

Molecular diagnostic applications of DNA methylation technology

by Dr Susan Cottrell

The methylation of CpG islands associated with growth control genes can contribute to cell division and carcinogenesis. By discovering sites of aberrant methylation in tumours, we can identify potential new biomarkers suitable for diagnostic and prognostic testing. Currently used research technologies, such as methylation-specific real-time PCR assays, are suitable for development into clinical platforms.

Biology of DNA methylation in carcinogenesis

CpG dinucleotides (cytosines immediately followed by guanines) are underrepresented in the human genome. However, clusters of CpGs, called CpG islands, are often found either overlapping or near gene regulatory regions. CpG islands range from around 500-2000 base pairs in length, and the CpG density in these regions approaches the level expected based on the genome frequency of the individual nucleotides. In normal tissues, most CpGs outside CpG islands have a methylated cytosine, while CpGs within CpG islands are unmethylated. During normal physiological processes, such as X chromosome inactivation or gene imprinting, CpG islands can however become methylated. Methylation of CpG islands is associated with conformational changes in chromatin that are correlated with silencing of the nearby gene. During X chromosome inactivation for instance, chromatin condensation eliminates the transcription from one of the X chromosomes in each female cell. However, CpG islands near some genes can become aberrantly hypermethylated during the process of carcinogenesis [Figure 1]. Hypermethylation, and therefore loss of gene expression, of genes involved in the control of cell division could easily give a cell a growth advantage. Some of these hypermethylation events, such as the methylation of Glutathione S-transferase Pi (GSTP1) in prostate cancer, are thought to be critical early events in carcinogenesis [1]. Whereas CpG islands become hypermethylated, CpGs outside islands often lose their methylation in tumours, leading to genome instability [Figure 1].

The genes affected by hypermethylation of CpG islands are involved in many different cellular processes, including cell division, apoptosis, hormonal regulation, and cell adhesion. All studied tumour types are affected by CpG island hypermethylation; however, research suggests that the spec-

trum of methylated genes is unique in each type of cancer. For instance, p15 is frequently methylated in leukaemias but rarely in solid tumours such as breast, prostate, and colon [2]. Due to the prevalence and specificity of methylation changes, aberrant methylation provides a compelling opportunity for molecular diagnostics. The information encoded in patterns of methylation in patient samples can be used to detect cancer early in its development and to determine the optimal treatment for each individual.

Role of methylation in molecular diagnostics

Methylation in the promoter region of a gene is often correlated with lack of expression. Due to this regulatory function, methylation of particular CpG islands is associated with many functional characteristics of cells and tissues. The majority of research has focused on methylation differences between neoplastic and non-neoplastic cells. While patterns of methylation can illuminate important biological differences between cancerous and non-cancerous tissues, sites of aberrant methylation can also be used as a marker for the presence of cancer.

In many instances, the detection of small numbers of tumour cells in an otherwise normal tissue sample could improve cancer diagnosis and prognosis. For instance, methylation has been used to detect lymph node micrometastases in patients with gastrointestinal and lung cancer [3]. Methylation of GSTP1 has been used to improve the detection of cancer in prostate cancer needle biopsies [4]. In these types of applications, methylation markers are valuable either because of the ability of methylation detection assays to find small numbers of neoplastic cells, or because of a field effect of methylation markers of cancer that extends beyond the histological boundaries of the lesion. If the specificity is sufficient, both of these possibilities could result in improved detection of otherwise hard to find cancer cells [Table 1].

Although the detection of tumour in a tissue sample is valuable, identification of small amounts of tumour-derived DNA in plasma, serum or other remote media could have a far greater clinical impact. Tumour-derived DNA can be detected in non-cellular fractions of blood, such as plasma and serum, as well as in substances derived from organ

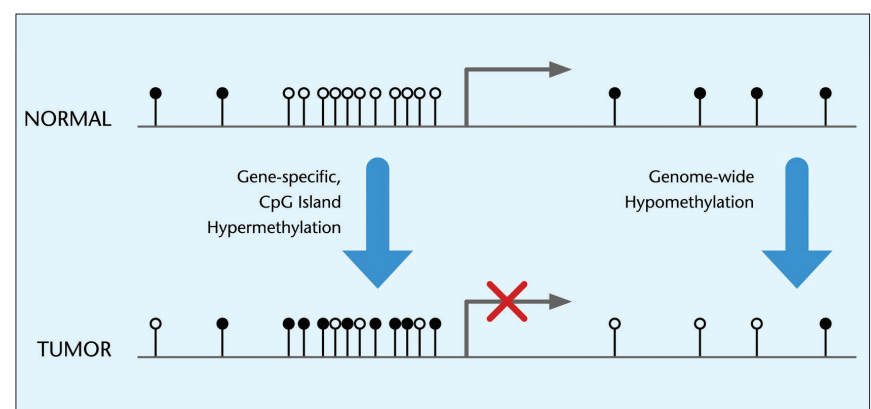


Figure 1. Patterns of methylation in normal and cancer cells. In non-cancerous cells, CpGs outside of CpG islands are often methylated, and CpGs within CpG islands are normally unmethylated. During the process of carcinogenesis, the overall levels of methylated cytosines go down, but CpG islands in regulatory regions of some genes become hypermethylated, leading to chromatin condensation and loss of gene expression.

lumens, such as faeces, urine and sputum [5]. After extraction of DNA from these samples, the methylation can be analysed to determine whether the pattern of methylation indicates the presence of tumour derived DNA. Thus, evidence of a tumour located in an organ such as the colon or lungs can be detected in a routinely obtained sample such as peripheral blood.

The detection of tumour DNA in blood has obvious value in the early detection of cancer. For a general screening assay, specificity will be crucial. Methylation markers in a blood-based screening assay must be almost completely unmethylated in peripheral blood cells, the primary contributor to non-tumour-derived DNA in serum or plasma. Furthermore, markers that are specifically methylated in certain types of cancers will allow for appropriate clinical follow-up. Through careful combination of markers, a panel can be constructed to achieve maximal sensitivity.

In addition to distinguishing cancer cells from non-cancer cells, methylation can also be used to stratify tumours into different prognostic or treatment response categories. Methylation of particular genes, such as Phosphoserine Aminotransferase [6] and PITX2 [7], can correlate with reduced disease-free survival. A diagnostic test based on this type of methylation analysis would allow clinicians to give their patients a clearer prognostic assessment and a more appropriate treatment plan. Similarly, methylation can also be used to predict response to certain treatments. MGMT methylation for instance, has been linked to the response of gliomas to alkylating agents [8]. The level of methylation of a gene, as opposed to the presence or absence of methylation,

| Gene | Disease | Application |
|-----------------|-----------------|---|
| GSTP1 | Prostate Cancer | Detection of tumours in serum/plasma/urine Detection of cancer cells in biopsies |
| MGMT | Glioma | Prediction of response to alkylating agents |
| PSAT1; PITX2 | Breast Cancer | Prognosis |

Table 1. Three promising DNA methylation markers for clinical application.

GSTP1: Glutathione S-Transferase Pi

MGMT: O(6)-methylguanine-DNA methyltransferase

PSAT 1: Phosphoserine aminotransferase

PITX2: Pituitary homeobox protein 2

will most likely be the prognostic indicator or predictor of drug response. Therefore, while detection of methylation in serum or plasma requires an analytically sensitive assay and a clinically specific marker panel, successful tumour stratification will require quantitative methylation measurement.

Technology for methylation analysis

Candidate methylation markers have been discovered in many ways. Techniques such as methylation-specific arbitrarily primed PCR, Methylated CpG island Amplification (MCA), Differential Methylation Hybridisation (DMH), and Restriction Landmark Genomic Scanning (RLGS) take advantage of methylation-specific restriction enzymes to scan the genome for aberrantly methylated CpG sites. The advantage of these methods is that they directly look for methylation differences. In contrast, candidates can also be identified indirectly using gene expression studies. Gene expression in cell lines treated with 5-azacytidine can be compared to mock-treated cell lines to find genes activated by this de-methylating agent. Some genes in the literature, such as known tumour suppressor genes with CpG islands, are also good candidates.

Further analysis of these marker candidates requires higher throughput methodology. By far the most commonly used assay in research labs is methylation specific-PCR (MSP) or the real-time version (MethyLight). The sample DNA is treated with sodium bisulphite to convert unmethylated cytosines to uracils, while methylated cytosines remain intact. In a gel based MSP assay, one set of primers amplifies the unmethylated version and one set amplifies the methylated version, and the presence of a band on a gel in each reaction determines the methylation state. In the real-time version, amplification with methylation specific primers and probes is normalised to the total amount of input DNA to determine the fraction of DNA methylated for each region of interest. Alternative marker analysis methods include oligonucleotide arrays, primer extension, and sequencing.

In many ways, methylation based markers are technically well suited for clinical use. In contrast to

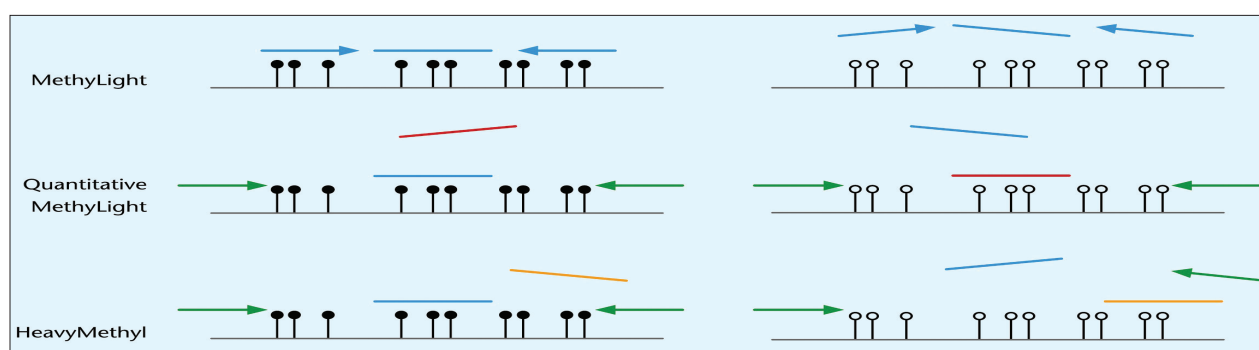


Figure 2. Real-time PCR assays for methylation marker detection and measurement. MSP-based MethyLight assays use primers and probes that bind, amplify and detect only the methylated (filled circles) version of the bisulphite treated target DNA. Quantitative MethyLight uses primers that amplify all target sequences, regardless of the methylation status. Competitive probes, one for the methylated targets (blue) and one for unmethylated targets (red), are used to measure the relative level of methylated target. HeavyMethyl uses an oligonucleotide blocker (yellow) to prevent binding of primers to unmethylated DNA. The methylated DNA is amplified and detected with a methylation specific probe.

RNA, DNA is a stable analyte. The methylation signal is not altered during the process of obtaining the clinical sample, and DNA is not rapidly degraded. Unlike protein markers, DNA methylation signals can be exponentially amplified by PCR or other methods to enhance sensitivity. Furthermore, short-term effects, such as circadian rhythms, do not alter DNA methylation patterns.

The bisulphite conversion step is thought to be the Achilles heel of methylation research. At this point, there are many alternative bisulphite conversion protocols used in research labs, and most are relatively time-consuming and labour intensive. In preparation for clinical use, the process needs to be shortened, the throughput must increase, and the results must continue to be robust and consistent. Since the quality of conversion is crucial to the performance of the assay, quality control measures must be in place to ensure complete conversion of patient DNA samples.

A methylation measurement or detection assay suitable for the clinic must be high-throughput (particularly for screening assays), quantitative, sensitive, and reasonably priced. MethyLight assays are suitable candidates to meet these criteria. These assays can detect a few copies of methylated target in a background of several thousand copies of unmethylated target. The real-time probe ensures that the

amplified product is the correct target and also allows for quantitative comparison of methylation levels. Integration of a MethyLight assay into a clinical lab would only require the presence of a real-time PCR platform. In addition to the MSP version of MethyLight, similar assays, such as HeavyMethyl [9] and Quantitative MethyLight [6], are also suitable candidates [Figure 2].

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