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Detection strategies for methylated and hypermethylated DNA

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HIGHLIGHTS

- We present the importance of aberrant DNA methylation in disease diagnosis
- We review the most recent research on detection of DNA methylation
- We assess optical and electrochemical methods for methylation analysis

ABSTRACT

DNA methylation plays an essential role in regulating cell growth and proliferation, and disease. Changes in aberrant DNA methylation are disease-specific, and, accordingly, the stage of disease progression can be anticipated. Aberrant forms of DNA methylation are recognized as biomarkers in various cancers. Thus, many research efforts recently focused on the detection of these epigenetics for both early cancer diagnoses and prognoses. Here, we provide the most important and relevant current developments while we discuss and assess the pros and cons of common detection strategies.

Keywords:
Aberrant DNA methylation
Cancer assay
Disease diagnosis
DNA hydroxymethylation
DNA hypermethylation
DNA hypomethylation
DNA methylation
Electrochemical method
Methylation analysis
Optical method

1. Aberrant DNA methylation and classical detection techniques

Epigenetics is the study of changes in the transmission of gene expression or cellular phenotype through cell division, which occurs without changing the DNA sequence. Epigenetics refers to genes being turned on or off by chemical reactions.

DNA methylation, the most important epigenetics, occurs upon the addition of a methyl group, -CH\textsubscript{3}, to the carbon 5-position of the DNA cytosine base catalyzed by DNA methyltransferases. DNA methylation occurs most often at cytosine-guanine sites (CpG dinucleotides) of gene promoters. Aberrant forms of DNA methylation are DNA hypermethylation and DNA hypomethylation. These relative terms imply excessive or
insufficient methylation compared to the standard DNA, respectively (i.e., they do not denote a different chemical structure at the nucleotide level). An additional common epigenetic modification of cytosine is DNA hydroxymethylation. Scheme 1 presents the structures of cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine.

DNA methylation plays an essential role in gene regulation, so it affects genomic imprinting [1], Alzheimer’s disease [2], silencing of microRNA genes [3], and X chromosome inactivation [4]. Aberrant DNA methylation reduces or increases the activity of genes on a cellular level. These changes are often inherited during the cell division, causing a disease on the organism level, such as schizophrenia [5], cardiovascular diseases [6], and various cancers [7], including lung cancer [8], ovarian cancer [9], colon cancer [10], breast cancer [11], urological cancer [12], sarcomas [13], Hodgkin lymphoma [14], and leukemia [15]. Tumor cells often exhibit methylation patterns that are substantially different from cells in healthy tissues (e.g., cancer is typically described by global as well as site-specific gene hypomethylation and hypermethylation) [16]. For brain tumors, it has even been shown that they are more often linked to aberrant methylation than to DNA copy changes [16].

Hydroxymethylcytosine as an intermediate in oxidation of DNA methylation, and – as an epigenetic modification – has been associated with the renewal of stem cells [17], neurodegenerative diseases [18] and cancer [19]. These examples highlight why aberrant DNA methylation can be used as a highly specific, sensitive biomarker for disease diagnosis and prognosis and why many research efforts in the past two decades have been dedicated to its detection. The potential use of methylated DNA biomarkers in clinical applications is advancing and has been reviewed previously [20–22]. Methylation biomarkers have been commercialized and are expected to take a decisive place in diagnostics and prognosis in the near future. For their successful implementation, data standards have yet to be established for the exchange of clinical research data.

An early method to detect DNA methylation relied on the digestion of genomic DNA with the restriction enzyme *HpaII* (*Haemophilus parainfluenzae*) followed by Southern blot analysis [23]. The Southern blot method relies on identifying specific sequences of DNA where DNA fragments separated on a gel are transferred directly to a second medium so that detection by hybridization occurs. Although straightforward, this method suffers from drawbacks, such as a limited availability of informative restriction sites, the occurrence of false positive results due to incomplete digestion, and the requirement for large amounts of high-molecular-weight DNA [24].

Frommer et al. made a major breakthrough in the analysis of DNA methylation by employing bisulfite treatment in which only unmethylated cytosine was converted to uracil [25,26]. In this method, primers are designed to overlap with the CpG site of interest, allowing determination of the methylation status (methylated or unmethylated). The sequence under study is subsequently amplified by PCR. However, the Frommer method requires large-scale sequencing of multiple plasmid clones to determine the overall methylation patterns.

Methylation-specific PCR (MSP) employs bisulfite treatment of DNA as a starting point for methylation analysis [27]. After such treatment, the intact DNA methyl groups react with specific primers. MSP is still the most common method in analysis of aberrant DNA methylation, as it has high sensitivity and requires only small amounts of DNA. It also allows the methylation status of any groups of CpG sites within a CpG island to be evaluated in a short time without using methylation-sensitive restriction enzymes. However, this method provides only qualitative data. The performance of MSP has been improved by a variety of PCR-based methods, such as real-time PCR [9], quantitative MSP [28], methylation-sensitive single-nucleotide-primer extension [29], and sensitive melting analysis after real-time MSP [30]. However, all of these methods suffer from not only uncertainty in completion of the bisulfite conversion but also limited coverage of CpG sites (a single CpG site is typically analyzed). These techniques are also subject to mispriming, as they rely solely on PCR.
amplification. Low concentrations of initial DNA templates (typical for DNA samples isolated from body fluids) and the use of high numbers of PCR cycles extend these limits.

Enzymatic regional methylation assays and methylation-sensitive single-nucleotide-primer extension (Ms-SNuPE) rely on radioactive labeling to quantify the regional CpG methylation density. Sodium bisulfite first reacts with the genomic DNA to convert unmethylated cytosine to uracil. Then, the desired target sequence reacts with PCR primers specific for bisulfite-converted DNA. The product is isolated and used as a template for the methylation analysis at the CpG site(s) of interest. Practically, radiolabeled products electrophorese on polyacrylamide gels under denaturing conditions and are visualized by exposure to an autoradiographic film or by phosphor-image analysis. Although MS-SNuPE is not widely used due to the use of radioactive isotopes, it works without restriction enzymes, and only small amounts of DNA are required.

MethyLight was introduced by Campan et al. to detect and to quantify DNA methylation sensitively in genomic DNA. This method relies on methylation-specific priming combined with methylation-specific fluorescent probing. Such a combination provides high throughput and real-time resolution. However, this method is expensive, as it requires both double-labeled fluorescent probes and quantitative PCR apparatus. Digital MethyLight, which utilizes MethyLight to examine a bisulfite-converted DNA sample distributed over multiple independent chambers, has shown to be more sensitive for the detection of a small number of methylated molecules in a large background of unmethylated DNA. Using digital MethyLight, cancer-specific DNA hypermethylation events have been identified in plasma samples of breast-cancer patients at the single-molecule level.

The methylation status can also be determined by other related classical techniques, including combined bisulfite restriction analysis (COBRA), array-based DNA methylation profiling, and microarray-based allele-specific extension. Several authors have reviewed these methods, to which we refer the interested reader.

Most classical techniques for assessing the genomic methylation and hypermethylation status focus on methylation-specific PCR and bisulfite-conversion analysis. Although these methods are mostly effective in mapping DNA methylation patterns, they are time consuming, require a large amount of DNA or radioactive labeling, damage the DNA, and result in false-positive results and significant error rates in PCR amplification; DNA extraction and bisulfite-conversion steps also lead to unreliable precision.

Recently, other techniques, relying mostly on electrochemistry and optical detection, were developed to overcome these limitations. In this review, we highlight such state-of-the-art research efforts, while we critically assess the pros and cons of current methods and provide an insight for the future research.

Table 1 summarizes the state-of-the-art detection strategies for DNA methylation and hypermethylation.

### 2. State-of-the-art detection methods for aberrant DNA methylation

#### 2.1. Strategies based on electrochemistry

Among all recent methods, electrochemistry offers distinct advantages in terms of simplicity, low cost, fast response time, and miniaturization. In principle, if both cytosine and methylated cytosine or any oligonucleotides have a sufficient potential difference, DNA methylation can be detected without any pretreatment. However, since DNA methyltransferase catalyzes and controls the DNA methylation reaction by transferring the methyl group from its cofactor S-adenosyl-L-methionine to the C-5 position of cytosine, the
development of an assay to measure its activity and inhibition can also be used to target DNA methylation [44].

Barton et al. reported a signal-on electrochemical method to assay human DNA methyltransferase activity [45]. They used a methylation-sensitive restriction enzyme (RsaI) with multiplexed DNA electrodes (modified with covalent methylene blue as a redox probe) to detect DNA methylation. Upon treatment of the electrode with active methyltransferase, the CpG methylation site within the recognition site of the enzyme was methylated. As a result, the methylated DNA was protected from restriction in subsequent treatment with the restriction enzyme. With the DNA intact, the redox probe signal was retained (signal-on). In absence of active methyltransferase, the DNA remained unmethylated, causing the signal-off results.

Taking advantage of the electrostatic repulsion between DNA and negatively charged indium tin oxide (ITO) microelectrode chips, Chen et al. recently developed a method for detection of DNA methylation and inhibitor screening [41]. The penultimate base, thymine, close to the 3′-terminal was tagged by methylene blue. Upon treatment with the methylation-dependent restriction enzyme (Dpn I Endonuclease), a good electrochemical response was obtained due to cleavage and aggregation of electroactive methylene blue-labeled fragments on the ITO electrode. In the absence of DNA methylation, the electroactive fragments were not generated, so a weak electrochemical response was obtained.

An electrochemical signal-off assay based on the electrostatic interactions between the electroactive signal transducer [Ru(NH$_3$)$_6$]$_{3+}$ and DNA strands was developed to measure DNA adenine methylation methyltransferase (Dam MTase) activity [46]. Single-stranded DNA1 (ssDNA) was initially self-assembled on a gold electrode via Au–thiol bonds, followed by hybridization with ssDNA2 (see Fig. 1). In the presence of Dam MTase, the resulting double-stranded DNA (dsDNA) was methylated. When the dsDNA was cleaved by methylation-responsive restriction endonuclease Dpn I, a large amount of electroactive probes were dissociated from the electrode, causing a decrease in the electrochemical signal.

Due to the conformational structure and very low diffusion coefficients of long DNA molecules, direct electrochemical detection of longer DNA molecules is difficult. To assay DNA methylation for longer CpG oligonucleotides, improvements in terms of sensitivity and stability against methyl-cytosine and cytosine are required. This can be achieved, for example, by taking the advantage of an endonuclease P1 enzyme to digest the longer target CpG oligonucleotide [47]. Quantitative detection of single cytosine methylation in relatively long sequences was also reported by optimizing the surface condition of a nanocarbon film electrode [48]. There, the oxygen-containing groups on the surface of a functionalized nanocarbon electrode reduced electrode fouling in the presence of biomolecules, while maintaining the intrinsic electrochemical properties.

One challenge in all these methods is the interference of thymine due to its oxidation potential being similar to that of 5-methylcytosine. Thus, for direct oxidation of DNA bases, an innovative approach was recently suggested, based on the stoichiometric relationship between purine and pyrimidine bases in DNA [49]. Distinct oxidation peaks for DNA bases were obtained at a glassy-carbon electrode modified with polypyrrole/carbon nanotubes. An oxidation peak potential difference of 180 mV was obtained between 5-methylcytosine and cytosine, whereas the oxidation peak of thymine overlapped with that of 5-methylcytosine. As the molar composition of guanine (as the complementary base of both 5-methylcytosine and cytosine) is equal to the sum of those of cytosine and 5-methylcytosine, the amount of 5-methylcytosine could be calculated.

Electrochemical protocols can also be designed to study various aspects of DNA cytosine methylation. For example, Voulgaropoulos et al. evaluated the methylation density and concomitant conformational changes in genomic DNA by combining the electrochemical measurements with thermal denaturation analysis [50].
DNA methylation sites can be specifically recognized by a family of proteins containing the conserved methyl-CpG binding domains (MBDs). Five MBD family members in mammals (i.e., MeCP2, MBD1, MBD2, MBD3, and MBD4 [51]) bind specifically to symmetrically methylated CpG motifs in dsDNA and have been used for highly sensitive electrochemical analysis of DNA methylation. The MBD-based assays do not require bisulfite treatment and PCR amplification, and can target all genomic regions [52]. MBDs have been used for methylated DNA precipitation [52] or direct detection of methylated DNA [53]. Combining the advantages of the specific MBD protein mentioned above with the electrochemical activity of the Coomassie brilliant blue G250, Ai et al. studied DNA methylation and DNA-methyltransferase activity [54]. They also used MeCP2 as a recognition unit for DNA CpG methylation in the design of another electrochemical immunosensor for both detection of DNA methylation and analysis of DNA methyltransferase activity [55].

Apart from enzymatic DNA methylation, exogenous alkylating chemical agents, such as methyl methane sulfonate, may also result in methylation of DNA bases, which is difficult to detect due to the small size and inertness of the methyl group [56]. The chemical methylation of DNA occurs directly at the ring nitrogens of the purine bases, which are weak acceptor sites for enzymatic DNA methylation.

Guo et al. developed a label-free photoelectrochemical sensor for the detection of methylated purines in DNA films using specific DNA repair enzymes [57]. These enzymes convert the DNA methylation sites into strand breaks on an ITO electrode where a Ru(bpy)$_2$(dppz)$_2$$^{2+}$ photoelectrochemical probe is used as the DNA intercalator [57]. The resulting photocurrent was correlated to the amount of 3-methyladenine produced in the presence of methyl methane sulfonate. Such strategies can be applied to the detection of other modified DNA bases using specific DNA repair enzymes.

2.2. Strategies based on ion-sensitive field-effect transistors (ISFETs)

In ISFETs, ions in solution act as the gate electrode of a transistor, so the detected electrical current from the sensor due to the changes in ion concentration can be sensitively detected. ISFETs are commonly employed as pH sensors to measure the H$_3$O$^+$ concentration in a solution. Toumazou et al. designed novel ISFET-based circuits for continuous computation of the ratio of methylated DNA to unmethylated DNA through chain extension [58,59]. Hydrolysis of pyrophosphate, PP$_i$, upon addition of nucleoside triphosphate (dNTP) to the DNA results in production of proton ions according to:

$$dNTP + DNA_n \rightarrow PP_i + DNA_{n+1} + Mg^{2+} + H^+$$ (1)

The resulting pH drop affects the gate-source voltage of the ISFET and can be directly detected. In this way, a single ssDNA probe was designed to identify the methylated regions. When the complementary region of DNA was incorporated, protons were released and a change in pH was observed. The level of methylation was determined using two ISFETs: one to detect the methylated DNA and the other the unmethylated DNA. The ratio of the methylated DNA to unmethylated DNA was recorded as a discrete output signal when methylation above a certain percentage was detected. ISFET-sensor devices can be integrated on CMOS chips for CMOS-based DNA-methylation arrays.

2.3. Strategies based on optical detection

A broad range of optical methods has been employed for the detection of DNA methylation. The fluorescence methods, such as near-infrared (NIR) fluorescence [71], and
fluorescence correlation spectroscopy [72], are the most common. Other methods include electrochemiluminescence (ECL) [73] and colorimetry [74].

2.3.1. Strategies based on fluorescence

Among fluorescence techniques, fluorescence resonance-energy transfer (FRET) is most common. FRET relies on the electron transfer between a donor fluorophore in an electronically excited state and a nearby acceptor chromophore located within a minimal spatial radius; it leads to a change in fluorescence intensity as a function of the donor-acceptor distance.

Due to their unique signal-amplification properties, cationic conjugated polymers (CCPs) are commonly used in FRET techniques for the detection of DNA methylation, as reviewed by Wang et al. [75]. CCPs (such as poly((1,4-phenylene)-2,7-[9,9-bis(6'-N,N,N-trimethyl ammonium)-hexyl fluorene] dibromide}), which are energy donors with a high fluorescence intensity in aqueous media, form a complex with oppositely charged fluorescein-labeled DNA through electrostatic interactions. CCPs can transfer energy to the acceptor fluorescein through FRET, resulting in significant optical amplification of fluorescein due to the overlap between the emission of CCPs and the absorption of fluorescein. Using this concept, Wang et al. developed a sensor that can determine the methylation status of CpG sites as low as 1% DNA methylation in human colon-cancer cells [42]. Fluorescein-labeled deoxyguanosine triphosphate (dGTP) was incorporated into the DNA probe by a single-nucleotide-base extension. Upon addition of CCP, an efficient FRET from CCP to fluorescein is observed while the strong electrostatic interaction between methylated DNA and CCP brings the fluorescein close to CCP.

Wang et al. improved the sensitivity of FRET for detection of DNA methylation using quantum dots as fluorophores and the enzymatic incorporation of multi receptors [76].

Wang et al. developed an assay for the detection of DNA hypermethylation and its effects on the transcription of a hypermethylated gene, p14ARF, in the cancer cell line LOVO (human colon adenocarcinoma cell line) [71]. The former was detected using a biotin–streptavidin system and the latter by an immunological system (anti-5-methylcytidine), both combined with a nylon membrane-based NIR fluorescence assay. The NIR fluorescence technique results in high sensitivity due to a low background autofluorescence.

Zhang et al. developed a method for detection of DNA methylation in lung-cancer cells using thermostable ligation and hyperbranched rolling-circle amplification (HRCA) for signal enhancement [60]. As an alternative to PCR, HRCA is an isothermal and exponential amplification technique derived from RCA working through a turn-by-turn cascade of primer extension and strand displacement (DNA polymerase replicates circularized oligonucleotide probes) [77]. HRCA has been widely employed for the detection of biomolecules due to its high sensitivity. The principle of a DNA methylation assay based on ligation-mediated HRCA, presented in Fig. 2, involves the following steps: bisulfite treatment, hybridization of methylated DNA with a linear padlock probe and the subsequent ligation of the linear padlock, purification of obtained circular padlock probes by digestion, and HRCA, whose products can be assessed by the fluorescent dye SYBR Green I. In presence of unmethylated DNA, neither HRCA nor a distinct fluorescence signal is observed, while, in presence of methylated DNA, both HRCA and fluorescence enhancements are observed.

Recently, Hosseini et al. developed a fluorimetric assay based on novel fluorescence DNA probe dipyridamole for the detection of DNA methylation in CpG islands of adenomatous polyposis coli, a tumor-suppressor gene [69]. They functionalized Fe3O4/Au nanoparticles with ssDNA via Au-thiol bonds at the 5'-end phosphate. After hybridization of ssDNA probes with both unmethylated and methylated complementary targets, they employed fluorescence spectroscopy to distinguish the methylated DNA. The detection mechanism relied on the interaction between dipyridamole and methylated/unmethylated DNA so that an increase in
the concentration of unmethylated ssDNA resulted in the enhancement of the fluorescence intensity, whereas an increase in the methylated ssDNA concentration resulted in quenching of the signal.

Fluorescence methods have also been employed to assay the activity of DNA-methyltransferase and to detect DNA methylation via methylation-sensitive cleavage coupled with nicking enzyme-assisted signal amplification [78] or a DNA intercalator dye coupled with an endonuclease and carbon nanomaterials [79].

2.3.2. Strategies based on electrochemiluminescence (ECL)

ECL relies on the light emission from electrochemically-generated intermediates that undergo a highly exergonic reaction. Kurita et al. employed ECL for quantitative determination of DNA methylation in a real sample without the need for amplification by PCR or bisulfite conversion [73]. Their strategy relied on labeling antimethyl cytosine antibodies with acetylcholinesterase and its subsequent addition to the single methylated cytosine in a DNA oligomer. Thiocholine produced in the enzymatic reaction of acetylcholinesterase with acetylthiocholine was preconcentrated on a gold-electrode surface via the formation of Au-thiol bonds. Upon applying a potential to the electrode, the light emission from a Ru(bpy)$_2^{2+}$ luminophore was measured in the presence of DNA methylation (see Fig. 3).

Li et al. developed a highly-sensitive ECL method to detect DNA methylation and assay methyltransferase activity using enzyme-linkage reactions [80]. They modified a gold-electrode surface by self-assembly of 5'-thiol-modified 32-mer ssDNA {tagged with ruthenium complex [Ru(bpy)$_2$(dcbpy)–NHS]}. After dsDNA was formed by hybridization with complementary DNA, all cytosine residues within the CpG sites of dsDNA were methylated by methyltransferase. The electrode was then treated by HpaII endonuclease to cleave unmethylated cytosines, leading to a decrease in the ECL signal. The ECL intensity depended on the methylation levels and the methyltransferase activity.

2.4. Strategies based on mass spectrometry (MS)

MS, originally, was mainly used to analyze the global DNA methylation content of a sample, i.e., to determine the overall 5-methylcytosine content or any changes that affect the entire DNA methylome [81]. The use of MS lowers the detection limit by several orders of magnitude compared to other methods. Enzymatic methylation of DNA cytosine residues in cultured human cells has been investigated by incorporation of stable isotopes and subsequent detection by MS [82].

Coupling electrospray ionization MS with liquid chromatography (LC) was used for determination of global DNA methylation levels in the lung tissue of lung-cancer patients [83]. An isotope-dilution LC/tandem MS method was developed for a direct analysis of 5-methylcytosine [84].

A powerful tool to address a variety of proteomic and genomic problems including epigenetics is matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) [85]. In MALDI, a matrix (usually a low-molecular-weight organic acid with a strong absorption at the laser-excitation wavelength) containing the embedded analyte molecule is desorbed into the gas phase and ionized by the matrix molecules using a short laser pulse. The ionized nucleic-acid molecules are then extracted with an electric field and separated by their time of flight to a detector (i.e., by their mass and charge) [86].

Boom et al. developed a method based on MALDI-TOF for analysis of DNA methylation employing base-specific cleavage of single-stranded nucleic acids [87]. They employed the IGF2/H19 imprinted region of the chromosome 11p15.5 as a model system, and they showed
that a single base-specific cleavage reaction can be used to determine the methylation sites and their ratios in the target region of interest.

Although MS is a very sensitive, reliable method to detect DNA methylation, it does not provide information about the methylation sequence.

2.5. Strategies based on nanopores

Recent advancement in nanotechnology has resulted in a plethora of research focusing on single-molecule detection. Among them, nanopore platforms provide powerful tools with the ambitious aim of electrical sensing of nucleic acids at the single-base level. Here, two reservoirs are separated by a porous membrane, and the electrical current across the membrane is sensitively detected. The membrane can be solid state with a single nanometer sized aperture fabricated (e.g., by a focused electron beam [88]); or, alternatively, in a biological nanopore sensor, the membrane consists of a lipid bilayer with a single α-haemolysin pore of a well-defined minimal inner diameter of 1.4 nm [89]. By applying an electric potential across the membrane, only the ionic current through the nanopore is detected. When a molecule immersed in solution traverses the narrow pore, ions are excluded, and a current blockage is detected. Thus, the exact magnitude of the current blockage provides information about the molecular structure.

Bashir et al. reported a single-molecule DNA methylation assay using solid-state nanopores [90]. They labeled the methylation sites of DNA with MBD1 proteins, resulting in ionic blockage current relative to unmethylated DNA.

Using biological nanopores, Gu et al. developed a protein nanopore sensor to distinguish between uracil and 5-methylcytosine in a DNA sequence [91]. They used Hg²⁺ to bind to both thymine-thymine and uracil-thymine mismatch. Such binding forms a reversible interstrand lock (MercuLock) that cannot be formed in the case of 5-methylcytosine-thymine mismatch. Although this method is suitable for detection of single-locus DNA methylation, it cannot be applied for genome-wide DNA methylation profiling as a high-throughput nanopore platform is yet to be established.

Gundlach et al. pulled ssDNA molecules through an engineered biological protein nanopore and found that ion currents passing through the pore not only reveal the exact location of DNA methylation sites with high accuracy but also allow the differentiation of hypermethylated and methylated bases [92]. Although operation at the single-molecule level is groundbreaking, nanopore techniques are not highly quantitative, require extensive measurement equipment, laborious procedures, and involve complex chemical labeling and amplification. These drawbacks currently prohibit their use in a clinical setting [91].

2.6. Other detection strategies for DNA methylation

2.6.1. Quartz-crystal microbalance (QCM)

A QCM is a mass sensor based on the piezoelectric properties of quartz crystals oscillating at a particular resonant frequency. Once molecules adsorb to its surface, the related mass change is most sensitively detected as a change in its resonant frequency.

Ma et al. combined HpaII-PCR with a QCM for DNA-methylation detection [70]. A small amount of genomic DNA was digested by HpaII followed by PCR. As methylated DNA was not affected by HpaII digestion, PCR amplification occurred for methylated sequences only. PCR products were subsequently injected into a QCM sensor and hybridized on the QCM-probe surface, resulting in a QCM-frequency shift. For methylated DNA, a large amount of PCR product hybridized with the DNA probe on the chip surface, leading to a significant shift in the resonant frequency (see Fig. 4). For unmethylated DNA, PCR amplification was ineffective and induced a negligible shift.
2.6.2. Bulge-specific immuno-recognition

Recently, immunochemical [93] and immunohistochemical methods [94] for detecting methylcytosine with anti-methylcytosine antibody were reported. These methods employ fluorescence polarization combined with capillary electrophoresis [95], magnetic microspheres [96], a nitrocellulose membrane [97], or a DNA microarray [98] for assessing the methylation level.

The first sequence-selective determination of methylcytosine in DNA with an anti-methylcytosine antibody was reported by Kurita and Niwa [99]. This assay relied on the affinity measurement between target cytosine or methylcytosine in a bulge region and the anti-methylcytosine antibody followed by hybridization with a bulge-inducing probe DNA. Due to having a single bond (between deoxyribose and phosphate), the methylcytosine in the bulge can rotate freely outward and thus be recognized by an anti-methylcytosine antibody, whereas the methylcytosine in a non-target region pairs with guanine and turns inward, being unavailable for recognition by the antibody. Thus, this bulge-specific technique can selectively distinguish whether or not the target cytosine is methylated without any effects from the status of other cytosine methylation in the non-target region.

2.6.3. Opto-fluidic ring resonators (OFRRs)

Fan et al. employed OFRRs as one of the most sensitive optical label-free sensors to date for the analysis of DNA methylation [67]. The OFRR is a thin-walled glass capillary in which light circulates repeatedly (i.e., resonates) along the ring surface. Adsorption of analytes at the inner surface of the OFRR leads to a shift in the detected resonant wavelength. Both MBD2 protein and anti 5-methylcytidine antibodies were used to capture the methylated oligonucleotides at the inner surface of the resonator. Accordingly, the OFRR’s response to artificially methylated ssDNA and dsDNA was recorded as a function of the number of methylated cytosines and DNA concentration. The antibody had a stronger affinity for the ssDNA, whereas MBD2 bound better with dsDNA.

3. Conclusion

The current research trends in cancer diagnosis have mostly relied on multiplex detection of protein biomarkers mostly from blood samples. The main challenge in detection of protein biomarkers is that an array of biomarkers should be simultaneously detected for specific cancer diagnosis, as most biomarkers are common among different cancers. The discovery of aberrant DNA methylation as specific cancer biomarkers has broadened the possibilities for diagnosis of cancer and other diseases. Compared to protein biomarkers, aberrant DNA methylation can be quantified in different samples, such as frozen tissues and formalin-fixed, paraffin-embedded samples, and body fluids, such as urine, plasma, and serum.

The combination of different pretreatments followed by various analytical tools has resulted in a plethora of techniques for the detection of aberrant DNA methylation. Each approach has its own strengths and weaknesses. Most methods and developments are complex and require expensive set-ups. Electrochemical strategies recently received a lot of attention for DNA-methylation detection due to their simplicity, easy miniaturization, low cost, and their fast response time.

DNA methylation has been studied more than hypermethylation and hypomethylation; but, in principle, when combined with statistical tools, DNA methylation strategies can be extended to the detection of hypermethylation and hypomethylation.

Although various technologies have been developed for the use of aberrant DNA methylation as disease biomarkers, their use is still at the research level and none yet meets
the strict demands of hospital laboratories. Nonetheless, we foresee that, in the near future, the recent technological advances together with advanced statistical methods will lead to the precise analysis of aberrant DNA methylation, so that epigenomics will be used for diagnosis of complex diseases.

References


Captions

**Scheme 1.** Comparison of cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine

Fig. 1. An electrochemical assay for DNA methylation and DNA methyltransferase activity. The sensor is based on self-assembly of thiolated ssDNA1 on an Au electrode, followed by hybridization of DNA2 to form dsDNA containing a specific recognition sequence of Dam MTase and Dpn I. When the sensor is exposed to solution of [Ru(NH₃)₆]³⁺, an ON state signal is recorded. Methylation by Dam MTase and further cleavage by Dpn I results in an OFF state signal. [Reprinted from [46] with permission from Elsevier].

Fig. 2. Ligation-mediated hyperbranched rolling circle amplification (HRCA) assay for detection of DNA methylation. [Reprinted with permission from [60], ©2012, American Chemical Society].

Fig. 3. Electrochemiluminescence (ECL) principle for the detection of methyl-cytosine. [Reprinted with permission from [73], ©2012, American Chemical Society].

Fig. 4. A quartz-crystal microbalance (QCM) assay for DNA methylation. (a) Methylated DNA was not digested with *Hpa*II, amplified by PCR, and the products were detected by QCM. (b) Unmethylated DNA was digested with *Hpa*II and was not amplified by PCR. [Reprinted with permission from [70], ©2013 American Chemical Society].

**Table 1.**
State-of-the-art detection strategies for DNA methylation and hypermethylation

<table>
<thead>
<tr>
<th>Detection strategy</th>
<th>Target analyte</th>
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<tr>
<td>Optical</td>
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<td>Human lung cancer</td>
<td>0.8 fM</td>
<td>Ultrahigh sensitivity and specificity, without restriction enzymes and PCR amplification, multi-step process, time-consuming</td>
</tr>
<tr>
<td>Photoelectrochemical</td>
<td>DNA methylation</td>
<td>Synthetic oligonucleotides</td>
<td>35 fM</td>
<td>High photon-to-current conversion efficiency, without bisulfite treatment, distinguishes single-base mismatches</td>
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<td>Electrochemical</td>
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<td>Synthetic CpG oligonucleotides (20-mer and 60-mer)</td>
<td>Not mentioned</td>
<td>Very simple and inexpensive, no bisulfite treatment or separation process</td>
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<td>Liquid chromatography tandem mass spectrometry</td>
<td>Global DNA methylation</td>
<td>Normal and tumor tissues of colorectal cancer</td>
<td>0.05 pg</td>
<td>Simple derivative process, inexpensive global DNA methylation</td>
</tr>
<tr>
<td>DNA microarray</td>
<td>DNA methylation</td>
<td>Hepatocellular carcinomas cell lines and normal blood samples</td>
<td>0.125 μM</td>
<td>Inexpensive but laborious, quantifies regional methylation density, not able to determine the methylation status of individual CpG sites</td>
</tr>
<tr>
<td>Methylation-specific PCR (MSP)</td>
<td>DAPK gene hypermethylation</td>
<td>Tissue and serum samples of ovarian cancer and leiomyoma patients</td>
<td>Not mentioned</td>
<td>Inexpensive, widely used, risk of false negative results, tedious process polyacrylamide gel</td>
</tr>
<tr>
<td>Method</td>
<td>DNA methylation</td>
<td>Target DNA</td>
<td>Sensitivity</td>
<td>Notes</td>
</tr>
<tr>
<td>------------------------------------</td>
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<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Surface enhanced Raman spectroscopy</td>
<td>DNA methylation</td>
<td>Artificial mixtures of methylated and unmethylated DNA</td>
<td>3 pM</td>
<td>High sensitivity, high accuracy, minimal amount of DNA, requires professional design</td>
</tr>
<tr>
<td>High-performance capillary electrophoresis</td>
<td>Global DNA methylation</td>
<td>Methotrexate-resistant A549 cells</td>
<td>1 µM</td>
<td>Good separation, simple to operate, requires low sample concentration</td>
</tr>
<tr>
<td>DNA microarray</td>
<td>DNA methylation</td>
<td>Genomic DNA of hepatocellular carcinomas cell lines</td>
<td>0.125 µM</td>
<td>Suitable for high-throughput analysis of multiple samples or target regions, amount of clinical samples, the method is not adequate</td>
</tr>
<tr>
<td>Quantitative methylation-specific PCR</td>
<td>TFPI2 methylation</td>
<td>Colorectal cancer patients’ sera</td>
<td>Not mentioned</td>
<td>Detection of small amounts of tumor DNA, great sensitivity and sensitivity</td>
</tr>
<tr>
<td>Opto-fluidic ring resonators (OFRR)</td>
<td>DNA methylation</td>
<td>Artificially methylated ssDNA and dsDNA</td>
<td>1 nM</td>
<td>Without pretreatment or conversion, simple operation, low sample volumes, the method is label-free biosensor</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>RASSF1A promoter methylation</td>
<td>Ovarian cancer tumor tissue and serum</td>
<td>Not mentioned</td>
<td>Requires expensive equipment to perform high-throughput analysis of genes, high sensitivity, not suitable for discovery</td>
</tr>
<tr>
<td>Reduced representation bisulfite sequencing</td>
<td>Global DNA hypermethylation</td>
<td>Down Syndrome placenta villi samples</td>
<td>Not mentioned</td>
<td>Requires large amounts of purified DNA, labor-intensive procedures, costly apparatuses</td>
</tr>
<tr>
<td>Fluorimetric nanobiosensor</td>
<td>DNA methylation</td>
<td>Synthetic sequence of APC gene</td>
<td>0.31 fM</td>
<td>Very low detection limit, simple operation, sensitive to small amount of tumor DNA</td>
</tr>
<tr>
<td>Quartz crystal microbalance (QCM)</td>
<td>DNA methylation</td>
<td>p16 and GALR2 genes in two cell lines</td>
<td>20 nM</td>
<td>Requires small sample amount, simple operation, antibody, good performance, high sensitivity and linear dynamic range</td>
</tr>
</tbody>
</table>