The implications of heterogeneous DNA methylation for the accurate quantification of methylation

DNA methylation based biomarkers have considerable potential for molecular diagnostics, both as tumor specific biomarkers for the early detection or post-therapeutic monitoring of cancer as well as prognostic and predictive biomarkers for therapeutic stratification. Particularly in the former, the accurate estimation of DNA methylation is of compelling importance. However, quantification of DNA methylation has many traps for the unwary, especially when heterogeneous methylation comprising multiple alleles with varied DNA methylation patterns (epialleles) is present. The frequent occurrence of heterogeneous methylation as distinct from a simple mixture of fully methylated and unmethylated alleles is generally not taken into account when DNA methylation is considered as a cancer biomarker. When heterogeneous DNA methylation is present, the proportion of methylated molecules is difficult to quantify without a method that allows the measurement of individual epialleles. In this article, we critically assess the methodologies frequently used to investigate DNA methylation, with an emphasis on the detection and measurement of heterogeneous DNA methylation. The adoption of digital approaches will enable the effective use of heterogeneous DNA methylation as a cancer biomarker.

KEYWORDS: biomarker ■ cancer ■ *CDKN2B* ■ digital PCR ■ high-resolution melting ■ *MGMT* ■ minimal residual disease ■ molecular diagnostics

Thomas Mikeska[†1,2], Ida LM Candiloro[1,2] & Alexander Dobrovic[†1,2]

[1]Molecular Pathology Research & Development Laboratory, Department of Pathology, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett Street, Melbourne, Victoria 8006, Australia
[2]The University of Melbourne, Victoria, Australia
[†]Authors for correspondence:
Tel: +61 3 9656 1807
Fax +61 3 9656 1460
alexander.dobrovic@petermac.org
thomas.mikeska@petermac.org

DNA methylation has been recognized to play an important role in developmental biology, aging and cancer etiology [1–5]. Many genes are deregulated by DNA methylation in cancer [6,7]. Aberrant DNA methylation associated with certain genes has attracted considerable interest as a potential biomarker for the early detection of disease onset, prognosis and choice of treatment, and the monitoring of disease after therapy [8–10].

In mammals, DNA methylation occurs principally at CpG dinucleotides. CpG dinucleotides are unevenly distributed throughout the genome and the majority are normally methylated [11]. Some regions of the genome show a high CpG density spanning hundreds to thousands of base pairs, and are termed CpG islands [12]. These CpG islands are often associated with the promoter regions of genes and are then generally unmethylated [13]. If the promoter CpG islands become methylated, either as part of a developmental or pathological process, this leads to the formation of a repressive chromatin complex and the gene is silenced [14].

Heterogeneous DNA methylation

DNA methylation is usually analyzed in the context of a PCR amplicon generated from bisulfite treated DNA. Each CpG position in each of the template molecules can be either unmethylated or methylated. For the amplicon, fully methylated means that all the tested CpG positions in the amplicon are methylated. Similarly, (fully) unmethylated means that all the CpG positions in the amplicon are unmethylated.

Methylation heterogeneity can arise at several levels. At the simplest level, it has been used to refer to a mixture of fully methylated and unmethylated alleles. A homogeneous mixture of cells may contain both unmethylated and fully methylated alleles, such as is the case for imprinted genes, such as *H19* [15]. Alternatively, a heterogeneous mixture of cells may comprise methylated and unmethylated alleles in varying proportions.

In this article, we will reserve the term heterogeneous methylation for the specific context where multiple alleles, which differ in the pattern of methylated and unmethylated CpG sites, are present. The term epialleles can be useful to describe these multiple alleles. Each unique pattern of DNA methylation for a given genomic sequence, including fully methylated and unmethylated, would comprise one of the possible epialleles that can exist in a sample.

Figure 1 shows all eight possible epialleles for a region comprising three CpG positions. It should be noted that it is impossible to distinguish the DNA methylation scenarios shown in Figure 1A & 1B by methodologies (e.g., pyrosequencing) that can quantify methylation at individual CpG sites.

future medicine part of fsg

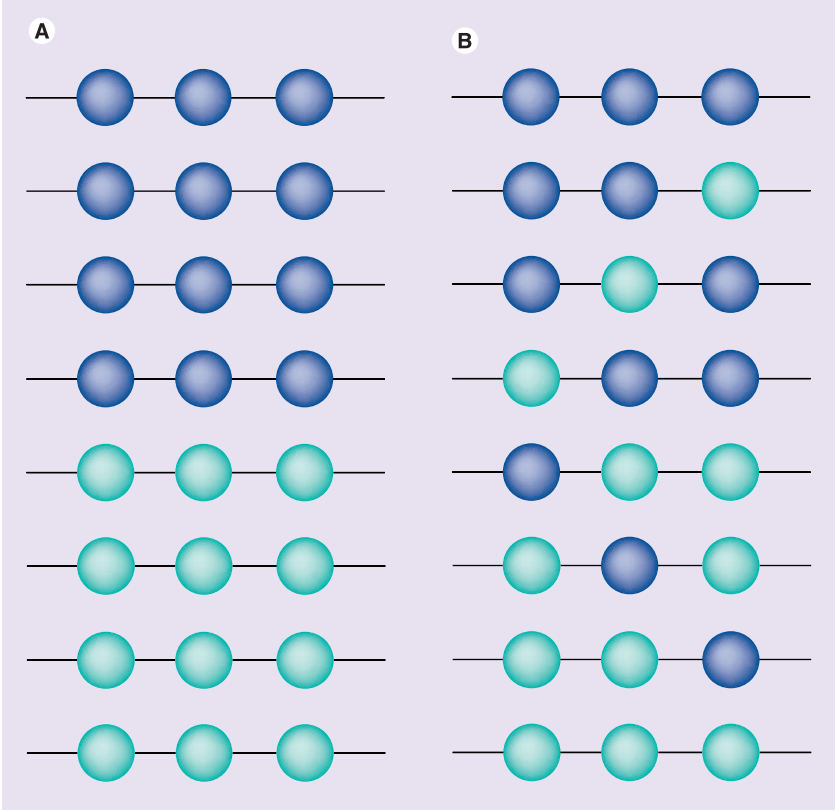

Figure 1. Two types of heterogeneous methylation. Each horizontal line represents an epiallele where the DNA methylation patterns of the region of interest contain three CpG sites. Green and blue circles are used to indicate unmethylated and methylated CpG sites. **(A)** A mixture of fully methylated and (fully) unmethylated epialleles. **(B)** Epialleles with all possible methylation patterns. Note that in **(A)** and **(B)**, the total amount of methylated CpG sites are equivalent but the fraction of methylated epialleles are markedly different.

If a CpG island becomes aberrantly methylated in cancer, heterogeneous methylation is frequently observed. The evidence for heterogeneous DNA methylation has largely come from bisulfite sequencing of single clones, for example for the DNA repair gene *MGMT* [16], the *RB1* tumor suppressor gene [17], the cell cycle progression inhibitor *CDKN2B* (*p15*) [18–20] and the proapoptotic gene *DAPK1* [21]. The spectrum of heterogeneous methylation usually varies between different samples. This has recently been illustrated for the *CDKN2B* (*p15*) gene, where acute myeloid leukemia (AML) samples are heterogeneously methylated to different extents [22].

The biology of heterogeneous methylation is incompletely understood. Heterogeneous DNA methylation profiles may result from heterogeneity within a sample [23]. However, it remains unclear whether this heterogeneity derives from multiple independent origins of methylation, a gradual accumulation of changes in DNA methylation during semiconservative replication of the DNA methylation pattern, or whether there is a continual flux of DNA methylation patterns within the mitotic progeny of a cell. The DNA methylation level may also increase for some gene loci during aging [24–26] and tumor progression [27].

One question that has not yet been resolved is how much DNA methylation is needed for recruitment of the repressive transcriptional machinery? For *CDKN2B*, a proportion of 30–40% of the potentially methylated CpG sites has been estimated, but the question remains open as this may well depend on the gene and the region of the promoter being examined [19].

The continually growing interest in investigating DNA methylation has led to the development of multiple approaches to detect DNA methylation at specific gene loci. Furthermore, the increasing use of DNA methylation as a biomarker of disease means that accurate quantification of the fraction of methylated alleles is of compelling importance. It is especially important if DNA methylation is to be used as a biomarker of response to therapy where quantitative estimations are desirable.

Methodology also plays a key role in deciding whether to call a locus methylated. Some loci of clinical importance may show low DNA methylation levels in normal tissues of some individuals, or may be heterogeneously methylated in cancer, or both. For example, methylation of the *MGMT* locus is an important predictive biomarker when certain alkylating chemotherapeutic drugs are used [28,29]. However, commonly used methodologies such as methylation-specific PCR (MSP) (reviewed later) are unreliable in the assessment of *MGMT* promoter methylation [30]. This derives from the heterogeneous methylation that this locus often shows [16], the low level of DNA methylation in the normal tissues of some individuals [31] and technical issues in the reliable performance of MSP. The region of a CpG island that is tested for DNA methylation may also play a crucial role [32–34], highlighting the importance of preliminary work to determine the likelihood of the methylation of particular regions or positions being more important or informative than others [34,35].

The analysis of DNA methylation has many traps for the unwary. In particular, the impact of methodology in measuring DNA methylation is often ignored. This is largely a consequence of the fact that most assays do not differentiate homogeneous and heterogeneous methylation.

In this article, we will assess the problems raised by heterogeneous DNA methylation for methodologies commonly used to investigate methylation at single gene loci. It is of considerable importance to know and understand the limitations of available approaches in order to interpret the data generated from a particular experiment.

Methods

All the methods described in this article use bisulfite-treated DNA as the starting material in PCR reactions. It is common to refer to a PCR product deriving from a single set of primers as an amplicon. In the case of heterogeneous DNA methylation, the PCR product is a complex mixture of many amplicons and heteroduplexes between those amplicons. In the interest of simplicity, however, we will use the word amplicon even when there are obviously many amplicons.

Several technical issues are common to most methods. A poor bisulfite conversion rate will result in an overestimation of the calculated DNA methylation level and will subsequently influence the accuracy of the calculated DNA methylation by increasing the background. Poor conversion is particularly critical when MSP-based techniques, which detect methylation based on primer binding, are being used.

A potential PCR amplification bias [36,37] towards either methylated or unmethylated templates further complicates DNA methylation analysis. The bias may apply to different extents for all methods utilizing methylation independent PCR primers (i.e. PCR primers that do not contain CpG sites). The inclusion of a limited number of CpG sites at the 5′-end of the primers allows the use of temperature dependent selection for methylated sequences to compensate for the PCR bias to unmethylated sequences [38].

Outline of methodologies

After PCR amplification of bisulfite modified DNA, a methylated CpG dinucleotide remains as a CpG, whereas an unmethylated CpG becomes a TpG. The different methylation assessment techniques then take advantage of this difference.

There are four broad groups of approaches depending on the type of primers and the type of analysis (FIGURE 2). The first group uses primers that are specific for the methylation status of the template, whereas the other groups use methylation independent primers that are intended to amplify the bisulfite modified DNA irrespective of its former methylation status.

The first group comprises methods that specifically amplify sequences based on their DNA methylation status (FIGURE 2A). It includes MSP and its quantitative offshoots, including MethyLight and sensitive melting analysis after real time-MSP (SMART-MSP).

The second group comprises scanning methods that differentiate methylated, partially methylated and unmethylated sequences on the basis of their different physicochemical properties (FIGURE 2B). Single-strand conformation analysis utilizes the different single strand conformations formed by sequences of slightly or markedly different base compositions, whereas denaturing HPLC, denaturing gradient gel electrophoresis and high-resolution melting (HRM) measure the melting behavior of DNA duplexes.

The third group comprises of methodologies that investigate the methylation level of one or more CpG sites in a particular PCR product using a sequence dependent approach (direct bisulfite sequencing, bisulfite pyrosequencing, mass spectrometric approaches, methylation sensitive-single nucleotide primer extension [MS-SNuPE] and combined bisulfite restriction analysis [COBRA]). The DNA methylation level for a given CpG site is displayed as an average of its methylation over all the different epialleles amplified during PCR (FIGURE 2C).

The final group of approaches examines the methylation of individual PCR products (FIGURE 2D), either cloned from PCR products or digitally generated from single templates, most commonly by using sequencing or HRM-based methodologies. The investigation of multiple DNA methylation patterns can be used to generate a DNA methylation pattern profile for a PCR product mixture if desired, but is more useful for the analysis of the complexity of methylation patterns and for the quantification of methylated epialleles.

Bisulfite sequencing of clones (bisulfite genomic sequencing)

This belongs to the final group of approaches, but as sequencing of individual clones was the first PCR-based methodology for the analysis of bisulfite modified DNA and remains a *de facto* gold standard, it is appropriate to begin with the consideration of this methodology. The amplicons generated during PCR amplification are cloned and individual clones are analyzed by Sanger sequencing [39]. Each CpG position of a single PCR amplicon of a single clone provides a binary answer; the CpG position is either methylated or unmethylated. Each clone gives the methylation pattern of a single epiallele.

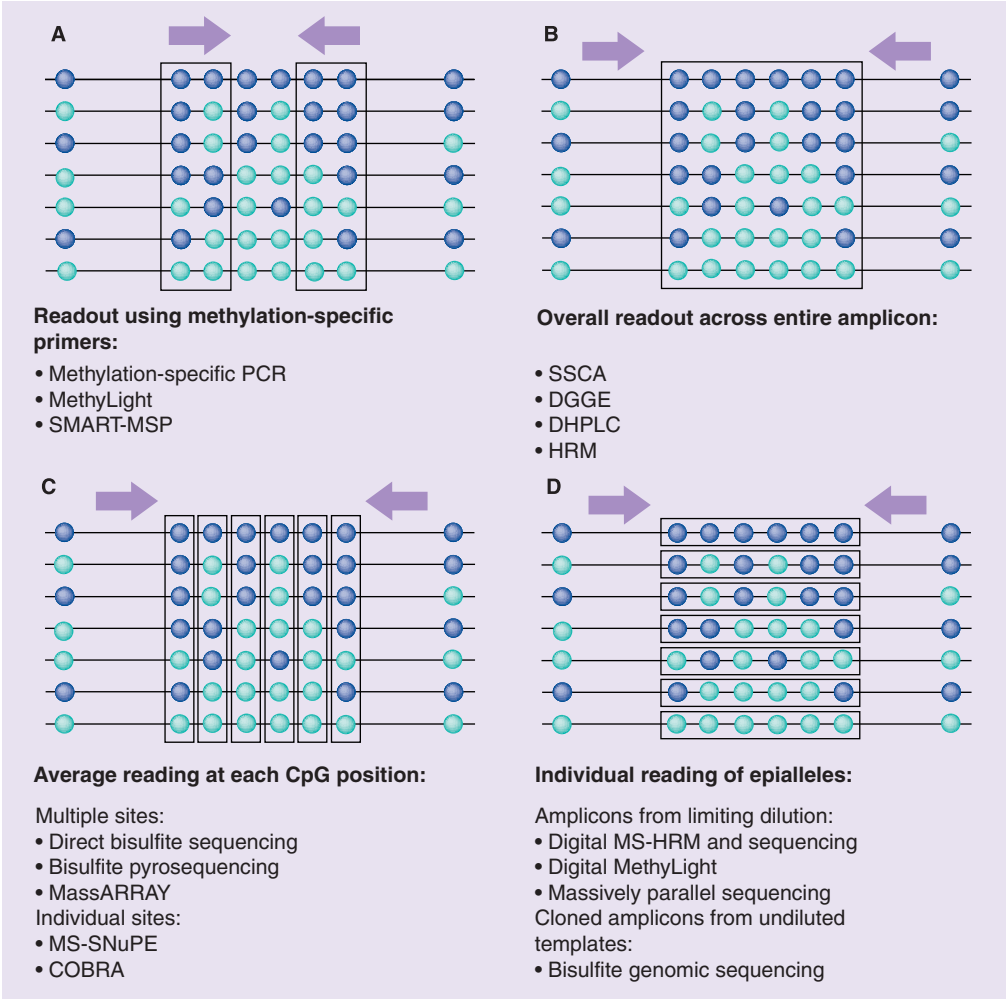

Figure 2. Techniques used to examine DNA methylation. Each horizontal line represents an epiallele. Green and blue circles are used to indicate unmethylated and methylated CpG sites. The positions of the horizontal arrows represent the regions for primer placement, whereas the boxes indicate which (and how) CpG sites are analyzed by the different methodologies. **(A)** MSP and its derivative techniques normally examine DNA methylation only in the primer (and, where applicable, probe) binding sites. Primers are designed to amplify either methylated or unmethylated DNA, but not a combination of both. The output from such techniques gives a single value, which is not necessarily a true reflection of the actual amount of methylation present when it is heterogeneous. The methods in **(B–D)** use methylation independent primers to amplify bisulfite modified DNA regardless of methylation status. **(B)** Scanning techniques can be useful for the identification of heterogeneously methylated DNA, but cannot quantify it accurately, or in some cases, at all. **(C)** These techniques analyze methylation at each individual CpG site (occasionally as several adjacent CpG sites for MassARRAY® [Sequenom, CA, USA]), and give an average methylation value for each CpG site over the entire amplicon population. MassARRAY and sequencing techniques are able to assess CpG sites across entire amplicons, however, MS-SNuPE and COBRA are used to assess a subset of CpG dinucleotides. **(D)** These methods give a result at an individual epiallele level. There are two strategies for achieving this: cloning or digital PCR following limiting dilution of templates. The latter has the advantage of avoiding PCR bias effects.
COBRA: Combined bisulfite restriction analysis; HRM: High-resolution melting; MSP: Methylation specific PCR; MS-SNuPE: Methylation sensitive single nucleotide primer extension; SMART-MSP: Sensitive melting analysis after real time-methylation-specific PCR; SSCA: Single-strand conformation analysis.

The analysis of many clones provides detailed information to characterize the heterogeneity of a sample. Furthermore, this approach allows the quantification of individual epialleles as well as for each CpG position among the epialleles. The data analysis can be assisted by the appropriate software tools [40,41].

It is a vexed question as to how many individual clones need to be analyzed to provide a comprehensive overview of a sample with

heterogeneous DNA methylation. Sequencing of clones is expensive and labor intensive. As the cost is dependent on the number of clones analyzed, usually less than 20 clones are sequenced per sample. In most cases this will be insufficient, as heterogeneously methylated samples are usually complex in nature. The information obtained therefore is accordingly compromised by stochastic effects. It should also be noted that the clones are derived from amplified PCR products and thus any PCR bias will be reflected in the clonal population.

Methods determining individual CpG methylation levels in PCR products

These methods determine the DNA methylation level at each CpG position of the totality of the PCR products generated during PCR amplification. Direct bisulfite sequencing, bisulfite pyrosequencing and MassARRAY® (EpiTYPER®) (Sequenom®, CA, USA) of PCR products allow the estimation of DNA methylation levels as an average for each CpG position. COBRA and MS-SNuPE examine a more limited range of CpG sites. These methods may indicate methylation homogeneity or heterogeneity when individual CpGs are methylated to different extents, but do not provide single allele resolution. As a consequence, the fraction of methylated epialleles is underestimated when heterogeneous methylation is present, as the methylation frequency at each CpG site is less than the frequency of methylated epialleles (FIGURE 1).

Direct bisulfite sequencing

Direct bisulfite sequencing of PCR products allows the semiquantitative to quantitative estimation of DNA methylation level as an average for each CpG position [39,42]. The DNA methylation level is calculated from the base proportions represented by different fluorescent dye signals in Sanger sequencing by capillary electrophoresis. It also allows ready detection and quantification of incomplete conversion by the examination of residual cytosines at the non-CpG cytosine positions.

When a mixture of unmethylated and fully methylated templates or a heterogeneously methylated sample is subjected to direct bisulfite sequencing, the four-dye electropherograms may show an increasingly poor peak quality with increasing nucleotide number, which usually complicates quantitative analysis because of the sequence-dependent differences in mobility of the different amplicons [43]. Finally, the peaks may get so broad that they tend to split. As a consequence, the four-dye electropherogram is a result of the overlapping and interfering individual electropherograms of the unmethylated and fully methylated epialleles or the heterogeneously methylated epiallele subpopulations.

By contrast, direct bisulfite sequencing of fully methylated or unmethylated amplicons results in a relatively clean four-dye electropherogram, therefore delivering a reliable nucleotide sequence and clear information about the DNA methylation level.

It must also be taken into account that the different fluorescent dyes of the labeled dideoxy terminator nucleotides (ddCTP/ddTTP for the sense strand and ddGTP/ddATP for the antisense strand) show different quantum efficiencies, resulting in different relative peak heights [43]. A sequence specific context and different efficiencies for the terminator incorporation may further cause peak heights different from the theoretical ratio.

An adaptation that addresses this issue and allows greater sensitivity (~10%) uses the sequencing of only C/T bases in the sense strand or A/G bases in the antisense strand. This approach improves the resolution of fluorescent peaks generated during gel separation, removing idiosyncrasies introduced when using multiple fluorescent dyes [44].

The detection limit of sequence variants by Sanger sequencing is approximately 10–20% of variant bases [45–47]. A similar detection limit has been reported for methylated bases [44,48]. For heterogeneously methylated amplicons, one would expect sequencing to underestimate the fraction of epialleles. The resulting electropherogram is mainly a result of the predominant epialleles and, due to averaging, the total amount of DNA methylation is therefore underestimated.

Bisulfite pyrosequencing

Bisulfite pyrosequencing is a sequencing-by-synthesis technique that has become increasingly popular for the analysis of DNA methylation as it provides relatively accurate quantification of methylation at individual CpG positions in a cost-effective manner [49–51]. The DNA methylation ratio is calculated from the signals observed for the two incorporated nucleotides, which determines the methylation status of a CpG position in a strand dependent manner (e.g., C/T on the sense strand and G/A on the antisense strand).

Bisulfite pyrosequencing is to some extent similar to direct bisulfite sequencing, but as it is not a mobility based method, the presence of

heterogeneous DNA methylation does not cause loss-of-phase of the sequencing information. Also the quantification of the DNA methylation level is more accurate. Finally, the detection limit at each CpG position is about 5% [52,53], and is therefore more sensitive than the detection limit of approximately 10–20% for direct bisulfite sequencing. This remains less sensitive than optimally desired for a biomarker.

Bisulfite pyrosequencing is somewhat disadvantaged by the relatively shorter length of sequence that can normally be analyzed (~100 bp [54,55]), but in many cases this is more than compensated by its ability to read cleanly at the beginning of a sequence, which is of paramount importance when short fragments are being analyzed, such as is recommended when analyzing DNA from formalin-fixed paraffin-embedded tissues.

While it has been claimed that bisulfite pyrosequencing is suitable for the analysis of heterogeneous methylation because of its more accurate quantification [54], the quantitative results for the consecutive CpG positions analyzed reflect the average methylation level per CpG position. Thus, although heterogeneous DNA methylation can often be identified, the fraction of heterogeneously methylated epialleles cannot be quantified.

DNA methylation analysis via mass spectrometry

The commonly used mass spectrometry methodology is Sequenom MassARRAY (EpiTYPER). This method is based on base specific cleavage of single stranded RNA from amplicons generated during PCR amplification of bisulfite treated DNA. The mass of the resulting fragments is dependent on the methylation status of the amplicon's CpG dinucleotides. The cleavage products are detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [56].

The methylation ratio for a single CpG position or a CpG unit is calculated from the signal intensities of the resultant cleavage products. The redundancy of certain cleavage products allows cross validation and the reliable determination of methylation levels [56]. DNA methylation levels are presented as a (semi)-quantitative result for a single CpG, or for multiple CpG positions, as an average of all PCR products generated during PCR amplification.

The MassARRAY approach delivers similar results to direct bisulfite sequencing but with a higher DNA methylation detection sensitivity. The sensitivity is similar to that of bisulfite pyrosequencing but the length of sequence analyzed can be much greater [57]. Nevertheless, the coverage of CpGs within the PCR product analyzed is somewhat restricted owing to the inability to differentiate the masses of certain cleaved products. Some cleavage products may also contain multiple CpGs (as many as six or seven), which are then unable to be resolved. Usually, up to 80% of all CpG positions of an amplicon can be interrogated within a single cleavage reaction, delivering a DNA methylation profile that is near complete [56].

MS-SNuPE

Methylation sensitive-single nucleotide primer extension is an adaptation of the single-nucleotide primer extension assay, which was originally introduced to type SNPs [58]. It is used to quantify the relative levels of cytosine and thymine at single CpG sites, essentially treating CpG sites as SNPs. Primer extension is performed on an aliquot of a PCR product generated from bisulfite treated DNA. The primer is placed immediately adjacent to the C of a CpG position. When the primer is extended, the inserted nucleotide depends on the DNA methylation present at that particular CpG position on the template. The calculated ratio is an average DNA methylation value at the CpG position of all different PCR products present. The DNA methylation ratio is quantitative and can be calculated by different detection systems utilizing radioactivity [59,60], peak heights in a chromatogram [61] or fluorescence [62].

A major constraint of this approach is the limited access to certain CpG sites of the PCR product. The typical high CpG density of many regions limits the positions for an appropriate extension primer design, which should preferably bind regardless of methylation status. MS-SNuPE may be multiplexed [59,61,62], but this approach is rarely used. A multiplex primer extension reaction is often not feasible, which limits the investigation of DNA methylation to a single CpG position. When only a single CpG dinucleotide is investigated, the determination of heterogeneity of DNA methylation is not possible and the overall methylation can be underestimated.

COBRA

Combined bisulfite restriction analysis is based on digestion with certain restriction endonucleases of the amplicons present after PCR amplification of bisulfite treated genomic DNA [63]. The digestion reaction is separated by gel electrophoresis and the restriction patterns allow an estimation or calculation of the DNA methylation level of one or

more CpG position(s) as an average of the different PCR products generated during amplification. Depending on the restriction endonuclease and the monitoring system used, the digestion pattern delivers qualitative to semiquantitative results.

The limited number of cutting sites for a particular restriction endonuclease in an amplicon allows the analysis of one or a few CpG positions, which provides a limited overview of the DNA methylation profile and pattern present in a sample. This problem can be partly solved by using several restriction endonucleases, but this approach is limited by the relatively large amounts of PCR product required per enzyme. Although methylation heterogeneity for different CpG sites recognized by the same restriction endonuclease can be observed, quantification is limited.

The estimated methylation at a given CpG position is dependent on the cutting efficiency of the restriction endonuclease chosen. This can be highly dependent on the sequence context. An impaired cutting efficiency will lead to an underestimation of the DNA methylation level.

A further complication pertinent to heterogeneous products is the formation of heteroduplexes within the PCR products. As the restriction endonuclease examines both strands of DNA, heteroduplexes result in lower efficiencies, and therefore underestimation of DNA methylation [64].

Methylation sensitive HRM

In methylation sensitive-HRM (MS-HRM), the detection of DNA methylation is based on the use of a fully saturating double stranded DNA binding dye to monitor the different melting profiles derived from different samples and appropriate controls after PCR amplification of bisulfite treated DNA [65]. Methylated epialleles melt later than unmethylated epialleles as a consequence of the greater number of hydrogen bonds in CpG dinucleotides compared with TpG dinucleotides.

Methylation sensitive-HRM is an adaptation of the earlier melting curve methodology [66]. The melting profile of a sample is compared with melting profiles of controls where the methylation level is known. Homogeneous and heterogeneous DNA methylation can be distinguished by their characteristic melting profiles. MS-HRM has effectively replaced earlier but now rarely used DNA methylation scanning methodologies, such as single-strand conformation analysis [67], denaturing HPLC [68,69] and bisulfite denaturing gradient gel electrophoresis [18].

With MS-HRM, the DNA methylation level of a homogeneously methylated sample can be estimated in a semiquantitative to quantitative manner and the detection limit can be in the range of 0.1–1% [70]. However, the presence of heterogeneous DNA methylation results in a complex melting profile that does not allow the ready estimation of the amount of methylated epialleles; the result is largely qualitative.

Methylation-specific PCR

Methylation-specific PCR was the first of the nonsequencing bisulfite-based methods and remains a widely used DNA methylation analysis method. In its original form, two PCR reactions with primer pairs specific for fully methylated and unmethylated sequences were used for the amplification of bisulfite-treated genomic DNA, and the PCR products were analyzed by gel electrophoresis [71].

Currently, it is common for only methylation specific primers to be used. The result obtained is qualitative and the band intensity of the reactions cannot be used to estimate the amount of methylated alleles. However, as the method is nonquantitative, even low background levels of DNA methylation may give a positive result. Due to this and other potential traps (reviewed in [72,73]), the results of this easily performed test should be interpreted with caution. In particular, incomplete conversion will increase the risk of false-positive results.

Whereas, with fully methylated and unmethylated templates, it is desirable to run MSP under highly stringent conditions to select against incompletely converted templates, this will also select against templates that are partially methylated in the primer recognition sequences.

MethyLight

MethyLight is the name commonly given to quantitative analysis of DNA methylation using a TaqMan® (Applied Biosystems, CA, USA) fluorescent probe to measure amplification in real-time. The most common procedure for MethyLight is based on MSP and utilizes methylation specific primers in combination with an internal methylation specific TaqMan fluorescent probe, and we will refer specifically to this version in the subsequent text [74–76]. The probe not only makes MSP quantitative by allowing real-time analysis but also reduces the risk of false-positive results due to incomplete conversion, as it generally contains one or more additional CpG sites.

When analyzing a homogeneously methylated sample, the estimation of the DNA methylation level by MethyLight is highly quantitative. However, heterogeneous DNA methylation will influence the annealing of both the primers and the probe in different and complex ways and the result will be at best only semiquantitative. Furthermore, the probe adds a strong bias towards the detection of fully methylated templates.

It was estimated that methylation can be detected by MethyLight in a 10,000-fold excess of unmethylated epialleles [76]. Nevertheless, heterogeneous methylation patterns will hamper any true quantification and reduce the sensitivity. Such sensitivities also require the input of a sufficient amount of bisulfite modified DNA.

A digital (PCR amplification from single templates) version of MethyLight has been developed using limiting dilution of templates [77]. This counting based approach retains some of the disadvantages of MethyLight for the analysis of heterogeneously methylated DNA, whereas PCR bias *per se* is not an issue, there may be partially methylated templates that either do not amplify or are not detected by the probe. Moreover, individual epialleles are not distinguished they are either amplified or not amplified.

More recently, fluorescent dyes have been used to monitor PCR amplification in real-time [78]. Most of these methods use SYBR® Green (Applied Biosystems, CA, USA) as the fluorescent dye, but SMART-MSP [73] uses a fully saturating double stranded DNA binding fluorescent dye, which not only allows real-time analysis but also enables investigation of the MSP product by HRM. Fluorescent approaches are quantitative when dealing with homogeneously methylated sequences and when normalized relative to a control assay. They do not use a probe and thus the initial PCR needs very careful optimization. SMART-MSP is quality controlled via a post-PCR HRM analysis. If the stringency of the primer annealing is lowered, it can be used to amplify heterogeneously methylated templates, but as with MSP, care is needed not to amplify false-positives at the same time. In some cases, the false-positives can be recognized by HRM.

DNA methylation as a cancer biomarker

The optimal cancer biomarkers

Currently, the best cancer biomarkers are those that are not methylated in normal healthy tissues and are relatively homogeneously methylated both at early stages and as the tumor progresses. A thorough study for each cancer biomarker in normal tissues, particularly in peripheral blood, from a series of normal individuals needs to be performed. Furthermore, the biomarkers should be studied in both tumor and adjacent normal tissues in their target cancers.

Heterogeneous DNA methylation patterns introduce particularly difficult problems when investigating low level DNA methylation. This is an important consideration when heterogeneously methylated regions are used as biomarkers for detection of early and minimal residual disease.

Some DNA methylation biomarkers have already been adopted as disease markers in various studies. DNA methylation of *MLH1* and *BRCA1* in normal tissues indicates a predisposition to the development of colorectal and breast cancer, respectively [79,80]. *CDKN2A* (*p16*) in sputum has been used to screen patients at increased risk of lung cancer [81] and *GSTP1* is an exceptionally clean biomarker for early detection of prostate cancer [82].

Digital PCR approaches enable the use of heterogeneously methylated biomarkers

If homogeneously methylated biomarkers are available, all the methodologies discussed are capable of producing useful quantitative results, except for the nonquantitative MSP approaches. However, the current and probably future paucity of consistently homogeneously methylated biomarkers means that heterogeneously methylated biomarkers usually need to be used, for example, *CDKN2B* in myelodysplasia [54]. Whereas averaging methodologies provide some information as to whether a locus is methylated (Figure 2C), only digital methodologies that provide information regarding individual epialleles are really adequate for accurate quantification. With digital methodologies, all methylated genes that are unmethylated in control tissues become of potential utility as epigenetic biomarkers.

Digital PCR was first used to detect somatic mutations present in genomic DNA [83]. The digital approach has now been applied for DNA methylation analysis and allows the analysis of single template epialleles, eliminating the potential PCR amplification bias seen in bisulfite sequencing of single clones that are cloned from PCR products rather than individual templates [22,43,77,80].

Digital PCR depends on limiting dilution. The templates in the sample to be investigated are diluted to the point where the majority of wells

contain either no template or only a single template copy according to the Poisson distribution. Methylation-independent bisulfite-specific primers are used to allow the amplification of each different epiallele regardless of its DNA methylation status. PCR is performed on single molecules, therefore generating a homogeneous amplicon population in each well or tube. Positive amplification is detected by a variety of methods and these wells can subsequently be sequenced [43,77].

All the methods described in the section titled 'Methods determining individual CpG methylation levels in PCR products' can be used for analysis of epialleles cloned by digital PCR. Sanger sequencing is most commonly used but pyrosequencing and perhaps MassARRAY (EpiTYPER) are likely to be used in the future.

The question of how many individual epialleles need to be analyzed to provide a comprehensive map of a sample with heterogeneous DNA methylation patterns, that we previously raised for bisulfite sequencing of cloned PCR products applies for these approaches also. The problem with sequencing all the digital clones is that, like bisulfite sequencing of cloned PCR products, the costs of sequencing may be too high to generate sufficient data for a comprehensive analysis, especially when a considerable fraction of epialleles may be unmethylated.

Digital methylation-sensitive HRM (dMS-HRM) screening, where the PCR amplification of a limiting dilution of templates is followed by HRM analysis, is a versatile digital approach [22,80]. HRM will distinguish PCR amplification positive wells from primer dimers, and can simultaneously not only count the methylated alleles but also assess the extent of DNA methylation of each epiallele (Figure 3). Only methylated epialleles need to be sequenced if the patterns of DNA methylation need to be analyzed. In addition, melting profiles that do not meet the quality control criteria (e.g., more than one template present in one reaction well) can easily be identified by the shape of the melting profile and discarded prior to analysis.

An emergent digital methodology is methylBEAMing (where BEAM stands for beads, emulsion, amplification and magnetics) [84]. The amplification occurs in microdroplets in an emulsion, the subsequent analysis of the PCR amplicons trapped on beads is by probes corresponding to fully methylated and unmethylated amplicons. Although these probes may cross hybridize to partially methylated amplicons, so far the methodology has not been adapted for comprehensive analysis of heterogeneously methylated epialleles.

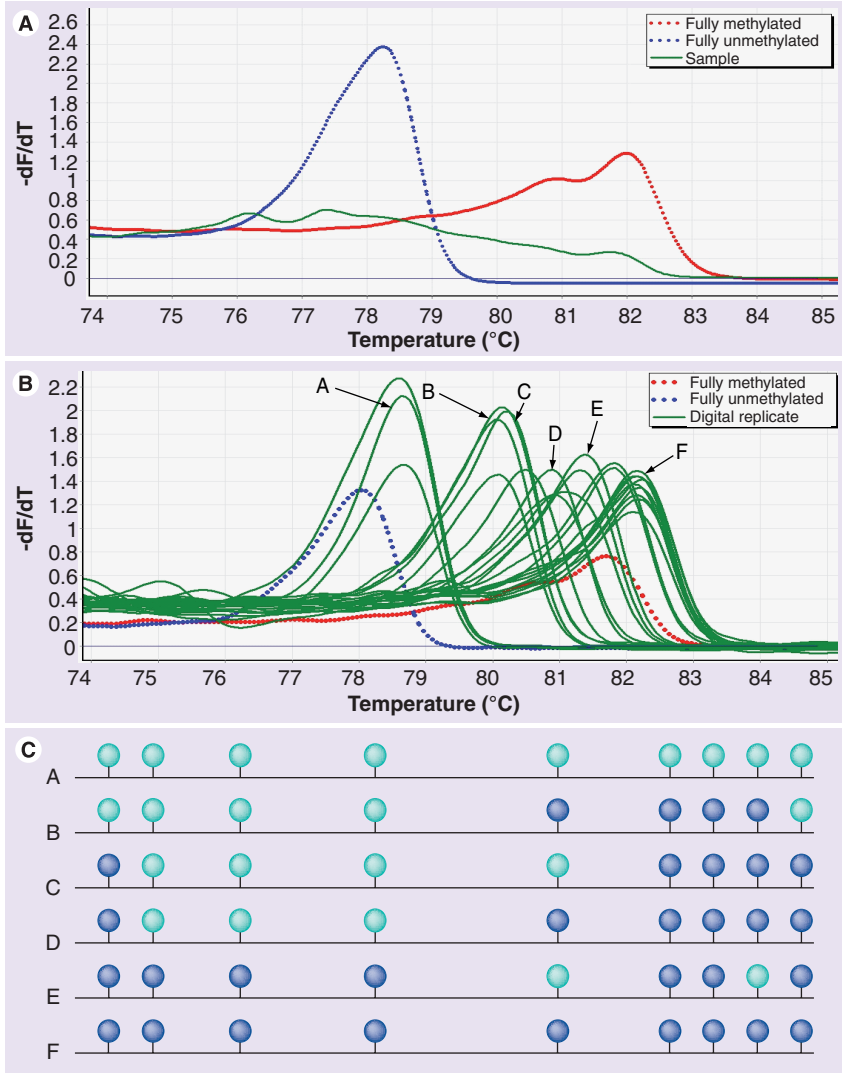

Figure 3. Digital methylation-sensitive high-resolution melting analysis of the *CDKN2B* (*p15*) gene. (A) Methylation-sensitive high-resolution melting (MS-HRM) melting profile for an acute myeloid leukemia (AML) sample compared with fully methylated and unmethylated controls. Due to the formation of heteroduplexes between closely related epialleles, MS-HRM cannot quantify heterogeneous DNA methylation but can identify it due to the absence of specific methylated and/or unmethylated peaks. In this case, the profile extends into the region of the fully methylated control without actually forming a distinct methylated peak. **(B)** Digital MS-HRM is able to reveal the nature of DNA methylation present. The spectrum of methylation in this case is weighted towards the more heavily methylated epialleles. Using digital MS-HRM to clone templates avoids PCR bias and can serve as a prescreening process before selecting 'clones' for sequencing. **(C)** Representation of the sequence of selected 'clones' using green and blue circles to indicate unmethylated and methylated CpG sites, respectively. Representatives of increasingly more methylated epialleles are shown. The letters A–F correspond with the melting profiles shown in **(B)** from which they derive. Sequencing reveals the exact DNA methylation pattern for each cloned epiallele.
Adapted from [22].

Massively parallel sequencing of heterogeneously methylated biomarkers

Massively parallel sequencing can be used to investigate heterogeneous methylation by deep

sequencing of PCR amplicons. It is directly analogous to bisulfite sequencing of single clones except that the 'cloning' of the PCR product is carried out by limiting dilution in an emulsion. It must be noted that this is intrinsically different from limiting dilution of templates prior to PCR amplification as used in digital MS-HRM, as PCR bias may still occur and lead to selective representation of epialleles.

The first report of this technology used pooled PCR products to analyze multiple amplicons in leukemia and lymphoma samples [85]. Massively parallel sequencing has subsequently been used to analyze the methylation of candidate biomarkers in breast cancer [86] and of the *MLH1* gene promoter in endometrial cancer [23]. These publications revealed heterogeneous patterns of methylation at the promoters of these genes that would have been difficult to detect by any other methodology, and confirmed that heterogeneous methylation is not only a technical problem but also an interesting phenomenon worthy of further study in order to increase our overall understanding of DNA methylation.

Conclusion

If heterogeneous DNA methylation is not taken into account, the correct interpretation of methylation data will be compromised, in particular in any instance where quantification is of importance. In this article, we have shown that different methodologies which can be fairly unanimous in the detection and quantification of fully methylated templates can lead to divergent results when heterogeneously methylated samples are analyzed.

Heterogeneous DNA methylation cannot be fully characterized without a method that allows the direct visualization of individual clones. Only in such cases can the entire population of heterogeneously methylated epialleles be quantified. The best current solution is a limiting dilution digital approach. The use of single templates allows amplification without PCR bias. Furthermore, subsequent DNA sequencing allows the detection of templates with a poor bisulfite conversion, which then can be excluded from the analysis.

Future perspective

The main impediment to the adoption of digital approaches is the expense of performing multiple PCR reactions. As the technologies improve to allow the analysis of multiple replicates in a cost-effective manner, digital analysis will become more routine and enable the adoption of heterogeneously methylated regions as cancer biomarkers.

Costs will come down owing to increasing miniaturization of the reaction vessels and consequent reduction in the amount of reagent needed. The use of nanodrops of liquid in an emulsion as reaction vessels, as has been used for methyl-BEAMing and for massively parallel sequencing, is likely to become more common.

Executive summary

Introduction

- DNA methylation is often heterogeneous.
- Heterogeneous methylation makes the fraction of methylated alleles difficult to adequately quantify. This compromises its use as a biomarker.

Methods

- There are many methodologies used to examine locus specific DNA methylation.
- Most of these methodologies have limitations when it is desired to use a heterogeneously methylated region as an epigenetic biomarker.

DNA methylation as a cancer biomarker

- Currently, the optimal epigenetic biomarkers are those where the normal state is unmethylated, and the disease state is completely methylated. In this case, several existing methodologies can adequately quantify the level of methylation.
- Digital methodologies using limiting dilution of templates most accurately quantify individual epialleles when heterogeneous methylation is present.
- Digital methylation-sensitive high resolution melting can accurately represent the extent of DNA methylation. It can reduce the cost of sequencing by indicating clearly unmethylated alleles.
- Massively parallel sequencing of PCR products from bisulfite modified DNA displays heterogeneous methylation based on multiple epialleles but suffers from a potential PCR bias.

Conclusion

- An understanding of heterogeneous methylation is vital for the use of DNA methylation as a cancer biomarker.
- Digital approaches counting individual epialleles will yield the most informative results.

Future perspective

- The optimal approach for quantification of DNA methylation at an allelic level will be based on a high-throughput approach that avoids PCR bias by assessing individual methylated templates.

Ultimately, analysis at the single molecule level without the need of bisulfite treatment and/or PCR amplification may be the ideal scenario. This approach would allow the direct visualization of DNA methylation at single CpG resolution without the confounding issues of the degree of bisulfite conversion and of PCR bias.

Acknowledgements

The authors thank Denise O'Keefe, Nick Wong and Jeff Craig for critical reading and helpful comments on this manuscript.

Financial & competing interests disclosure

Alexander Dobrovic is the coinventor of intellectual property on MS-HRM and SMART-MSP held by the Peter MacCallum Cancer Centre (Australia) and the University of Aarhus (Denmark). We acknowledge the National Breast Cancer Foundation (Australia), the Cancer Council of Victoria, the Victorian Cancer Agency and the US Department of Defense for funding epigenetics projects which led directly to the consideration of the issues discussed in this manuscript. Views and opinions of, and endorsements by the authors do not reflect those of the US Army or the US Department of Defense. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Bibliography

1 Cedar H, Bergman Y: Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* 10(5), 295–304 (2009).

2 Esteller M: Aberrant DNA methylation as a cancer-inducing mechanism. *Annu. Rev. Pharmaccol. Toxicol.* 45, 629–656 (2005).

3 Feinberg AP, Tycko B: The history of cancer epigenetics. *Nat. Rev. Cancer* 4(2), 143–153 (2004).

4 Fraga MF, Esteller M: Epigenetics and aging: the targets and the marks. *Trends Genet.* 23(8), 413–418 (2007).

5 Issa JP: Aging, DNA methylation and cancer. *Crit. Rev. Oncol. Hematol.* 32(1), 31–43 (1999).

6 Shames DS, Minna JD, Gazdar AF: DNA methylation in health, disease, and cancer. *Curr. Mol. Med.* 7(1), 85–102 (2007).

7 Esteller M: Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* 16(Spec. No. 1), R50–R59 (2007).

8 Laird PW: The power and the promise of DNA methylation markers. *Nat. Rev. Cancer* 3(4), 253–266 (2003).

9 Levenson VV: Biomarkers for early detection of breast cancer: what, when, and where? *Biochim. Biophys. Acta* 1770(6), 847–856 (2007).

10 Tost J: DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Mol. Biotechnol.* 44(1), 71–81 (2010).

11 Ehrlich M, Gama-Sosa MA, Huang LH *et al.*: Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res.* 10(8), 2709–2721 (1982).

12 Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. *J. Mol. Biol.* 196(2), 261–282 (1987).

13 Saxonov S, Berg P, Brutlag DL: A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc. Natl. Acad. Sci. USA* 103(5), 1412–1417 (2006).

14 Esteller M: Epigenetics in cancer. *N. Engl. J. Med.* 358(11), 1148–1159 (2008).

15 Kerjean A, Dupont JM, Vasseur C *et al.*: Establishment of the paternal methylation imprint of the human *h19* and *MEST/PEG1* genes during spermatogenesis. *Hum. Mol. Genet.* 9(14), 2183–2187 (2000).

16 Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO: Graded methylation in the promoter and body of the O6-methylguanine DNA methyltransferase (*MGMT*) gene correlates with MGMT expression in human glioma cells. *J . Biol. Chem.* 269(25), 17228–17237 (1994).

17 Stirzaker C, Millar DS, Paul CL *et al.*: Extensive DNA methylation spanning the *Rb* promoter in retinoblastoma tumors. *Cancer Res.* 57(11), 2229–2237 (1997).

18 Aggerholm A, Guldberg P, Hokland M, Hokland P: Extensive intra- and inter-individual heterogeneity of p15ink4b methylation in acute myeloid leukemia. *Cancer Res.* 59(2), 436–441 (1999).

19 Cameron EE, Baylin SB, Herman JG: p15(ink4b) CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing. *Blood* 94(7), 2445–2451 (1999).

20 Dodge JE, List AF, Futscher BW: Selective variegated methylation of the p15 CpG island in acute myeloid leukemia. *Int. J. Cancer* 78(5), 561–567 (1998).

21 Aggerholm A, Hokland P: Dap-kinase CpG island methylation in acute myeloid leukemia: methodology versus biology? *Blood* 95(9), 2997–2999 (2000).

22 Candiloro IL, Mikeska T, Hokland P, Dobrovic A: Rapid analysis of heterogeneously methylated DNA using digital methylation-sensitive high resolution melting: application to the *CDKN2B* (p15) gene. *Epigenetics Chromatin* 1(1), 7 (2008).

23 Varley KE, Mutch DG, Edmonston TB, Goodfellow PJ, Mitra RD: Intra-tumor heterogeneity of MLH1 promoter methylation revealed by deep single molecule bisulfite sequencing. *Nucleic Acids Res.* 37(14), 4603–4612 (2009).

24 Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP: Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res.* 58(23), 5489–5494 (1998).

25 Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB: Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat. Genet.* 7(4), 536–540 (1994).

26 Nakagawa H, Nuovo GJ, Zervos EE *et al.*: Age-related hypermethylation of the 5′ region of *MLH1* in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. *Cancer Res.* 61(19), 6991–6995 (2001).

27 Pasquali L, Bedeir A, Ringquist S, Styche A, Bhargava R, Trucco G: Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. *Cancer Lett.* 257(1), 136–144 (2007).

28 Esteller M, Herman JG: Generating mutations but providing chemosensitivity: the role of O_6-methylguanine DNA methyltransferase in human cancer. *Oncogene* 23(1), 1–8 (2004).

29 Hegi ME, Diserens AC, Gorlia T *et al.*: *MGMT* gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* 352(10), 997–1003 (2005).

30 Preusser M, Elezi L, Hainfellner JA: Reliability and reproducibility of PCR-based testing of O_6-methylguanine-DNA

methyltransferase gene (*MGMT*) promoter methylation status in formalin-fixed and paraffin-embedded neurosurgical biopsy specimens. *Clin. Neuropathol.* 27(6), 388–390 (2008).

31 Candiloro IL, Dobrovic A: Detection of *MGMT* promoter methylation in normal individuals is strongly associated with the T allele of the RS16906252 *MGMT* promoter single nucleotide polymorphism. *Cancer Prev. Res.* 2(10), 862–867 (2009).

32 Bock C: Epigenetic biomarker development. *Epigenomics* 1(1), 99–110 (2009).

33 Deng G, Peng E, Gum J, Terdiman J, Sleisenger M, Kim YS: Methylation of *hMLH1* promoter correlates with the gene silencing with a region-specific manner in colorectal cancer. *Br. J. Cancer* 86(4), 574–579 (2002).

34 Mikeska T, Bock C, El-Maarri O *et al.*: Optimization of quantitative *MGMT* promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J. Mol. Diagn.* 9(3), 368–381 (2007).

35 Schuffler P, Mikeska T, Waha A, Lengauer T, Bock C: Methmarker: user-friendly design and optimization of gene-specific DNA methylation assays. *Genome Biol.* 10(10), R105 (2009).

36 Warnecke Pm, Stirzaker C, Melki JR, Millar DS, Paul CL, Clark SJ: Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucleic Acids Res.* 25(21), 4422–4426 (1997).

37 Wojdacz TK, Hansen LL: Reversal of PCR bias for improved sensitivity of the DNA methylation melting curve assay. *BioTechniques* 41(3), 274, 276, 278 (2006).

38 Wojdacz TK, Hansen LL, Dobrovic A: A new approach to primer design for the control of PCR bias in methylation studies. *BMC Res. Notes* 1, 54 (2008).

39 Frommer M, Mcdonald LE, Millar DS *et al.*: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl Acad. Sci. USA* 89(5), 1827–1831 (1992).

40 Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T: Biq analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* 21(21), 4067–4068 (2005).

41 Kumaki Y, Oda M, Okano M: Quma: Quantification tool for methylation analysis. *Nucleic Acids Res.* 36, W170–W175 (2008).

42 Clark SJ, Harrison J, Paul CL, Frommer M: High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* 22(15), 2990–2997 (1994).

43 Chhibber A, Schroeder BG: Single-molecule polymerase chain reaction reduces bias: application to DNA methylation analysis by bisulfite sequencing. *Anal. Biochem.* 377(1), 46–54 (2008).

44 Paul CL, Clark SJ: Cytosine methylation: quantitation by automated genomic sequencing and genescan analysis. *Biotechniques* 21(1), 126–133 (1996).

45 Bar-Eli M, Ahuja H, Gonzalez-Cadavid N, Foti A, Cline MJ: Analysis of *N-RAS* exon-1 mutations in myelodysplastic syndromes by polymerase chain reaction and direct sequencing. *Blood* 73(1), 281–283 (1989).

46 Collins SJ, Howard M, Andrews DF, Agura E, Radich J: Rare occurrence of *N-ras* point mutations in philadelphia chromosome positive chronic myeloid leukemia. *Blood* 73(4), 1028–1032 (1989).

47 Do H, Dobrovic A: Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low level mutations in cancer biopsies. *Mol. Cancer* 8, 82 (2009).

48 Lewin J, Schmitt AO, Adorjan P, Hildmann T, Piepenbrock C: Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. *Bioinformatics* 20(17), 3005–3012 (2004).

49 Colella S, Shen L, Baggerly KA, Issa JP, Krahe R: Sensitive and quantitative universal pyrosequencing methylation analysis of CpG sites. *Biotechniques* 35(1), 146–150 (2003).

50 Tost J, Dunker J, Gut IG: Analysis and quantification of multiple methylation variable positions in CpG islands by pyrosequencing. *Biotechniques* 35(1), 152–156 (2003).

51 Uhlmann K, Brinckmann A, Toliat MR, Ritter H, Nurnberg P: Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. *Electrophoresis* 23(24), 4072–4079 (2002).

52 Dejeux E, Audard V, Cavard C, Gut IG, Terris B, Tost J: Rapid identification of promoter hypermethylation in hepatocellular carcinoma by pyrosequencing of etiologically homogeneous sample pools. *J. Mol. Diagn.* 9(4), 510–520 (2007).

53 Lillycrop KA, Phillips ES, Torrens C, Hanson MA, Jackson AA, Burdge GC: Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR α promoter of the offspring. *Br. J. Nutr.* 100(2), 278–282 (2008).

54 Brakensiek K, Wingen LU, Langer F, Kreipe H, Lehmann U: Quantitative high-resolution CpG island mapping with pyrosequencing reveals disease-specific methylation patterns of the *CDKN2B* gene in myelodysplastic syndrome and myeloid leukemia. *Clin. Chem.* 53(1), 17–23 (2007).

55 Gharizadeh B, Nordstrom T, Ahmadian A, Ronaghi M, Nyren P: Long-read pyrosequencing using pure 2'-deoxyadenosine-5'-o'-(1-thiotriphosphate) sp-isomer. *Anal. Biochem.* 301(1), 82–90 (2002).

56 Ehrich M, Nelson MR, Stanssens P *et al.*: Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc. Natl Acad. Sci. USA* 102(44), 15785–15790 (2005).

57 Coolen MW, Statham AL, Gardiner-Garden M, Clark SJ: Genomic profiling of CpG methylation and allelic specificity using quantitative high-throughput mass spectrometry: critical evaluation and improvements. *Nucleic Acids Res.* 35(18), E119 (2007).

58 Kuppuswamy MN, Hoffmann JW, Kasper CK, Spitzer SG, Groce SL, Bajaj SP: Single nucleotide primer extension to detect genetic diseases: experimental application to hemophilia b (factor IX) and cystic fibrosis genes. *Proc. Natl Acad. Sci. USA* 88(4), 1143–1147 (1991).

59 Gonzalgo ML, Jones PA: Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* 25(12), 2529–2531 (1997).

60 Gonzalgo ML, Jones PA: Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). *Methods* 27(2), 128–133 (2002).

61 El-Maarri O, Herbiniaux U, Walter J, Oldenburg J: A rapid, quantitative, non-radioactive bisulfite-SNuPE- IP RP HPLC assay for methylation analysis at specific CpG sites. *Nucleic Acids Res.* 30(6), E25 (2002).

62 Kaminsky ZA, Assadzadeh A, Flanagan J, Petronis A: Single nucleotide extension technology for quantitative site-specific evaluation of metC/C in GC-rich regions. *Nucleic Acids Res.* 33(10), E95 (2005).

63 Xiong Z, Laird PW: Cobra: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 25(12), 2532–2534 (1997).

64 Uejima H, Lee MP, Cui H, Feinberg AP: Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. *Nat. Genet.* 25(4), 375–376 (2000).

65 Wojdacz TK, Dobrovic A: Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* 35(6), E41 (2007).

66 Worm J, Aggerholm A, Guldberg P: In-tube DNA methylation profiling by fluorescence melting curve analysis. *Clin. Chem.* 47(7), 1183–1189 (2001).

67 Bianco T, Hussey D, Dobrovic A: Methylation-sensitive, single-strand conformation analysis (MS-SSCA): a rapid method to screen for and analyze methylation. *Hum. Mutat.* 14(4), 289–293 (1999).

68 Baumer A, Wiedemann U, Hergersberg M, Schinzel A: A novel MSP/DHPLC method for the investigation of the methylation status of imprinted genes enables the molecular detection of low cell mosaicisms. *Hum. Mutat.* 17(5), 423–430 (2001).

69 Deng D, Deng G, Smith MF *et al.*: Simultaneous detection of CpG methylation and single nucleotide polymorphism by denaturing high performance liquid chromatography. *Nucleic Acids Res.* 30(3), E13 (2002).

70 Wojdacz TK, Borgbo T, Hansen LL: Primer design versus PCR bias in methylation independent PCR amplifications. *Epigenetics* 4(4), 231–234 (2009).

71 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA* 93(18), 9821–9826 (1996).

72 Cottrell SE, Laird PW: Sensitive detection of DNA methylation. *Ann. NY Acad. Sci.* 983, 120–130 (2003).

73 Kristensen LS, Mikeska T, Krypuy M, Dobrovic A: Sensitive melting analysis after real time methylation specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. *Nucleic Acids Res.* 36(7), E42 (2008).

74 Lo YM, Wong IH, Zhang J, Tein MS, Ng MH, Hjelm NM: Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. *Cancer Res.* 59(16), 3899–3903 (1999).

75 Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW: CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res.* 59(10), 2302–2306 (1999).

76 Eads CA, Danenberg KD, Kawakami K *et al.*: Methylight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* 28(8), E32 (2000).

77 Weisenberger DJ, Trinh BN, Campan M *et al.*: DNA methylation analysis by digital bisulfite genomic sequencing and digital methylight. *Nucleic Acids Res.* 36(14), 4689–4698 (2008).

78 Chan MW, Chu ES, To KF, Leung WK: Quantitative detection of methylated *SOCS-1*, a tumor suppressor gene, by a modified protocol of quantitative real time methylation-specific PCR using SYBR green and its use in early gastric cancer detection. *Biotechnol. Lett.* 26(16), 1289–1293 (2004).

79 Gazzoli I, Loda M, Garber J, Syngal S, Kolodner RD: A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the *MLH1* gene in normal tissue and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumor. *Cancer Res.* 62(14), 3925–3928 (2002).

80 Snell C, Krypuy M, Wong EM, Loughrey MB, Dobrovic A: BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. *Breast Cancer Res.* 10(1), R12 (2008).

81 Palmisano WA, Divine KK, Saccomanno G *et al.*: Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res.* 60(21), 5954–5958 (2000).

82 Brooks JD, Weinstein M, Lin X *et al.*: CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol. Biomarkers Prev.* 7(6), 531–536 (1998).

83 Vogelstein B, Kinzler KW: Digital PCR. *Proc. Natl. Acad. Sci. USA* 96(16), 9236–9241 (1999).

84 Li M, Chen WD, Papadopoulos N *et al.*: Sensitive digital quantification of DNA methylation in clinical samples. *Nat. Biotechnol.* 27(9), 858–863 (2009).

85 Taylor KH, Kramer RS, Davis JW *et al.*: Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Res.* 67(18), 8511–8518 (2007).

86 Korshunova Y, Maloney RK, Lakey N *et al.*: Massively parallel bisulphite pyrosequencing reveals the molecular complexity of breast cancer-associated cytosine-methylation patterns obtained from tissue and serum DNA. *Genome Res.* 18(1), 19–29 (2008).