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The MethDet: a technology for biomarker development

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Abstract

Early detection and diagnosis of a disease in its presymptomatic form has to rely on biomarkers, and multiple laboratories are involved in their development and validation. In this article, we describe our work on a platform technology for a genome-wide analysis of DNA methylation while still using a small amount of sample – a biopsy, a section from a formalin-fixed paraffin-embedded tissue or a small volume (0.4 ml) of plasma from blood. This technology (methylation detection or MethDet) allows genome-wide association studies similar to the analysis of single-nucleotide polymorphisms. Instead of mostly static genetic differences, the MethDet technology tests disease-dependent changes of epigenetic makeup, which is closely related to the gene expression pattern of a disease. The MethDet assay has the capacity to utilize highly fragmented DNA (e.g., cell-free circulating DNA from plasma) to identify disease-specific changes, effects of treatment or changes in the disease activity.

Keywords

biomarker; cancer; cell-free circulating DNA; differential diagnosis; DNA methylation; genome-wide; multiple sclerosis; predictive; prognostic

Biomarkers

A biomarker is ‘a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working Group [1]).

The need for precise biomarkers in modern medicine is unquestionable. This need stems from less-than-efficient techniques for early disease detection that are too complicated, too inaccurate or too expensive for population-wide screening. The latter consideration is especially important as the population ages and healthcare budgets are posed to explode. Not all early detection techniques are based on biomarker analysis – according to the Biomarkers Definitions Working Group, a biomarker has to be ‘objectively measured and evaluated’ [1]. By this definition, subjective assessments of a radiologist (e.g., mammography) or a pathologist (biopsy or cytology sample), while extremely important for disease detection, are not biomarkers by themselves. In this context, prostate-specific antigen, cancer antigen 19–9 and 125 are true diagnostic biomarkers, even though their utility for early disease

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detection is rather limited. The concept of personalized medicine – a treatment that is tailored to the needs of an individual – requires predictive biomarkers to stratify patients for therapy. In many cases, these biomarkers form an integral part of the companion diagnostics packages essential for the development of more effective drugs. Biomarkers for monitoring of drug efficacy and prognosis of outcomes complete the known biomarker universe.

Development of the MethDet assay

Development of the methylation detection, or MethDet assay, began with the search for a stable substrate that reflected long-term changes in gene expression. We wanted to exclude short-term fluctuations that could be attributed to variations in the normal physiology, such as circadian changes or changes induced by the menstrual cycle. The pioneering work of several groups [2–10] suggested that DNA methylation had the optimal combination of substrate stability and links to long-term changes in gene expression, which could have been harnessed to build a diagnostic biomarker. Two major issues had to be resolved – location and technique, or where and how to analyze DNA methylation. A tissue-based biomarker had very limited applications for diagnosis if the goal was early detection of an unknown primary tumor because the target tissue was unknown (the situation is quite different when the location of the tumor is established). Fortunately, cell-free circulating DNA in blood [11–13] had offered a distal reflection of tumor-related system-wide changes, thus resolving the first part of the ‘where’ question. The second part was determined by our (always) incomplete knowledge of DNA methylation, its mechanisms and targets, suggesting that any predetermined set of genes would be suboptimal and a broader search for genome-wide associations was required to develop a useful biomarker. Thus, from its very beginning, the MethDet technology targeted the analysis of multiple genes by microarrays in the discovery phase with the understanding that the clinical test would evaluate only the best genes using much simpler techniques. When the MethDet was planned, the choice of techniques for the analysis of DNA methylation was limited to enzymatic methods employing methylation-sensitive restriction enzymes and chemical conversion methods based on bisulfite modification. At that time, bisulfite-based approaches were the mainstream of methylation analysis of a few defined promoters when an abundant sample was available [14–17]. The need for an abundant sample is still the most significant limitation of bisulfite-based techniques owing to inherent problems of bisulfite conversion – degradation of DNA by harsh chemical treatment [18] and reduced complexity of DNA after conversion, which contains A, T and G with only a few C. Moreover, DNA strands after conversion are no longer complementary, and sequence differences can lead to PCR amplification bias [19]. Enzymatic methods did not have these limitations and could be used for the analysis of multiple fragments that had recognition sites for a methylation-sensitive restriction enzyme of choice [20–22]. A combination of methylation-sensitive restriction enzyme with PCR amplification and microarray readout seemed to fulfill requirements for low sample input, sensitive detection of differential methylation and high-throughput analysis of targets.

Different approaches to methylation analysis now available are summarized in Figure 1 [23]. First, methylated and unmethylated copies of a fragment have to be made different before any amplification, because amplification will make both copies identical by substituting methylated cytosines with their unmethylated counterparts in the amplified product. One way to differentiate methylated and unmethylated fragments is by introducing sequence differences using either bisulfite conversion or digestion with a methylation-sensitive restriction enzyme. Another possibility is physical separation using selective retention of methylated fragments by methylated DNA-binding proteins or antimethylated cytosine antibodies. The most important part of methylation analysis is completed at this time, because the rest of the procedure (amplification and detection) serves to uncover changes introduced by the differentiation step. From this perspective, various microarray-based

techniques or next-generation sequencing are just different means to visualize differences that have been already introduced. Compared with bisulfite modification [18] or affinity purification, digestion with a methylation-sensitive restriction enzyme results in high-quality, high-yield DNA with only a minimal chance of degradation or irretrievable loss. This is an important issue because of the possibility of a preferential loss of specific fragments, which may depend on DNA sequence, methylation or tertiary structure; such loss is very difficult to control when only small amounts of the initial sample are available. In addition, reaction with a restriction enzyme can be driven very close to completion, so that neither false-positive nor false-negative results are registered. The situation is different for chemical treatment by bisulfite, which (at a low rate) also modifies methylated cytosines [24]. Practically, this means that both false-negative (unmethylated cytosines remain unmodified) and false-positive (methylated cytosines that are nonetheless modified) results are always possible [15].

Affinity-based differentiation by antibodies or proteins that bind methylated DNA has inherent limitations of its own – similar to other affinity-based methods, the efficacy of binding depends on the density of methyl groups within the DNA fragment [25]. As a result, a large fragment with a single methylated cytosine will be retained poorly, while a small fragment that contains multiple methylation sites will have a much better chance of retention. Normalizing the length of fragments can partially resolve this problem, although unequal affinity of fragments with different methylation content still remains a problem [25].

While developing the MethDet assay, we encountered several problems mostly linked to the small amount of input sample. These problems started with the seemingly simple task of DNA isolation – all of the tested column-based techniques produced unacceptable losses of the input sample. Fortunately, a DNAzol®-based technique of the Molecular Research Center, Inc. (OH, USA) proved successful, with losses estimated to be below 20% of the input sample (we are cognisant that the labor-intensive DNAzol procedure is suboptimal for a diagnostic clinical laboratory, so an alternative approach will have to be designed). Another task – measurement of isolated DNA – was resolved by the PicoGreen® assay, which allowed accurate and stable measurements down to 50 pg/ml.

Digestion of isolated DNA by a methylation-sensitive restriction enzyme also presented some problems. The first and foremost was selection of the enzyme, which had to be able to digest unmethylated fragments completely. This meant that type IIe restriction enzymes [26], and HpaII in particular [27], could create problems – these enzymes required simultaneous binding of two recognition sites, one being the target for cleavage and the other acting as an activator of enzymatic activity [28]. This suggested that the complete digestion of an unmethylated fragment was unlikely and a background of undigested DNA was to be expected. In addition, the methylation-sensitive enzyme had to be a frequent cutter, minimizing the overall constraint of the enzyme-based methylation test, which was by its nature limited to the analysis of the recognition sites for a selected enzyme. After testing several candidates, we chose Hin6I, an isoschizomer of HhaI and a type IIp restriction enzyme [101].

Once the enzyme was selected, reaction conditions had to be optimized to ensure deep digestion of very small quantities of DNA. After several unsuccessful attempts, we identified a slight elevation of temperature and an increase in pH that produced the deep digestion we sought – no visible band in the digested part of the sample after PCR and a bright band in the undigested control. Later on, these conditions had to be revisited to facilitate the analysis of heterogeneous samples.

Finally, amplification and signal detection steps had to be designed. Amplification of a limited number of fragments (MethDet-56) could be performed with fragment-specific primers flanking selected sites for Hin6I and used in multiplexed PCR reactions. This decision imposed a number of limitations on primer design, assembly of primers for multiplexing and the number of fragments amplified in each reaction. In addition, the need for a second round of amplification became obvious, so a second set of primers for nested PCR was designed. In hindsight, this step should have been avoided because fragment-specific primers limited the number of fragments that could be analyzed. Instead, sequence-independent amplification was adopted for genome-wide CpG analysis (details of the procedure will be disclosed in the pending patent application).

Unique features of the MethDet technology

Unique features of the MethDet technology in its current version are its capacity to routinely analyze samples with very low DNA concentrations while performing a comprehensive analysis for all elements in each sample. The MethDet assay can test 0.2 ng of DNA (compared with the minimum of 1.0 μg for the Illumina Infinium Methylation assay) and works well with fragmented DNA from formalin-fixed paraffin-embedded tissues or cell-free circulating DNA from blood (the Illumina website states that 'The Illumina platform works best with relatively intact, high-quality DNA. For the Infinium Assay, we recommend fragment sizes of at least 2 kb.'[102]). By contrast, the MethDet technique works best with approximately 500 base pair fragments when the chance to encounter several Hin6I sites in a fragment is lower. As a result, the MethDet assay is in a unique position to offer genome-wide analysis for any type of samples – from tissue culture to fresh-frozen and formalin-fixed, paraffin-embedded tissues, saliva, sputum, spinal fluid, blood and urine. This wide-ranging capacity is tempered by the dependence on methylation-sensitive restriction enzymes, which provide data on the methylation status of recognition sites but cannot evaluate methylation outside of them.

Currently, two clinical-grade biomarkers are being developed for pancreatic cancer and multiple sclerosis (MS). In the pancreatic cancer project, a panel of blood-based biomarkers is sought for differential diagnosis of ductal adenocarcinoma, chronic pancreatitis and intraductal papillary mucinous neoplasm. In the MS project, the goal is to differentiate patients with a clinically isolated syndrome who later develop definitive MS from those who do not. Validation of these biomarkers will be performed using a clinical-grade platform rather than a microarray-based discovery system to reduce expense and simplify transition to inter-laboratory and prospective testing. Successful validation will provide irrefutable proof that blood-based biomarkers for differential diagnosis can be developed using the MethDet assay.

Potential applications of the MethDet assay

Potential applications of the MethDet assay are manifold – it can be used to detect differences of methylation in virtually any situation. On the diagnostic side, blood-based methylation biomarkers can be used for the early detection of disease and its differential diagnosis; several projects indicate that inflammatory processes, benign disease and cancer produce different patterns in the blood [29,30]. Likewise, precancerous conditions and cancer are described by different methylation patterns in cell-free circulating DNA (advanced adenomas of colon and atypical ductal hyperplasia of breast) [Cassinotti E, Melson J, Liggett T *et al.*; Manuscript Submitted], while different histological subtypes of the disease can also be distinguished by blood analysis (adenocarcinoma and squamous cell carcinoma of the lung) [Liggett T, Shrestha S, Melnikov A; Manuscript Submitted].

The MethDet assay can be applied in different areas – we have used it for detection of MS [31], while reports of methylation differences in other diseases (e.g., Parkinson's [32,33], Alzheimer's [34], schizophrenia and other psychiatric disorders [35], and drug addiction [36]) indicate that their blood-based detection and monitoring will also be possible. It is also likely that DNA methylation patterns reflect environmental and nutritional effects [37,38], creating yet another potential application of the MethDet assay.

Treatment-induced changes indicate that the MethDet technology can be used to monitor recurrence (in preparation) and treatment [29] by analyzing blood, which will be valuable for different diseases. Moreover, treatment-induced changes suggest that emerging resistance to a specific drug is likely to alter methylation patterns, thus providing a way to detect resistance before it becomes clinically obvious. Finally, the existing preliminary data strongly suggest that predictive biomarkers for a specific treatment regimen can be detected in excised tissues [Melnikov A, Shrestha S, Levenson V; Unpublished Data], thus expanding the applications of the MethDet assay to personalized medicine and companion diagnostics.

Factors limiting practical application of the MethDet assay

Currently, two steps can present a challenge for routine use of the clinical-grade test – one is the isolation of DNA, which is still a manual process and requires experience, and the second is pre-amplification of isolated DNA, which is performed after treatment with Hin6I to increase the amount of DNA and make the analysis of multiple genes possible. New developments in the automatic isolation of miniscule amounts of DNA without potentially devastating selective losses are being tested; once such a system is identified, it will probably require regulatory approval for clinical applications. Preamplification should be easily transferrable to one of the existing small-volume liquid handlers.

We expect that this test will be inexpensive, in the range of US$200–800, depending on the features analyzed. This should facilitate adoption by medical providers, individual patients and the insurance industry, although upcoming changes in the medical field make these predictions rather uncertain. Commercialization of this test will require forward-looking investor(s) to complete validation and clinical trials and to obtain regulatory approval.

Expert commentary & five-year view

Rapid development of novel techniques for systemic analysis produces new candidate biomarkers much faster than any time previously. Next-generation sequencing [39], autoantibodies [40,41], metabolomics [42], glycomics [43] and other 'omics' technologies are bound to provide valuable biomarkers. Assuming that none of them will solve all possible problems, the most challenging task will be the selection of the best available biomarkers and the integration of their informative value into a clinically and economically viable assay. It appears unlikely that a technically complicated multistep test with elaborate analysis will be readily adapted by a clinical laboratory, even if the test itself is inexpensive. A genetic test might not be suitable for early disease detection either, since the presence of a specific mutation or a SNP indicates increased risk, rather than the actual presence of a disease. On the other hand, when combined with other biomarkers, a genetic test can improve selection of therapy for a particular patient or identify a group of patients who are most likely to benefit from a specific treatment. System-wide approaches are very likely to critically improve disease classification, which will lead to breakthroughs in mechanistic studies of disease origin, its development and prognosis. Similarly, system-wide approaches will improve our understanding of drug interactions, which will facilitate the development of better drugs with increased efficacy and reduced toxicity, albeit for a somewhat smaller group of patients. Importantly, the very process of drug development will most likely

become less expensive with the early elimination of ineffective leads and better selection of target populations of patients. It is highly probable that DNA methylation analysis by the MethDet technique will play a major role in these advances in the next 5 years, once several important tasks have been completed:

- Development of a clinical-grade test for rapid validation of discovered biomarkers. The current version of the clinical assay is based on quantitative PCR with the comparison of threshold cycle values of Hin6I-treated and untreated parts of the sample. The difference of eight to ten cycles is considered significant (Figure 2);
- A software system for data analysis will be designed, because otherwise the objective test will require subjective evaluation of the results;
- To make adoption easier, the test should produce a simple binary result at each classification step. For example, early diagnosis should identify healthy individuals and patients with as-yet unspecified disease, then patients with benign disease should be separated from those with inflammatory and malignant lesions, and then each of these has to be identified. To a certain extent, this process should be similar to the taxonomic classification of a species;
- Validation is the final multistep process that can make or break the biomarker assay. Analytical and clinical validations are required to confirm the performance of the test, and then the clinical value of the results is established in different laboratories with retrospectively collected samples [44]. Once confirmed, the test can be advanced to prospective studies.

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Key issues

- The methylation detection technology (or MethDet) technology has grown from its humble beginnings of gel-based analysis to genome-wide testing of all CpG islands in the human genome.
- The process has been adopted for testing of samples with very low DNA content, including fine-needle biopsies.
- These changes allow genome-wide analysis of CpG islands using cell-free circulating DNA from blood.
- Methylation of this DNA reflects the presence of different diseases in a disease-specific pattern.

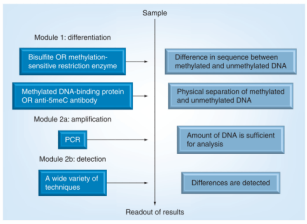

Figure 1. Stages of DNA methylation analysis

Module 1 produces differences between methylated and unmethylated sequences. These differences can be either sequence-based (bisulfite modification or restriction digest) or rely on physical separation (antibody against methylated cytosine and methylated DNA-binding protein). Detection of differences is carried in Module 2, which relies on PCR amplification to increase the amount of DNA (Module 2a) for analysis. Analysis of differences is performed in Module 2b using a wide variety of techniques – from sequencing to microarrays to mass spectrometry.

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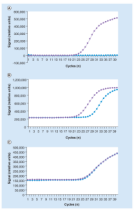

Figure 2. Clinical-grade assay based on quantitative PCR (Taqman®)
(A) Unmethylated fragment. **(B)** Partially methylated fragment. **(C)** Methylated fragment. Circles represent undigested control DNA; diamonds represent Hin6I-digested DNA.