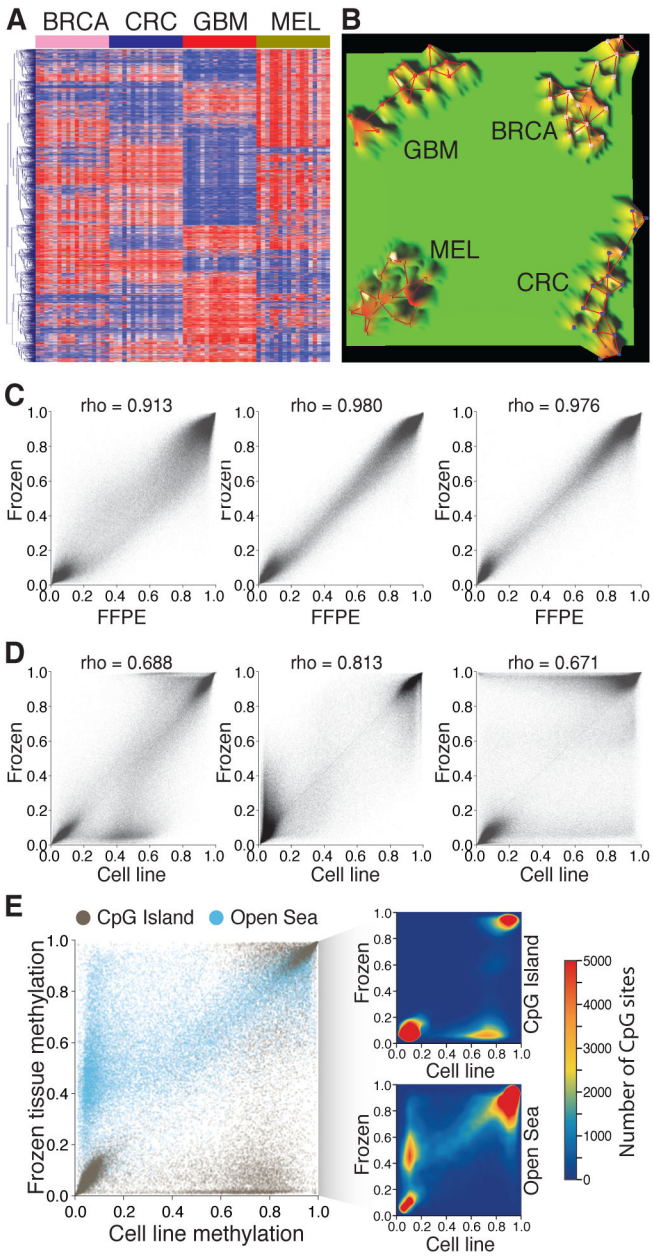

Figure 3. Strengths and weaknesses of DNA methylation as a cancer biomarker

A–B: DNA methylation profiling can identify the tumor types. **A-** Heat map showing the unsupervised hierarchical cluster analysis using the DNA methylation level of the top 2,000 most variable CpG sites in four different cancer types: breast cancer (BRCA), colorectal cancer (CRC), glioblastoma (GBM), and melanoma (MEL). **B-** Terrain maps generated with the same DNA methylation signatures showing four separate conglomerates of samples representing the four different tumor types. **C–E:** Culture conditions induce genome-wide variations in DNA methylation level. **C-** Correlations between DNA methylation levels of three pairs of melanoma brain metastases stored as frozen and as formalin-fixed paraffin embedded tissues. **D-** Correlations between DNA methylation levels of the same three pairs

of melanoma brain metastases stored frozen and established as cell cultures. **E-** Comparison of DNA methylation levels according to the CpG context of the assessed CpG sites. CpG sites located in CpG islands and in open sea regions are represented. The density plots represent the variations observed in CpG islands (upper) and open sea (lower).
CpG: Cytosine – guanine.