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DNA methylation as a universal biomarker

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Abstract

Cell-free circulating DNA carries not only tumor-specific changes in its sequence but also distinctive epigenetic marks, namely DNA methylation, in certain GC-rich fragments. These fragments are usually located within the promoters and first exons of many genes, comprising CpG islands. Analysis of DNA methylation using cell-free circulating DNA can facilitate development of very accurate biomarkers for detection, diagnosis, prediction of response to therapy and prognosis of outcomes. Recent data suggest that benign and inflammatory diseases have very specific methylation patterns within cell-free circulating DNA, which are different from the pattern of a malignant tumor of the same organ. In addition, specific methylation patterns have been detected for cancers of different organs, so a differential diagnosis of site-specific cancer appears feasible. Currently, cancer-related applications dominate the field, although methylation-based biomarkers may also be possible for other diseases, including neurodegenerative and psychiatric disorders.

Keywords

biomarker; cancer; cell-free plasma DNA; detection; diagnosis; methylation; prediction; prognosis; treatment

Methylated cytosine, the ‘fifth base of DNA’, is attracting increasing attention as a potential biomarker. Existing evidence indicates that abnormal methylation can be used for detection and diagnosis of disease, prediction of response to therapeutic interventions and prognosis of outcome. In this brief article, I will address recent advances in the development of DNA methylation-based biomarkers. A number of excellent reviews have been published recently on methylation and cancer detection [1–3] and, therefore, the main emphasis here will be on biomarkers for cancer diagnosis (discrimination between malignant and nonmalignant disease), biomarkers for treatment monitoring and prediction of response, and biomarkers for prognosis. Besides cancer, DNA methylation also appears to be useful for detection and diagnosis of other diseases, including psychiatric and neurodegenerative disorders [4–6]. As any testing for detection and diagnosis should preferably be noninvasive, I will concentrate on recent advances in blood-based DNA methylation analysis for the detection and diagnosis of

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cancer. Finally, I apologize in advance to my colleagues in the field, whose work unfortunately has had to have been left outside the scope of the review, owing to space limitations.

Challenges to methylation analysis in clinical samples

A multitude of techniques reviewed recently by Delvenne *et al.* has been developed for assessment of methylation in tissues and biological fluids [7]. For the most part, these techniques have two components (Figure 1) – first, a biological or chemical module to differentiate methylated and unmethylated fragments, either by physically separating them or by changing their sequence. Bisulfite modification converts unmethylated cytosines to uracils, making the sequence of methylated and unmethylated fragments different. Methylation-sensitive restriction enzymes selectively destroy unmethylated DNA, so that only methylated fragments remain available for detection. Physical separation can be achieved by methylated DNA-binding proteins or antibodies against methylated DNA [7]. The second module (Figure 1) serves to amplify the differences and detect them by a variety of techniques from mass spectroscopy [8] to microarrays [9–11] to different combinations of amplification and detection by PCR [7].

While analysis of methylation in homogeneous samples can be relatively straightforward, heterogeneity of clinical specimens pose a major hurdle to data analysis. Compared with cultured cells, clinical specimens contain a mixture of components, with each cell bringing its own methylation peculiarities to the final readout. The composition of this mixture also changes with time, so that each specimen is more than likely to be unique with different proportions of the same fragment methylated in different samples. Sampling variability imposes another level of complexity that has to be considered; even the adjacent sections of the same tumor will have slightly different composition and thus quantitatively different methylation patterns. Finally, the natural history of cancer can bring together cells with different degrees of neoplastic transformation that will have different levels of methylation of the same sequence. For obvious reasons, a threshold has to be established; by its very nature, such a threshold will probably be artificial and only remotely related to the underlying biological complexity of developing cancer. Artificial thresholds are especially important when different techniques are used to assess methylation in a given fragment.

Methylation can also be detected using the bisulfite modification procedure [12], which converts unmethylated cytosines to uracils while leaving methylated cytosines intact (Figure 1). Ensuing changes in DNA sequence are then detected by a variety of methods. The bisulfite technique has its limitations, including a significant – up to 95% – loss of DNA during bisulfite treatment [13,14], which depends on multiple poorly controlled factors, including DNA concentration [15]. If milder conditions are used, there is a significant danger of incomplete conversion leaving questions about the methylation status of some cytosines [16,17]. Biased efficacy of PCR amplification of bisulfite-modified DNA has also been noted [18], suggesting another possible problem for clinical sample analysis. On the other hand, any CpG site can be analyzed, so for abundant and homogeneous samples, bisulfite modification can provide a comprehensive picture of methylation. In clinical samples, however, the amount of DNA is usually very small, the DNA itself is heterogeneous and this heterogeneity is likely to be variable. In this situation, bisulfite-based techniques are difficult to perform and less informative [19–21]. As a result, bisulfite-based quantitative measurement of biomarkers in clinical samples is more demanding than a simple PCR amplification after treatment with a methylation-sensitive restriction enzyme.

Blood-based DNA methylation for cancer diagnosis

Cancer diagnosis usually implies identification of cancer in either an asymptomatic individual or a patient with uncertain illness. In this respect, diagnosis of skin melanoma is less complicated owing to, for example, the highly accessible location of the tumor, its characteristic appearance and, hence possibility of visual assessment. Diagnosis is also relatively uncomplicated when certain signs of the disease are detectable (e.g., a palpable lesion in the breast) and a diagnostic biopsy can be done. The situation becomes more complicated if symptoms are less well defined. For example, ovarian cancer has to be differentiated from benign lesions, cysts and inflammation of ovary, pelvis and colon [22]. Similarly, pancreatic cancer has to be differentiated from chronic pancreatitis, ulcers of the duodenum and stomach, biliary stricture and benign or malignant liver tumors [23]. Even more difficult is diagnosis of disease in asymptomatic individuals because of multiple uncertainties regarding the nature of the disease, its location and whether the disease is present at all. In this respect, cancer screening is a typical example of looking for a black cat in a dark room without even knowing if the cat is really there. For obvious reasons, tissue analysis does not work – who would agree to have multiple biopsies of different organs just to learn that there is no tumor, and please come back next year?

Fortunately, biological fluids, especially blood, contain molecules originating in many different tissues of the body, so different molecules have been tested as systemic biomarkers. From this point of view, proteins are probably the most investigated components of the blood and a number of first-generation biomarkers have been discovered (e.g., CA19–9 [24], CA125 [25] and prostate-specific antigen [26]). Insufficient specificity and sensitivity of these biomarkers stimulated further investigations and cancer-specific autoantibodies have emerged as potentially the most promising protein-based biomarkers developed recently [27]. Apparently, biological amplification of autoantibodies by the immune system can generate a strong signal very early in the pathological process, replacing its instrumental amplification (e.g., by mass spectrometry).

Well-developed techniques for amplification of nucleic acids make them an obvious target for biomarker development, although RNA-based biomarkers in blood have not been pursued until recently owing to the chemical instability of RNA in aqueous solutions. It appears that stability is not a limiting factor for miRNA, so new miRNA-based biomarkers are likely to emerge soon [28]. By contrast, DNA-based biomarkers developed using cell-free circulating DNA (cfcDNA) in blood have been used successfully for prenatal diagnosis [29], with applications for cancer detection and diagnosis, and monitoring of treatment efficacy starting to emerge [30–33].

Initially, a high concentration of cfcDNA in some cancer patients suggested that a simple measurement of cfcDNA level could be used as a marker. Unfortunately, high variability in abnormal cfcDNA concentrations prevented it from becoming more than a secondary marker [34]. Increase in cfcDNA due to inflammation [35] indicated that concentration of cfcDNA was most probably a general sign of excessive cell death rather than a specific indication of malignant growth.

When tumor-specific mutations were discovered in cfcDNA, early cancer diagnosis based on mutation detection in cfcDNA in blood seemed possible. So far, this possibility remains unrealized – while this DNA can be used to characterize tumors [36], it does not indicate the specific location of the tumor. Moreover, low representation of mutated sequences early in the disease and technical problems with mutation discovery by whole-genome sequencing reduce the near-term potential of a mutation-based early diagnosis. A tumor-specific mutation that appears early and can uniquely characterize the primary tumor has not yet been found.

Another type of tumor-specific modification (methylation) can be detected in cfcDNA. This does reflect primary tumor [37–39], presenting an alternative opportunity for biomarker development. In this case, tumor-specific changes are limited to specific sequences (DNA methylation predominantly occurs in CpG islands within promoters of many genes [40]), so uncertainties associated with the localization of mutations are somewhat diminished. In addition, a simple enrichment (e.g., treatment of heterogeneous DNA with a methylation-sensitive restriction enzyme) can make even minute amounts of abnormally methylated DNA the dominant fraction in the sample (Figure 2). Thus, technically, diagnosis of cancer by blood analysis appears to be possible. Can biology create a problem?

Biologically, cancer diagnosis by blood analysis has two elements: detection of the site of the disease (e.g., if the disease is located in the lung, ovaries or other parts of the body) and identification of the nature of the disease (e.g., is it a benign lesion, inflammation or a malignant growth?). A lesion detected by imaging can be either benign or malignant; if molecular analysis cannot differentiate between these possibilities, its value is dramatically reduced. Similarly, an ideal test should produce different results for tumors and chronic inflammation, which can sometimes be a precursor to neoplasia. For instance, Barrett's esophagus can advance to esophageal cancer, while chronic pancreatitis to pancreatic cancer. In such situations, it is essential to have well-defined diagnostic parameters that recognize the molecular make-up of inflammatory disease as different from the make-up of cancer. Existing results are controversial [41–43], suggesting that differentiation of nonmalignant and malignant conditions may be possible if the right set of promoters is analyzed.

Potential for cancer detection based on abnormal methylation in cfcDNA has been demonstrated for different cancers, including prostate [43–45], breast [45], gastric [46], testicular [32] and bladder [47] cancers, and melanoma [48]. In most cases, however, detection is based on the analysis of the same set of promoters for different diseases with results most frequently expressed as a ratio of hypermethylation relative to healthy controls. It appears that technical challenges prevent simultaneous analysis of multiple promoters in each sample of cfcDNA and development of efficient detection algorithms similar to the Significance Analysis of Microarray and Prediction Analysis of Microarray (SAMPAM) algorithm developed for tissue analysis [49].

Recognizing that differential detection is the essential part of molecular diagnostics, we compared methylation profiles of cfcDNA from blood of patients with pancreatic cancer and chronic pancreatitis [50], and patients with ovarian cancer and benign disease [Liggett TE *et al*., Manuscript in preparation] using our customized microarray-based methylation detection platform MethDet-56 (56 promoters analyzed in each sample for proof-of-principle studies [39]). In both cases, nonmalignant diseases produced specific methylation patterns that were very different from patterns of malignant diseases. Importantly, methylation patterns were unique to the analyzed disease, raising hopes that cfcDNA can be used to identify the site and the nature of the disease. Thus, benign, inflammatory and malignant diseases could be differentially identified, suggesting that molecular diagnosis based on methylation analysis of cfcDNA is possible. Validation of the identified biomarkers in blinded samples is in progress.

DNA methylation for treatment monitoring, prediction of response & prognosis of outcome

Treatment monitoring

If the central dogma of epigenetics is correct and methylated promoters are silenced, while unmethylated ones can be active, then DNA methylation reflects, albeit imprecisely, the patterns of gene expression. If we extend this reasoning to drug effects, an interesting picture

emerges. Consider that drug activity changes some conditions in the body, and these conditions are reflected in changes of gene expression [51]. If regulation of expression and DNA methylation are indeed linked, a change in the DNA methylation pattern may be observed, not only for drugs that inhibit methylation-related functions [52], but also for any drug that is physiologically active.

Indeed, different drugs tested with cultured cells in the author's laboratory (e.g., amitriptyline and phenobarbital) revealed that drug-specific changes in methylation profiles are detectable soon after drug application [Liggett TE *et al*., Manuscript in preparation]. Similarly, drug-specific profile changes have been identified in cfcDNA of patients treated with different drugs (used as monotherapy) [Liggett TE *et al*., Manuscript in preparation], suggesting that an active compound that alters gene expression may induce changes in cfcDNA methylation. These observations expand potential application of the methylation profiling from detection and diagnosis to treatment monitoring through detection of drug-specific changes in patients' cfcDNA. At the same time, they may open the possibility of early discovery of resistance, which may manifest either as a reversal of drug-induced changes or as induction of another layer of changes, this time specific for resistance.

Prediction of response

DNA methylation patterns predictive of response to specific drugs and drug combinations started to emerge several years ago. Arguably the most well-established marker is methylation of the *MGMT* promoter in patients with glioblastoma multiforme, which predicts response to alkylating agents [53–55]. Methylation of other genes is predictive of response to treatment in ovarian [56,57], breast [58,59], gastric [60] and esophageal [61] cancers and melanoma [62], so the utility of methylation profiling for detection of innate resistance and, thus, correct stratification of patients for treatment gradually enters the mainstream of clinical decision making [54,56,63,64].

The emerging area of pharmacoepigenomics will probably need to address the issues of cell type-specific response to drugs, which can make translation of methylation patterns from cell culture to cfcDNA of patients very challenging, if at all possible. It is most likely that direct translation will prove to be too complicated and correlative studies with patients' specimens will be required before clinically relevant predictive biomarkers for different drugs are available [64].

Prognosis of outcome

A similar question gradually develops in tumor tissue analysis. DNA methylation profiling has been used to prognosticate clinical outcomes of bladder [65] and colorectal cancers [66], and recurrence of breast cancer after anthracycline therapy [59] and bladder cancer after IL-2 treatment [67]. While recurrence after treatment may involve changes in DNA methylation induced by the treatment itself, recurrence associated with pre-treatment profiles suggests that tumors with a certain methylation make-up are prone to be more aggressive and thus less susceptible to treatment [66]. It remains to be seen whether this methylation make-up is tumor-specific; it may be present in normal tissues as well, defining individual reactions not only to therapy, but also to other external stimuli. In this respect, genome-wide analysis of methylation in different tissues of the same individual may open new and unexpected venues of investigation.

Methylation changes in other diseases

The current model states that gene-expression patterns are different in healthy tissues and in disease. If DNA methylation profiles indeed reflect – however imprecisely – specific features

of a gene-expression pattern, we can expect to find disease-specific methylation profiles not only in cancer but also in many other diseases. While at first glance this hypothesis may appear extreme, there is a significant amount of evidence that supports the possibility of specific methylation patterns in genetic disorders, neurological and psychiatric diseases, and even in infection.

Abnormal methylation in genetic disorders can be either a direct result of errors in epigenetic imprinting (e.g., Beckwith–Wiedemann syndrome, Angelman syndrome [68], Russell–Silver syndrome [69]) or an indirect effect of mutations in proteins that bind methylated DNA (e.g. the autism spectrum disorder Rett syndrome) [70]. Both imprinting and DNA binding are likely to involve many individual elements – imprinted loci in one case and different proteins in another – that can be virtually independent, so it is safe to assume that abnormal methylation can be linked to other inherited abnormalities with as yet unknown foundations. From this standpoint, detailed analysis of DNA methylation patterns may help with disease diagnosis, classification and possibly treatment.

Neurodegenerative [71] and psychiatric [72,73] disorders ranging from Alzheimer's [74,75] to schizophrenia [76] and depression [77] appear to have disease-specific methylation patterns as well. Certain regions of the brain in patients with multiple sclerosis have abnormally methylated genes [78] and brain tissue of patients with epilepsy shows disease-specific methylation [4,79]. In the authors' laboratory, a test for abnormal DNA methylation of cfcDNA in treatment-naive patients with relapsing-remitting multiple sclerosis has revealed a specific profile that can be further developed into a specific biomarker [50]. Interestingly, this profile changes significantly during clinical exacerbations of the disease, suggesting that analysis of cfcDNA may assist in early detection of attacks before major brain damage has occurred.

Alterations in DNA methylation induced by drugs (pharmacoepigenetics) can be used to modulate activity of the disease. A recent observation indicates that in certain neurological disorders with expansion of trinucleotide repeats, DNA methylation can modulate stability of the repeats, thus changing the severity of the disease and indirectly implicating methylation in its natural history [80].

Finally, recent data indicate that DNA methylation may be involved in synaptic plasticity and is required for memory retention [6]. These observations open the widest possible space for methylation analysis – from prediction of risk to diagnosis and treatment of the disease on one hand, and to evaluation of higher brain activity on the other hand.

Infection-induced changes in gene expression suggest that DNA methylation may also play a role. Indeed, aberrant methylation has been described for virus-induced hepatitis [81], gastritis induced by *Helicobacter pylori* [82], and cervical dysplasia caused by human papillomavirus infection [83], among others. In these cases, however, it is difficult to separate methylation related to inflammatory response from methylation induced by the specific pathogen. Our work with cfcDNA from patients with chronic pancreatitis indicates that an inflammation-specific methylation pattern can be developed, suggesting that changes of gene expression due to inflammation are sufficient to change methylation of specific genes [50].

Conclusion

Methylation profiles of cfcDNA provide large sets of correlative data that can be used for development of specific and accurate biomarkers. These biomarkers are starting to find their application in cancer (for detection, diagnosis, prediction, prognosis and monitoring) but DNA methylation may be a fertile ground for search of other biomarkers and clinical assessment of other diseases.

Expert commentary

Methylation of cfcDNA is a promising approach for the development of robust and accurate biomarkers for detection, diagnosis, prediction and prognosis of different diseases. The major challenge to the discovery of biomarkers is the dearth of efficient techniques for methylation analysis in cfcDNA. Such techniques have to combine high sensitivity and accuracy with tolerance to inherent heterogeneity of clinical samples and be able to measure methylation at multiple sites within the same sample. The origins of cfcDNA are unclear, so the development of mechanistic biomarkers is currently impossible. This, however, does not preclude the discovery of accurate correlative biomarkers that might become extremely valuable in clinical practice.

Five-year view

Development of new analytical techniques for genome-wide methylation analysis using small clinical samples will open a multitude of possibilities for biomarker development. These possibilities will be limited only by the access to well-characterized clinical cohorts and by problems with clinical assessment required to put such cohorts together. Genome-wide methylation results for individual patients will overcome this limitation by creating massive sets of data, which will allow statistics-based discovery of new molecular features that will be used for patient stratification and even gradual substitution of clinical and pathological evaluation. In 5 years, blood-based methylation analysis will be used for population-wide cancer screening, for personalized therapy and for risk assessment, while methylation-based efficacy markers will serve as surrogate end points for drug discovery.

Key issues

- Cell-free circulating DNA in blood has DNA methylation patterns specific for different diseases.
- These patterns are different for different diseases.
- These patterns can be determined and used for biomarker development.
- Differential diagnosis based on methylation analysis of cell-free circulating DNA is possible for different types of cancer and benign proliferative and/or inflammatory diseases.
- Similarly, methylation-based biomarkers can be developed for neurodegenerative diseases.
- Methylation patterns may change in response to treatment, which can be used for monitoring of response.
- Origins of cell-free circulating DNA are unclear, so biomarkers are inevitably correlative.
- DNA methylation analysis by bisulfite modification has significant drawbacks that prevent its widespread application for the development of methylation biomarkers.

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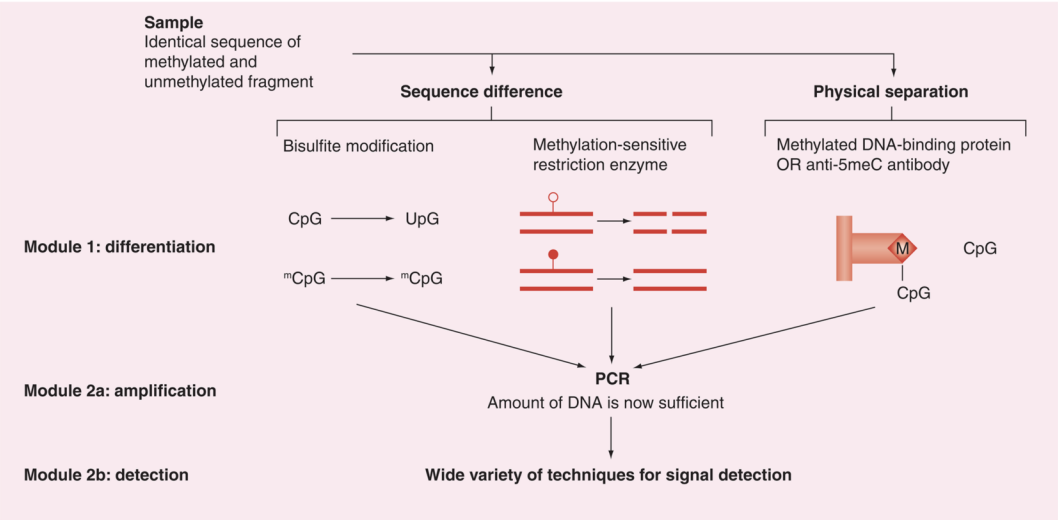

Figure 1.
General schema of DNA methylation analysis.

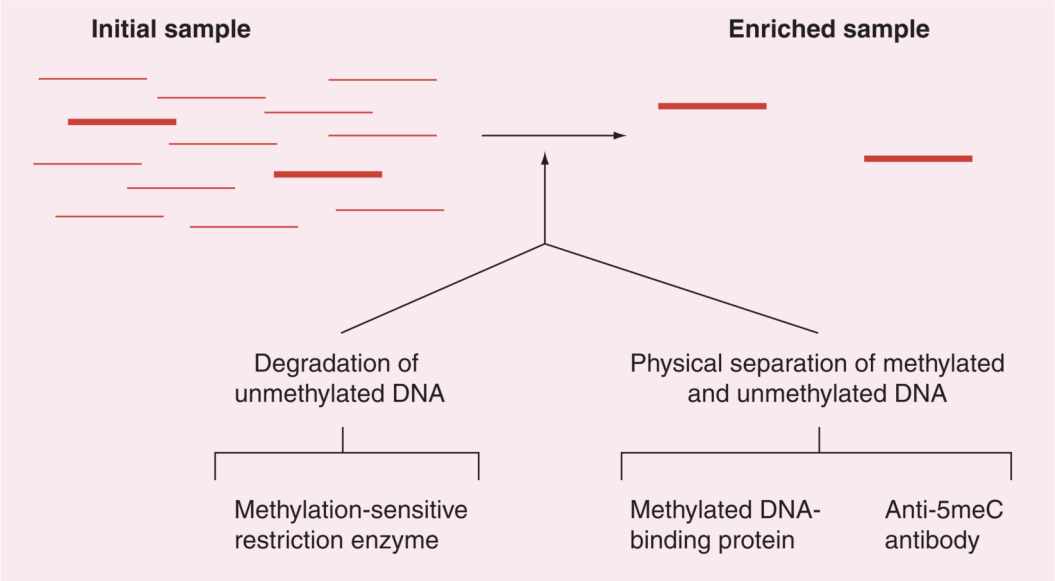

Figure 2.
Enrichment of methylated DNA can be achieved by enzymatic degradation or physical separation.