



Eagles report: Developing cancer biomarkers from genome-wide DNA methylation analyses

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Abstract

Analyses of DNA methylation in human cancers have identified hypermethylation of individual genes and diminished methylation at repeat elements as common alterations, and have thereby provided important mechanistic insights into cancer biology as well as biomarkers for cancer detection, prognosis and prediction of therapy responses. The techniques available in the past were best suited for investigations of individual candidate genes and sequences, whereas recently developed high-throughput techniques promise to generate unbiased and comprehensive surveys of DNA methylation states across entire genomes. In this minireview we give a short overview of established and novel techniques and outline some major questions that can now be addressed to develop further cancer biomarkers and therapies based on DNA methylation.

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DNA METHYLATION CHANGES IN HUMAN CANCERS

Epigenetic alterations are now firmly established as causative factors in the development and progression of human cancers, especially changes in DNA methylation. In mammalian cells, methylation of DNA bases occurs almost exclusively at the 5'-position of cytosines and in adult somatic cells it is essentially only present at cytosines in the dinucleotide cytosine-guanosine (CpG). Since their discovery in the 1980's, DNA methylation changes in human cancers have mostly been analyzed on a gene-by-gene basis, i.e. metaphorically speaking, from a frog perspective. Now, novel techniques which have developed over the last decade provide the means to survey DNA methylation in a more comprehensive fashion across the genome. This eagle's view of the DNA "methylome" of various normal and cancer cells is expected to generate ample opportunities for the development of diagnostic, prognostic and predictive biomarkers to be used in clinical oncology. In this minireview, we will give a brief overview of DNA methylation changes in human cancers, describe current and emerging techniques for their analysis, with their respective strengths and limitations, and outline possible future developments.

In the human genome, CpG-sites occur at only one

quarter of the expected frequency in general and the remaining CpG-sites are often clustered in CpG-islands. Many of the approximately 30000 CpG-islands straddle the transcriptional start sites of genes. Most CpG-islands are never methylated under physiological conditions, not even during germ cell or embryonic development. In contrast, isolated CpG-sites within genes or intergenic regions are often methylated. In particular, most of the CpG sites located in repeat sequences within the genome are densely methylated in normal somatic cells. Many repeat sequences originate from ancient retroviruses (human endogenous retroviruses, HERV) or simpler endogenous retroelements (SINEs and LINEs). Methylation of such retroelements helps to constrain their activity. In addition to repeat sequences, selected single copy genes can be methylated in normal cells, among them some important determinators of pluripotency and cell lineage^[1]. Here, methylation aids in restricting their expression to certain cell types. Finally, DNA methylation of specific sequences helps in the selection of a single allele for expression, as in imprinted genes, and in choosing one active gene out of a cluster, e.g. in the immune system and presumably in the brain^[2,3].

This elaborate pattern of DNA methylation is disturbed in almost every human cancer. There are principally two types of changes, which occur quite independent of each other. In the case of DNA hypermethylation, methylation appears at otherwise unmethylated CpG-islands. CpG-island hypermethylation is usually associated with silencing of the affected gene promoter^[4]. In the case of DNA hypomethylation, methylation is lost from retroelements and single-copy genes, permitting transcription of normally repressed sequences^[5]. As one might predict, abnormalities in DNA methylation patterns are generally more pronounced in high-stage and high-grade cancers. Nevertheless, research over the last two decades has revealed a high degree of specificity in DNA methylation changes between different cancer types and among cancers of the same type at different stages of progression. These differences can be exploited for cancer detection and classification with respect to subtype and prognosis. For instance, the *GSTP1* gene is hypermethylated in approximately 90% of all prostate cancers^[6]. Additionally, certain methylation changes are associated with differential response to specific therapies and can be used as predictive biomarkers. A prominent example is hypermethylation of the *MGMT* CpG-island which indicates silencing of the gene. The *MGMT* gene encodes the methyl-O-guanosyl DNA methyltransferase, which reverses and repairs DNA alkylation. Accordingly, gliomas with *MGMT* hypermethylation can be predicted to respond more favorably to the alkylating drug temozolomide^[7].

Analyses of DNA methylation changes in cancer diagnostics have unique advantages resulting from the very biochemical properties of this epigenetic mechanism. First, DNA *per se* is a rather stable molecule. Second, methylation at the 5'-position of cytosine is chemically stable, is difficult to establish except by enzymatic transfer and equally difficult to reverse in the absence of specific enzymes. Third, the turnover of methylcytosine at most

genomic sites is, under most conditions, slow. As a consequence of these properties, DNA methylation changes can be robustly analyzed in a large variety of biological samples, including biopsies, body fluids, and archival material^[8]. With modern techniques, minute amounts of DNA can be analyzed, permitting high sensitivity. In addition, since most CpG-islands are never methylated, hypermethylation in CpG-islands is often highly specific for tumor cells. Importantly, analyses from clinical samples and body fluids especially should always include appropriate quality controls to avoid artefacts resulting from, e.g. limited amounts of DNA or fixation.

In practice, a limitation to the specificity of DNA hypermethylation assays arises from the tendency of some CpG-islands to become hypermethylated in aging, inflamed and preneoplastic tissues, albeit usually not quite as densely as in full-blown malignancies^[9]. Another practical limit to the sensitivity of DNA hypermethylation assays is set by the heterogeneity of the changes within one tumor type and within individual tumors. Typically, a particular CpG-island is hypermethylated in a fraction of all cases and not all CpG-sites within the island are affected to the same extent in each individual tumor cell. Cases like *GSTP1* hypermethylation in prostate cancer, where practically all CpGs within a CpG-island are methylated in almost all tumors of one entity constitute fortunate exceptions (Figure 1). In such cases, the sensitivity of a methylation assay will strongly depend on the technique used (as discussed below) and its specificity may suffer, if hypermethylation of the gene is also present in preneoplastic tissue. Finally, tumor tissues comprise a mixture of several different cell types including stromal, endothelial and immune cells, in addition to the actual cancer cells. Although most CpG-islands are completely unmethylated in all cell types, methylation can display considerable specificity at some CpG-islands, in particular at their "shores"^[10] and even more so at CpG-dinucleotides outside CpG-islands^[11].

ESTABLISHED TECHNIQUES FOR ANALYZING DNA METHYLATION CHANGES IN HUMAN CANCERS

Many currently used assays for DNA methylation are based on bisulfite conversion of DNA^[12]. Bisulfite attaches itself to the C-6 of the cytosine ring. Subsequently, under alkaline conditions, the sulfonated cytosine is deaminated and desulfonated to uracil. The methyl group at the C-5 position prevents sulfonation and, therefore, methylcytosine remains the same. When polymerase chain reaction (PCR) is performed on the modified DNA, uracil results in thymine and methylcytosine in cytosine. Sequencing of the PCR products yields a reliable picture of the DNA methylation pattern at each CpG in the amplified sequence and is the current gold standard for DNA methylation analysis. Sequencing can be performed directly on the PCR product or after cloning. Direct sequencing yields an overall picture of the methylation pattern across several hundred bases, but standard Sanger

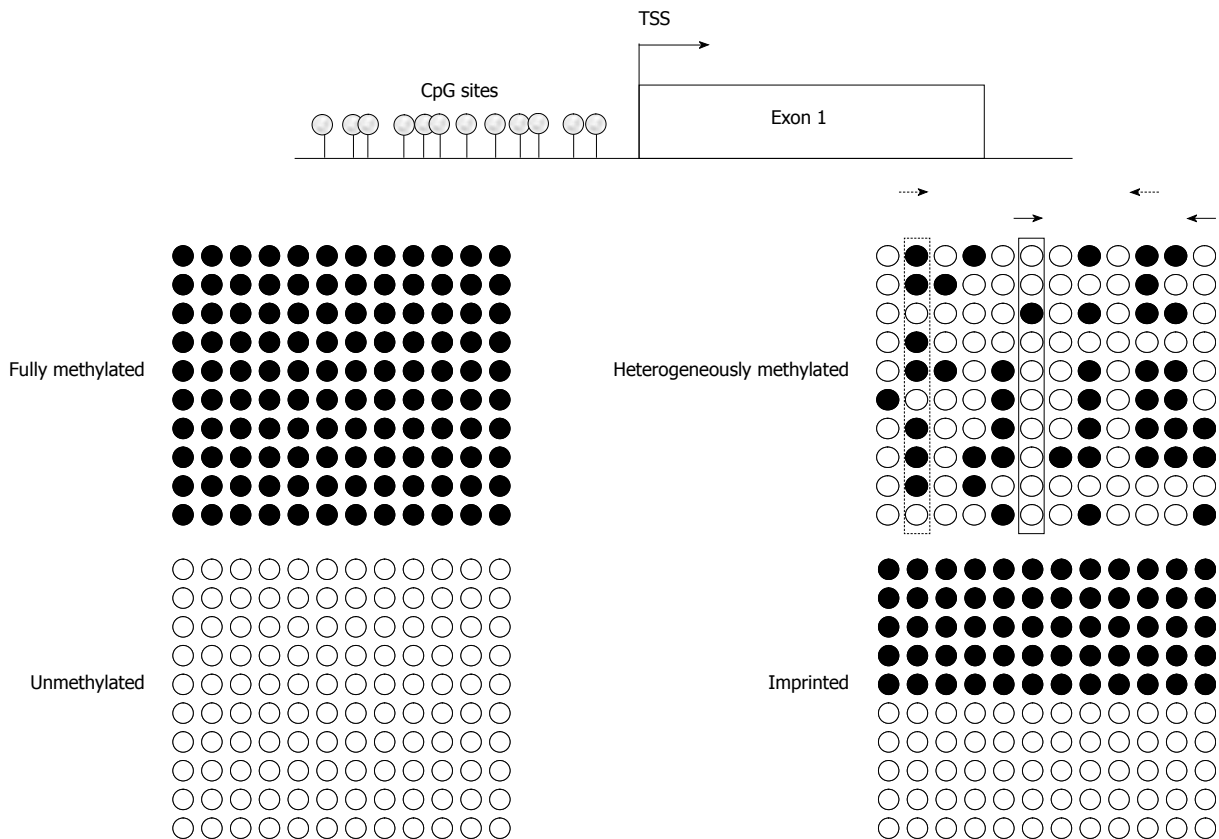


Figure 1 Assaying various methylation states by different techniques. Idealized bisulfite sequencing results of cytosine-guanosine (CpG)-sites in a proximal promoter region (TSS denoting the transcriptional start site), showing fully methylated, unmethylated, heterogeneously methylated and typical imprinted (differentially methylated) states. Each circle corresponds to a single CpG site, each line to one cloned allele. Filled circles represent methylated and empty circles unmethylated cytosines. Bisulfite sequencing gives an excellent indication of methylation state at every single CpG site, but quantification requires an adequate number of investigated clones. Pyrosequencing, methylcytosine-binding domain (MBD)- or methylation-specific polymerase chain reaction (MS-PCR)-based assays will detect 100% methylation at fully methylated and 0% methylation at unmethylated sequences, respectively. Imprinting results in methylation values of 50% at every single CpG site in pyrosequencing. In addition, MS-PCR-assays will detect signals for methylated and unmethylated DNA. For heterogeneously methylated DNA, bisulfite sequencing yields quite clear results, if a sufficient number of clones are used. In contrast, pyrosequencing will identify high methylation values (dotted rectangle) for several CpG sites and low values (solid rectangle) for other sites, whereas MBD-based analyses lead to intermediate overall methylation values. The results of MS-PCR methods depend critically on the chosen CpG-sites and may vary enormously even with small changes in the PCR stringency. For example, MS-PCR assay using primers interrogating CpG sites 2 and 10 (dotted arrow) yields higher methylation values compared to an assay aimed at CpG sites 6 and 12 (solid arrows).

sequencing is not quantitative. Pyrosequencing of PCR products allows a quantitative measure of average methylation at each individual CpG site, but only over relatively short stretches of DNA (up to 100 bases). Sequencing of multiple clones provides the most precise information and can reveal heterogeneous methylation at individual sites or the presence of differentially methylated alleles at imprinted genes. However, this technique is relatively lengthy and expensive.

Many exploratory studies on clinical samples have been performed by the methylation-specific PCR (MS-PCR) technique^[13]. Here, PCR primers are chosen to selectively fit either the methylated or unmethylated sequence of interest after bisulfite treatment. Hypermethylation events at CpG-islands result in the appearance of a PCR product with the methylated-specific primers, whereas healthy tissues yield a PCR product only with the unmethylated-specific primers. The PCR products can be visualized on a gel or can be quantitatively determined by real-time PCR using fluorescence probes. In particular, the MethylLight variant of MS-PCR, which employs selective

Taqman type probes for detection of the PCR products, is in widespread use^[14,15]. The MethylLight method can be designed to avoid the potential problem of bias introduced by using different primer pairs in MS-PCR. MS-PCR or MethylLight are excellent techniques to detect or even quantify methylation that is homogeneously absent or present, but they can result in highly variable results at partially and heterogeneously methylated sequences.

Many problems of applying DNA methylation analysis in clinical practice arise from the heterogeneous character and distribution of methylation changes on the one hand and of partially altered methylation in preneoplastic tissues on the other hand. For cancer detection, assaying multiple methylation markers increases sensitivity and helps to discriminate against changes caused by aging, inflammation and preneoplasia. For prognosis and classification, multiple markers are mandatory to cover biological heterogeneity arising during cancer progression and due to histological subtypes. For therapy prediction, using multiple markers could allow prediction of the response to alternative therapies. The most straightforward way to analyze multiple

CpG sites and sequences is multiplexing of individual assays. The MethyLight technique can be straightforwardly automated and has been employed to measure methylation at dozens of genes across hundreds of samples in a semi-quantitative fashion^[16]. More recently, mass spectrometry has been developed for automated analysis of DNA methylation at multiple sites in large sample sets. Typically, the analyses exploit differences in nucleotide molecular mass following bisulfite treatment^[17,18]. Conceivably, these or other automatable multiplex techniques may form the basis of DNA methylation assays in clinical routine use in the near future. For instance, some of the difficulties arising from the heterogeneous composition of tumor samples may be solved by digital PCR combined with high resolution melting (HRM) or pyrosequencing methods as discussed by Mikeska *et al.*^[19]. Obviously, however, multiplex assays interrogate a predefined set of sequences and cannot provide a global and unbiased overview of DNA methylation patterns and their changes in cancers.

Detection of DNA hypomethylation in cancer poses still another set of challenges. Hypomethylation occurs foremost at retroelements and other repetitive sequences in the genome. These repeats are similar, but not identical and methylation at retroelements in particular can be heterogeneous in normal cells and the decrease in cancer cells at various CpG-sites variable. Hypomethylation at single-copy sequences is likewise often partial and heterogeneous. In general, DNA hypomethylation accrues in many cancers with tumor progression^[20]. Therefore hypomethylation alterations might be exploited as biomarkers of prognosis, but unfortunately the specificity and sensitivity of assays for hypomethylation in cancer are so far limited. Global and comprehensive surveys of DNA methylation in cancer might help to identify specific hypomethylation changes useful as biomarkers.

NOVEL TECHNIQUES FOR ANALYZING DNA METHYLATION CHANGES IN HUMAN CANCERS

Several techniques have now come close to providing comprehensive surveys of the methylation pattern of normal and cancerous cells and tissues.

Some of these techniques are also based on bisulfite modification of DNA. The most straightforward approach to screening whole genomes for methylation changes would be genome-wide high-throughput sequencing following bisulfite conversion, which has become a realistic prospect with the advent of novel high-throughput (“deep”) sequencing techniques^[21]. The main obstacles prohibiting - at present - a broad use of this technique are the high fraction of repeat sequences in the genome and the reduced complexity of the DNA after bisulfite treatment due to the conversion of many cytosines to uracils. Both factors complicate the assignment of sequences from the sequencing “reads” to the genome. These same factors have also limited the use of arrays in DNA methylation analysis, although, e.g. bead-based arrays have already provided some

promising results^[22,23]. It is expected that these technical and bioinformatic obstacles will be overcome in the near future. For the time being, many researchers avoid them by investigating only more easily accessible parts of the genome isolated from the rest, e.g. by digestion with certain restriction enzymes and fractionation. The great advantage of bisulfite sequencing is that it yields information on the methylation of individual CpGs, deep sequencing with sufficient coverage (meaning multiple sequencing of the same segment) can even provide quantitative information at heterogeneously methylated sites.

In the meantime, techniques based on the biological recognition of methylcytosine by enzymes or antibodies - rather than on its chemical properties as in the bisulfite reaction - have made great advances. The oldest technique of this kind is the use of restriction enzymes that cut DNA dependent on its methylation status^[24]. Methylation differences result in differentially sized fragments that can be detected by Southern blotting or in the loss of a PCR product spanning the restriction site. Based on this simple principle, a variety of techniques have been developed in the past to explore differential methylation at a larger scale, such as 2D gels or hybridization to arrays after differential amplification of cut and uncut sequences^[25,26]. A novel technique for genome-wide methylation analysis, referred to as comprehensive high-throughput array-based relative methylation (CHARM), uses the bacterial enzyme McrBC for cleaving of DNA dependent on its methylation state followed by array hybridization of the fragments^[27]. This technique was used to unravel cell lineage and tumor-specific methylation differences at CpG island shores^[10,28].

The more recently developed technique of methylation-dependent immunoprecipitation (MeDIP) exploits the affinity of a methylcytosine antibody to enrich methylated sequences from the genome. Differences between tumor cells and their normal counterparts can be identified by comparative hybridization of the selected sequences to arrays or by high-throughput sequencing^[18,29].

In the cell nucleus, methylated DNA is recognized by specific proteins such as MBD2 or MeCP2 *via* a specific domain, the methylcytosine-binding domain (MBD). This biological recognition mechanism is exploited by novel techniques. They use recombinant proteins containing an MBD fused to another protein that can be easily captured on beads or columns, such as an antibody Fc domain or a glutathione transferase. As in MeDIP, array hybridization or high-throughput sequencing can follow to characterize the selected DNA^[30,31]. A promising variation of this technique has entered research using proteins that recognize specifically unmethylated CpGs^[32]. This technique may facilitate the analysis of hypomethylation events.

All present-day capture techniques do not provide methylation information at single CpG resolution, but enrich methylated or unmethylated sequences. Another limitation is the need for a certain CpG density to achieve efficient precipitation or binding of methylated DNA^[31]. DNA with lower CpG-density will not be as reliably captured, even if fully methylated. Neither, however, requires

bisulfite treatment of DNA and capture techniques based on MBD proteins even work with native DNA which needs only to be fragmented.

DNA METHYLATION CHANGES IN HUMAN CANCERS: OPEN QUESTIONS

Together, these approaches promise to provide us with a more complete picture of DNA methylation patterns in normal cells from different cell types, tissues and developmental stages. They will reveal how much individuals differ from each other. The results will help to elucidate the physiological functions of DNA methylation, e.g. in the immune system and in the brain, which are intriguing but poorly understood. We hope to learn how DNA methylation changes in autoimmune, neurological, psychiatric and cardiovascular diseases. In the field of oncology, eagle-eye perspectives of DNA methylation patterns are expected to yield important mechanistic insights into cancer pathogenesis and to provide a robust basis for the rational development of methylation-based diagnostic assays and epigenetic targeted drugs.

One of the most burning questions is how many DNA methylation changes cancers may harbor. Extrapolating from current knowledge, it is anticipated that large differences will be detected among cancer types and subtypes. For instance, carcinogenesis in the prostate involves a coordinate change in the methylation of several genes including *GSTP1*, whereas methylation changes in bladder cancer appear to accrue in a rather stochastic fashion with cancer progression, the largest differences existing between papillary and invasive subtypes^[33]. In colorectal cancers, the molecularly defined CpG-island methylated phenotype (CIMP)+ cases are already known to contain an unusually large number of methylation changes, which may ultimately turn out to range into the thousands. In comparison, the average number of aberrantly methylated genes (primarily CpG-islands) across all cancers is speculated to figure in the lower hundreds, similar to the average number of point mutations within genes emerging from the first genome-wide sequencing projects^[34]. We should also find out which methylation changes occur across many cancers and which are relatively specific. From current knowledge, *RASSF1A* and *GSTP1* hypermethylation, respectively, are representatives of these kinds of changes^[35,36]. Moreover, the identification of cell-type specific methylation patterns may provide markers for estimating the cellular heterogeneity of tumors, or identifying specific cell populations, as has been proposed for regulatory T cells^[37]. Methylation signatures of tumor stem cells should be particularly interesting, not only for heuristic purposes, but also for prognosis and therapy. Already, DNA methylation profiles have been successfully established for embryonic stem cells^[21].

A second important question coming closer to being answered is what causes alterations of DNA methylation in cancer. Are the changes determined by systematic defects in the molecular network of factors directing and maintaining normal patterns or do they occur in a more

random manner by accidents, perhaps followed by selection for those changes lending a growth advantage to the tumor cells? The answer to this question is essential for the further development of epigenetic therapies. In the former case, therapies will rather be directed towards restoring normal methylation regulation. In the latter case, therapies should aim at reexpression of individual antitumor genes which have been inactivated by epigenetic accidents. In either case, detailed analysis of the underlying molecular mechanisms will have to follow on the large-scale surveys to accompany and support drug development. This research will also provide biomarkers for prediction and monitoring of therapy responses.

The third result of systematic surveys of genomic methylation will be a broad and robust basis from which candidates can be chosen for further investigation and development for clinical use as biomarkers for detection and classification. Experience from the past suggests that methylation analyses of single genes will only provide reliable biomarkers in exceptional cases. Evidently, only a very small part of cancer genomes has been analyzed for methylation so far and this conclusion could be wrong. However, we think that the development of an optimized set of methylation markers for each cancer or tissue may be the most promising strategy, because of maximum sensitivity and specificity, but also the potential to cover the different scopes of detection, prognosis and prediction. Costs for high-throughput analyses, especially DNA sequencing, are declining fast. It may, therefore, even be possible that appropriately standardized and controlled whole-genome analyses could replace multiplex assays for DNA methylation in the future. In any case, development and validation of an optimal set of methylation biomarkers will certainly be necessary for each cancer type individually.

Finally, large-scale methylation analyses of human cancers will provide an important database for researchers studying molecular mechanisms of pathogenesis. Hypermethylation events are often associated with gene silencing and some of the silenced genes may be functionally important^[4]. In this fashion, important pathogenetic processes in particular cancers may be identified and new targets for therapy may be developed. The same expectation applies to DNA hypomethylation which often occurs at genes associated with invasion and metastasis and leads to expression of cancer-testis antigens^[38]. It also may permit reactivation of retroelements that promote genomic instability^[5]. Therefore, anti-metastatic and immune therapies should benefit from insights into DNA hypomethylation, in particular.

Large-scale approaches have not only offered new possibilities for analysis of DNA methylation in cancer, but also for studying global patterns of gene copy number alterations and mutations as well as changes in expression of mRNAs and other RNA species. More and more, integrative analyses assessing changes at various levels are used to classify cancer subtypes^[39] or to identify aberrantly active molecular pathways^[40]. Analyses of DNA methylation are

expected to become integrated into these global analyses shortly. In particular, large-scale sequencing has revealed a peculiar landscape of point mutations in cancer^[41], in which a relatively small number of genes are frequently mutated in each cancer type and a larger number of others carry point mutations in only a few cases each. Most of the frequently mutated genes, such as *TP53* or *KRAS*, have indeed long been known. Even after integrating copy number alterations with such studies, fewer consistent and obviously functionally important genetic changes have emerged from a large number of recently published studies. Many researchers now suspect that epigenetic changes, termed epimutations, may account, to a large extent, for the elusive “drivers” of tumor development and progression. If so, targeting epigenetic changes may turn out to be even more valuable in cancer therapy than hitherto assumed. Integrating large-scale analyses of DNA methylation with those of other changes will help to address this issue.

Developments in science are often driven by the fruitful interplay between large-scale surveys and detailed analysis of individual factors and mechanisms, the synergism between frog and eagle approaches. In the 1980's, DNA methylation research encompassed both the analysis of single restriction sites at individual genes and global, albeit unspecific measurements of total methylcytosine content. Similarly, the comprehensive surveys of genomic methylation patterns are complemented by detailed molecular analysis of the interaction of multiple epigenetic regulators at individual genes during the establishment of DNA methylation and chromatin patterns. The development of DNA methylation assays for clinical oncology by analysis of candidate genes has clearly reached a limit. Likewise, the further development of epigenetic drugs for cancer treatment requires a broader understanding of the overall changes in DNA methylation. Thus, the reports of the eagles are eagerly awaited.

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