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CHAPTER 2

An overview of different techniques to detect hypermethylation

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ABSTRACT
For the past decade, abnormal patterns of DNA methylation have been recognized as epigenetic molecular changes in neoplasia. CpG islands in promoter regions of genes are targets for methylation, and if this occurs superfluously, gene transcription may be blocked. Methylation of tumor suppressor genes contributes to an immortalized phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth. It is known to be an early event in carcinogenesis of many different tumor types. DNA-methylation-based technologies have a promising future in research, clinical diagnostics and therapy. Different techniques spanning from genome-wide methylation content to methylation of single residues in specific genes have been developed in the past 20 years. In this review, we discuss some general themes in DNA methylation analysis, outline the basic principles of current key techniques and introduce some recently described techniques. We discuss the advantages and disadvantages of these techniques, including potential artifacts and pitfalls, and suggest some overall guidelines that may be instructive for a rational choice of methodology.
1. INTRODUCTION
Epigenetic modifications are defined as heritable changes in the pattern of gene expression other than alterations in nucleotide sequences. These include DNA methylation and histone modifications, which are known to regulate a wide range of physiological and pathological processes (1). In humans and other mammals, DNA methylation has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands (2), located in the promoter regions of many genes. DNA methylation of the promoter region can block gene expression and lead to loss of function. Transcriptional inactivation by methylation of the promoter region of tumor suppressor genes is an important mechanism in human carcinogenesis (3). Given the importance of DNA methylation in cancer and various other diseases, assays to measure DNA methylation are very useful in both research and clinical practice.

1.1. DNA methylation
DNA methylation occurs only at cytosines located 5’ to guanosine in the CpG dinucleotides. In contrast to the relative paucity of CpGs in the genome as a whole, these dinucleotides can be clustered in small stretches of DNA termed “CpG islands” (2). The catalytic mechanism of DNA methyltransferases has been proposed as being similar to that of thymidylate synthetase (4). In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The first DNA methyltransferase identified was revealed by Bestor in 1988 (5). This enzyme, now termed DNMT1, is a protein that contains 1620 amino acids and exhibits a 5- to 30-fold preference for hemi-methylated substrates. DNMT1 is the main enzyme in mammals, and is responsible for the post-replicative restoration of hemi-methylated sites to full methylation. Then DNMT2, DNMT3a and DNMT3b were identified by searches of expressed sequence tag databases (6). DNMT2 lacks the large N-terminal regulatory domain common to other eukaryotic methyltransferases and does not exhibit comparable DNA methyltransferase activity, whereas DNMT3a and DNMT3b are thought to be involved primarily in methylation of new sites, a process called de novo methylation (6).

DNA methylation alters chromosome structure (7;8); however, the mechanisms by which DNA methylation is translated into transcriptionally silent chromatin is still not clear. Although there are several hypotheses to explain the way by which DNA methylation is interpreted by nuclear factors, the active recruitment of methyl-CpG binding activities appears to be the most widespread mechanism of methylation-dependent repression. DNA methylation can promote the binding of proteins such as MeCP1, MeCP2, MBD1, MBD2, MBD3 and MBD4, which induce histone modification (9). Although MeCP1 was originally identified as a large multi-protein complex, MeCP2 is a single polypeptide with an affinity for a single methylated CpG. In mammals MBD1 and MBD2 are bona fide methylated DNA binding proteins and MBD3 is able to bind methylated DNA only in certain species. In the case of MBD4, this protein removes thymines from T:G mismatches resulting from deamination of 5-methylcytosine. This repair pathway contributes to mutation avoidance at methylated CpG dinucleotides (10). MeCP2 and MBD2 have been shown to participate in protein complexes that recruit transcriptional co-
repressors, chromatin remodeling proteins and histone deacetylases (11-13). Through such complexes, sites of DNA methylation could then target the formation of chromatin, including the deacetylated state of histones, which is typical for transcriptionally repressive domains (11-16).

1.2. DNA methylation in cancer
The importance of DNA methylation for gene expression, and especially for transcriptional silencing, is apparent in the exceptions to the rule that CpG islands associated with gene promoter regions are unmethylated. Methylation of some CpG islands in non-malignant tissues increases with age (17;18). Cancer-specific DNA methylation changes of individual gene have so far focused primarily on hypermethylation of CpG islands (19;20). It is known to be an early event in carcinogenesis of many different tumor types (21). Methylation of tumor suppressor genes contributes to an immortalized phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth. Until now, several types of tumor suppressor genes have been represented, such as cell cycle, DNA repair, hormonal response, cytokine signaling, transcription factor, p53 network and other pathways (22). But it is still unknown why some genes become hypermethylated in certain tumors, whereas others with similar properties remain unmethylated.

1.3. Clinical implication of DNA methylation in cancer
1.3.1. Hypermethylation of gene promoter as a diagnostic marker for cancer
In recent years, abnormal patterns of DNA methylation have been recognized as molecular changes in neoplasia (18) and represent excellent targets for novel diagnostic approaches. Only those methylation markers that are unmethylated in normal cells can be included in a diagnostic panel. Moreover, since hypermethylation of gene promoter is common in cancer, marker panels can be designed that over 70 percent of virtually all major types of cancer are positive (23). Table 1 summarizes DNA methylation markers that are reported in various tumor types.

1.3.2. Hypermethylation of gene promoter as a predictive marker in cancer therapy
CpG island hypermethylation of some genes is recognized as a predictor of response to treatment. For instance, DNA methylation of some tumor suppressor genes in breast cancer has been shown to be predictive of responsiveness to tamoxifen therapy (24). Gene inactivation by promoter hypermethylation may be the main reason of the loss of hormone response. A clinical example is the methylation and silencing of MGMT gene in gliobastoma that has been associated with an increased benefit from temozolomide treatment (25). The MGMT protein is directly responsible for repairing the addition of alkyl groups to the guanine base of the DNA. This base is the preferred target in the DNA of alkylating chemotherapeutic drugs such as procarbazine, streptozotocin, or temozolomide (25).

1.3.3. Reversal of gene silencing to prevent or treat cancer
Demethylation agents such as 5-azacytidine or 5-aza-2-deoxycytidine can reactivate the affected genes and restore production of the corresponding protein in cultured cancer cells (7). These findings make reversal of gene silencing a rational target for therapy. 5-Azacytidine was developed as an antineoplastic agent some 20 years ago, before its effects on DNA methylation
was known. Only after Taylor and Jones found that it had demethylation properties, its clinical activity could be explained (26). Recently, clinical trials with 5-aza-2-deoxycytidine are ongoing in AML. But it is still difficult to transfer this technique to human primary cancers because of the lack of cancer specificity. Global hypomethylation caused by 5-azacytidine may be associated with even greater chromosomal instability. So it is unclear whether demethylation agents can be used as an anti-cancer drug.

2. TECHNICAL IMPROVEMENTS IN DETECTING HYPERMETHYLATION

The detection of DNA methylation as novel biomarker in cancer research and diagnostics was revolutionized by two major discoveries. The first was the discovery of a very simple assay to visualize methylcytosine by treatment of genomic DNA with sodium bisulfite. Bisulfite treatment results in the conversion of cytosine residues into uracil, except methylcytosine residues that are protected against this treatment. In this way, by the use of sequence analysis methylated and unmethylated DNA can be distinguished (27). The second discovery was the development of methylation specific PCR (MSP). By taking advantage of the sequence differences within CpG islands of a promoter after bisulfite treatment dependent on methylation status, specific PCR primers can be designed that can distinguish methylated DNA from unmethylated DNA (28). From that time on, numerous different assays have been developed and used to study methylation as reviewed by Laird in 2003 (29). In the past few years, even more and more new techniques for detecting DNA methylation have emerged. Every technique has its own advantages and disadvantages. In the following sections some of these new techniques divided in 5 groups are described: global methylation analysis, marker discovery, qualitative analysis, quantitative analysis and complex informational analysis (summarized in table 2).

2.1 Global methylation analysis

Global methylation analysis used in a pool of DNA is usually performed by determining the ratio between 5-methylcytosine and cytosine. High performance liquid chromatographic (HPLC) and high-performance capillary electrophoresis (HPCE) are the earliest techniques to detect methylation. They can already accurately quantify the total amount of 5-methylcytosine. Alternative techniques have been developed afterwards, including assays based on the use of bacterial DNA methyltransferase or 5-methylcytosine specific antibodies.

2.1.1 HPLC

HPLC method is for the analysis of major and minor deoxyribonucleosides in DNA. The method offers good sensitivity, selectivity, precision and accuracy for the determination of all five deoxyribonucleosides without the use of harsh hydrolysis conditions, large DNA samples, difficult sample preparation procedures or in vivo labeling of DNA (30). According to standard procedures, total genomic DNA is hydrolyzed to deoxyribonucleotides (base + deoxyribose + phosphate) using a combination of deoxyribonuclease and nuclease P1. Following hydrolysis, deoxyribonucleotides are further converted into deoxyribonucleosides (base + deoxyribose) by treatment with alkaline phosphatase, and the products are then separated by standard reverse-phase HPLC. Cytosine and 5-methylcytosine can be identified and quantified by including
external standards of bases and monitoring UV absorbance at 254 nm. To avoid interference from RNA contamination, it is usually necessary to remove RNA from the nucleic acid preparations by enzymatic hydrolysis using a combination of ribonuclease A and ribonuclease T1.

2.1.2 HPCE
Based on HPLC, a new approach to evaluate the relative degree of genomic DNA methylation through the quantification of 2’-deoxynucleosides is proposed. Detection and quantification of 5-methyl 2’-deoxycytidine in genomic DNA has been performed using micellar HPCE with Ultra-Violet/Visible (UV/VIS) Spectrophotometry detection (31). This approach has been demonstrated to be more sensitive and specific than other HPCE methods for the quantification of DNA methylation degree and also to be more simple, rapid and cost-effective than other HPLC-based methods. The detection and quantification of nucleosides through enzymatic hydrolyses notably increases the specificity of the technique and allows its exploitation in the analysis of poorly purified and/or concentrated DNA samples such as those obtained from paraffin-embedded tissues.

2.1.3 TLC
Thin-layer chromatography (TLC) may be an useful alternative (32), if the equipment for high-resolution separation of hydrolyzed DNA is not available. In brief, DNA is cut at all CCGG sites using the restriction enzyme MspI, and the internal cytosine in these sites is then radioactively labeled using γ-labeled ATP and polynucleotide kinase. Following hydrolysis of the DNA to mononucleotides using nuclease P1, radioactively labeled cytosine monophosphate and 5-methylcytosine monophosphate are separated on TLC plates and identified and quantified as individual spots using a phosphorimager or X-ray film. The percentage of mC was calculated using the formula: % 5mC = 5mC/C * 100. Because only cytosines in CCGG sites are probed, this approach only provides a rough estimate of the ratio between 5-methylcytosine and cytosine.

2.1.4 SssI methyl acceptance assay
The enzyme SssI DNA methyltransferase is able to catalyse the de novo methylation of CpG sites (33) using the universal methyl donor S-adenosylmethionine (SAM). The principle of this assay is very simple: the enzyme is used to transfer a tritium-labelled methyl group from SAM to unmethylated cytosines in CpG sites of genomic DNA (34). The DNA is then immobilized on DEAE paper and the unincorporated SAM is washed off. The amount of incorporation may then be quantitated using a scintillation counter. The more radioactive the sample, the less CpG methylation there was in the DNA sample. This procedure can be used to quantitate small global changes in methylation; however, the absolute number of counts recorded can vary a great deal from experiment to experiment. This is because both the SAM and the SssI enzyme are somewhat unstable. If it is impractical to analyse all of the samples at the same time, an internal control should be used to normalise the data between days. Another important source of error in the SssI procedure is the measurement of DNA concentration. Genomic DNA can be extremely difficult to dissolve to homogeneity, and as a result, the apparent OD260 might not accurately reflect the amount of DNA included in the reaction. A useful way of obtaining a more
Different techniques for methylation detection

homogeneous solution is to digest the DNA with a restriction enzyme (e.g., EcoRI) that does not contain a CpG sequence in its recognition site (35).

2.1.5 Chloroacetaldehyde reaction

Oakeley et al. described a method for studying changes in genome-wide levels of DNA methylation by fluorescent labeling using chloroacetaldehyde (36). The DNA is first treated with sodium bisulfite. Then the bisulfite-treated DNA is depurinated under acidic conditions, and the purines are removed by silver nitrate precipitation or by column chromatography. Subsequent incubation of the sample with chloroacetaldehyde yields the intensely fluorescent ethenocytosine derivative of 5-methylcytosine, which can be quantified using a fluorimeter and used as a direct measure of the level of 5-methylcytosine in the genome. The chloroacetaldehyde assay is an attractive alternative to the SssI acceptance assay. Not least the use of stable and inexpensive chemicals and the avoidance of radioactivity are major advantages.

2.1.6 Immunological techniques

In 1980, a method (37) to identify 5-methylcytosine (m$^5$Cyt) in DNA immobilized on nitrocellulose paper by using antibody raised against m$^5$Cyt has been described. Sonicated and denatured DNAs were labeled at the 5’ end with [γ-32P] ATP by polynucleotide kinase. DNA was incubated with antibody against m$^5$Cyt, and the DNA-protein complex was collected on nitrocellulose paper. The paper was washed and dried, and radioactivity was measured. The method enables the identification of individual methylated sites in purified DNAs in the size range of single genes.

In 1997, an advanced method (38) to detect DNA methylation has been developed by confocal fluorescence microscopy using a monoclonal anti-5-methylcytosine (anti-m$^5$C) antibody and a polyclonal anti-histone H1 (antihistone) antibody as an internal standard. The specificity of the anti-m$^5$C antibody was demonstrated by a titration series against both single-stranded DNA and double-stranded DNA substrates in either the methylated or unmethylated forms. The antibody was found to show similar kinetics against both double- and single-stranded DNA, and the fluorescence was proportional to the amount of DNA used. No signal was observed with unmethylated substrates. The use of a confocal microscope makes these data independent of possible focusing artefacts.

2.2 Marker discovery

As hypermethylation of gene promoter is used as both a diagnostic marker and a treatment target in cancer, more new techniques for marker discovery were developed in the last 10 years. Restriction landmark genomic scanning (RLGS) was one of the first methods to be adapted for genome-wide methylation analysis. Other important tools for detecting altered patterns of DNA methylation across the genome involve variations of arbitrary primed PCR, such as methylation-sensitive arbitrarily primed PCR (MS-AP-PCR), methylated CpG island amplification representational difference analysis (MCA-RDA) and amplification of inter-methylated site (AIMS). These methods are particularly useful because arbitrary primed PCR is carried out using DNA templates that have been enriched for methyl sequences, resulting in preferential
amplification of CpG islands and gene-rich regions. However, all of these techniques require further validation by bisulphite genomic sequencing. Undoubtedly, one of the most efficient means of studying CpG-island methylation at a genome-wide scale involves novel technologies that make use of CpG-island and promoter microarrays on global sequencing (39). A widely used example of such an approach is differential methylation hybridization (DMH), which allows the simultaneous determination of the methylation levels of a large number of CpG-island loci. Later on, treatment DAC+/- TSA microarray, illumina and methylated DNA immunoprecipitation (methyl–DIP) also arise (40-42).

2.2.1 MS-AP-PCR
Gonzalgo et al. described MS-AP-PCR technique that uses methylation-sensitive restriction digestion coupled with AP-PCR to identify random but specific methylation changes at multiple sites in genomic DNA in a rapid and efficient manner (43). Restriction enzymes with different sensitivities to cytosine methylation in their recognition sites are used to digest genomic DNAs from primary tumors, cell lines, and normal tissues prior to arbitrarily primed PCR amplification (44;45). Fragments that showed differential methylation are cloned and sequenced after resolving the PCR products on high-resolution polyacrylamide gels. The cloned fragments are used as probes for Southern analysis to confirm differential methylation of these regions in tissues and cell lines.

MS-AP-PCR is a simple and reproducible fingerprinting method for screening the genome for regions of DNA that have altered patterns of DNA methylation associated with oncogenic transformation. Southern blotting, high-performance liquid chromatography analysis, and the methyl-acceptance assay have been used to study global levels of DNA methylation. None of these methods, however, can be used to isolate specific and unknown DNA sequences from genomic DNAs that are differentially methylated between normal and tumor tissues.

2.2.2 MCA-RDA
Toyota et al. developed a new technique called MCA-RDA. MCA allows for the efficient PCR amplification of methylated CpG islands (46), which can detect methylation of many genes, or to clone CpG islands differentially methylated in cancer. RDA is a subtraction technique that relies on hybridizing the two genomes of interest (tester and driver), followed by PCR amplification of tester sequences that did not hybridize with driver DNA. MCA was used to enrich for hypermethylated CpG islands, and RDA was used to identify those that are exclusively methylated in cancer.

An important application of MCA is the discovery of a novel gene hypermethylated in cancer. MCA coupled with RDA is a rapid and powerful technology for this purpose and compares favorably with other described techniques. In addition to the identification of genes hypermethylated in cancer, MCA could potentially be used to discover novel imprinted genes using parthenogenetic DNA, as well as novel X chromosome genes.

2.2.3 DMH
Huang et al. developed a novel array based method, called DMH, which allows a genome-wide screening of hypermethylated CpG islands in cells (39). Genomic DNA is pre-cut with a methylation insensitive enzyme, such as MseI. Linkers are then ligated to the digested DNA
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before it is incubated with the methylation-sensitive enzymes, BstUI and HpaII. The resulting digests are amplified by PCR and the products hybridized to an array of immobilized CpG islands.

DMH has at least three unique features. First, a high-density, DNA array-based screening strategy is applied in DMH. Second, all the genomic fragments screened by DMH contain multiple methylation–sensitive BstUI sites. This allows a more precise measurement of the frequencies and extent of methylation of the tested CpG island loci in the tumor genome. DMH is useful for a genome-wide screening of methylation in cancer and can be converted into a high-throughput analysis by implementing the microarray technologies mentioned before. Third, the genomic fragments are derived from a library specifically constructed to contain highly enriched CpG island sequences.

2.2.4 RLGS

RLGS is a method that provides the methylation status of thousands of unselected CpG islands in the genome within a single gel (47). Genomic DNA is initially digested with methylation-sensitive restriction enzymes, such as NotI, which recognize large CpG-rich sequences. These sequences usually occur in CpG islands and are cleaved only if the CpG dinucleotides are unmethylated. The digested DNA is radioactively labeled at the restriction sites using \([\alpha -32P]dCTP, [\alpha -32P]dGTP\) and DNA polymerase, digested with a second restriction enzyme, such as EcoRV, and then subjected to electrophoresis in an agarose tube gel (first-dimension separation). The agarose gel is then equilibrated in HinfI digestion buffer and the DNA digested in the gel with HinfI. The agarose gel is then placed horizontally across the top of a nondenaturing polyacrylamide gel, and the DNA is separated by electrophoresis in the second dimension. The gels are then dried and exposed to X-ray film or inspected using a phosphorimager.

The above approach produces a complex pattern of spots, in which a spot will be missing if a particular NotI site is methylated and has not, therefore, been recognized and cleaved. This spot may be stabbed from the corresponding control gel for further identification by sequence analysis. Alternatively, software systems have been developed for automated analysis of RLGS profiles. Differences between RLGS profiles have been used to identify imprinted genes and genes involved in disease states such as cancer.

The RLGS approach is suitable for simultaneous assessment of the methylation status and copy number of thousands of CpG islands. Such parallel analyses are critical for pattern elucidation within and between tumor types, and for estimating the overall influence of CpG-island methylation on the cancer-cell genome. The disadvantages include the labor-intensive and time-consuming procedure, the need for large amounts of starting DNA and good quality of DNA, and the difficult interpretation of the two-dimensional gel images.

2.2.5 Treatment DAC+/- TSA microarray

Suzuki et al. described a new microarray-based strategy that combines gene expression status and epigenetic regulation (41). The approach is based upon that silencing of hypermethylated genes in cancer is dependent on both methylation of dense CpG islands and histone deacetylase (HDAC) activity (48).
CDNA microarray technology is used to identify genes upregulated in cancer cell lines, after cells were treated with low-dose 5-aza-2′ deoxycytidine (DAC), which minimally blocks DNA methylation, in combination with trichostatin A (TSA) to inhibit histone deacetylase (HDAC). It is shown that the low dose of DAC used and the short treatment time for the cells resulted in only a few alleles being demethylated, and these alleles may have led to the upregulation of gene expression. This situation could diminish the sensitivity of a microarray screen. This gene screening technique has led to the identification of gene hypermethylation events that cluster within specific tumor types, and can simultaneously involve several members of a single gene family.

2.2.6 MSO
Gitan et al. developed a novel approach, called methylation-specific oligonucleotide (MSO) microarray, for detecting changes of DNA methylation in cancer (49). The method uses bisulfite-modified DNA as a template for PCR amplification, resulting in conversion of unmethylated cytosine, but not methylated cytosine, into uracil within CpG islands of interest. The amplified product, therefore, may contain a pool of DNA fragments with altered nucleotide sequences due to differential methylation status. A test sample is hybridized to a set of oligonucleotide (19–23 nucleotides in length) arrays that discriminate methylated and unmethylated cytosine at specific nucleotide positions, and quantitative differences in hybridization are determined by fluorescence analysis. A unique control system is also implemented to test the accuracy and reproducibility of oligonucleotides designed for microarray hybridization. This MSO microarray is applied to map methylated CpG sites within cell lines and tissue samples. MSO microarray is a promising technique for mapping methylation changes in multiple CpG island loci and for generating epigenetic profiles in cancer.

2.2.7 AIMS
To screen for tumor-specific alterations and to make a global assessment of methylation status in cancer cells, Frigola et al. modified the methylated CpG island amplification method to generate easily readable fingerprints representing the cell’s DNA methylation profile (50). The method is called AIMS. The method is based on the differential cleavage of isoschizomers with distinct methylation sensitivity. Specific adaptors are ligated to the methylated ends of the digested genomic DNA. The ligated sequences are amplified by PCR using adaptor-specific primers extended at 3’ end with two to four arbitrarily chosen nucleotidic residues to reduce the complexity of the product. Fingerprints consist of multiple anonymous bands, representing DNA sequences flanked by two methylated sites, which can be isolated and individually characterized. It is a novel method to screen for differential DNA methylation. The approach is feasible for the analysis of large series of samples. Moreover, a high number of sequence tags may be generated in a few experiments. Represented tags arise from the chromosomal regions that are richest in CpG islands and genes. AIMS appears to be a powerful tool for identifying new genes critical to carcinogenesis.
2.2.8 Illumina

Illumina's GoldenGate Methylation Solution is the first array-based platform that combines high sample throughput, high multiplexing, and single-site CpG resolution for DNA methylation profiling (40). It is an adaptation of a high-throughput single nucleotide polymorphism (SNP) genotyping system (51) to DNA methylation detection, based on genotyping of bisulfite-converted genomic DNA and PCR.

Briefly, bisulfite-treated, biotinylated genomic DNA was immobilized on paramagnetic beads. Pooled query oligonucleotides were annealed to the genomic DNA under a controlled hybridization program, and then washed to remove excess or mishybridized oligonucleotides. Hybridized oligonucleotides were then extended and ligated to generate amplifiable templates. Requiring the joining of two fragments to create a PCR template in this scheme provided an additional level of locus specificity. It is unlikely that any incorrectly hybridized allele-specific oligonucleotides (ASOs) and locus-specific oligonucleotides (LSOs) will be adjacent, and therefore should not be able to ligate after ASO extension. A PCR reaction was performed with fluorescently labeled universal PCR primers. The methylation status of an interrogated CpG site was determined by calculating $\beta$, which is defined as the ratio of the fluorescent signal from the methylated allele to the sum of the fluorescent signals of both methylated and unmethylated alleles. The $\beta$-value provides a continuous measure of levels of DNA methylation in samples, ranging from 0 in the case of completely unmethylated sites to 1 in completely methylated sites. The method provides not just a discrete measure of positive versus negative DNA methylation, but a continuous measure of levels of DNA methylation. So this technology can be applied to the high-throughput discovery and validation of potential biomarkers of cancer. Because methylation detection interrogates genomic DNA, rather than RNA or protein, it offers several technological advantages in a clinical diagnostic setting: (1) readily available source materials, particularly important for prognostic research, because DNA can be more reliably extracted than RNA from archival clinical samples for study; (2) capability for multiplexing, allowing simultaneous measurement of multiple targets to improve assay specificity; (3) easy amplification of assay products to achieve high sensitivity; and (4) the ability to detect a positive signal in tumors that arises from methylation inactivation of one allele of tumor-suppressor genes (52).

2.2.9 Methyl-DIP

Weber et al. developed an immunocapturing approach called Methyl-DIP to enrich methylated DNA and combine it with detection by DNA microarray (42). It is a technique based on chromatin immunoprecipitation using the ChIP-on-chip approach which has provided another important recent advance in the epigenomic profiling of cancer cells. In methyl-DIP, DNA is first fragmented by sonication and methylated fragments are then immunoprecipitated using a methylation-specific antibody against methyl-CpG-binding domain proteins (MBDs), which have a great affinity for binding to methylated cytosines, has been used to identify hypermethylated genes in mammary tumorigenesis. These fragments can then be hybridized to an array of proximal promoter regions to assess DNA methylation patterns specifically in gene-regulatory regions.
The key finding that DNA immunoprecipitated can be used as a probe for hybridization to genomic microarray platforms promises to simplify and universalize the analysis of the DNA methylome, because it allows the rapid identification of multiple CpG sites. However, CpG-island and tiling microarrays are available for lots of promoters from different companies, the entire human genome is not yet represented in any microarray. In addition, the need for whole-genome amplification before and after immunoprecipitation can introduce PCR biases.

2.3 Qualitative analysis

A lot of methods have been developed to assess the methylation status of specific genes in a broad range of applications. All current methods rely on treatment of genomic DNA with bisulfite and/or restriction enzymes prior to PCR amplification. Several of these methods were originally developed for detection of single-nucleotide polymorphisms (SNPs) and disease-causing mutations in genomic DNA, but later have been adapted for detection of bisulfite-induced sequence differences between methylated and unmethylated alleles.

2.3.1 MSRE - Southern

Isoschizomers of bacterial restriction endonucleases with different sensitivities to 5 methylcytosine have been widely used to determine the methylation status of cytosine at specific sites, which was called MSRE (methylation-sensitive restriction endonuclease). If large amounts of DNA are available (>10 μg), the digestion products may be analyzed by conventional Southern blotting. Essentially, the DNA digestion products are fractionated by electrophoresis in an agarose gel, blotted onto a nylon membrane and then hybridized with a radioactive probe representing the gene of interest (53). If the site(s) of interest is methylated, the bands generated with the methylation-sensitive isoschizomer will differ in size from those generated with the non-sensitive isoschizomer. The fraction of digestion-resistant DNA may be quantified by image analysis and is proportional to the degree of methylation present at that site (methylation level).

Methods based on the use of MSREs generally suffer from the limitation that they provide information only about CpGs within the cleavage sites of the specific enzymes. And MSRE-based analysis may be plagued by false positives because of incomplete digestion, resulting in the false conclusion that at least some cytosines in the target sequence are methylated. Furthermore, the sensitivity of Southern blotting analysis of MRSE-digested DNA depends on the hybridization background, but is usually ~10%.

2.3.2 Hpa II PCR

Methylation-sensitive restriction enzymes and Southern blot analysis are commonly used to assay for DNA methylation, usually requiring DNA from about 10^5 cells. However sensitivity adequate for only a few hundred cells is needed in many cases. Hpa II PCR is a sensitive PCR assay using primers that bracket a HpaII site; if the DNA is treated with HpaII prior to PCR, an amplified product is seen only from a methylated genomic template (54).

The two improvements of Hpa II PCR are quantitatively applicable to 100-300 cells, with as little as 10% DNA methylation detectable.
2.3.3 Bisulfite genomic sequencing

Bisulfite genomic sequencing protocol has been developed by Frommer et al. (27). The method is based on sodium bisulfite-mediated conversion of cytosine to uracil in single-stranded DNA, followed by PCR amplification of the resultant modified DNA. Exact methylation maps of single DNA strands from individual genomic DNA molecules can readily be established, where the position of each 5-methylcytosine is given by a clear positive band on a sequencing gel. It provides information about the methylation status of every cytosine residue within the target sequence. For highest resolution of methylation patterns, the PCR products are cloned into an appropriate plasmid vector followed by sequence analysis of a number of individual clones. While the experimental procedure is laborious and time-consuming, interpretation of data is simple. Alternatively, the PCR product may be sequenced directly to provide an average across all molecules in the sample. The major disadvantages of cloning and sequencing are that a high number of clones have to be sequenced for reliable results, and that artifacts relating to PCR infidelity, incomplete bisulfite conversion, or bisulfite conversion of 5-methylcytosine to thymine become significant. Direct sequence analysis is less prone to artifacts but does not provide information about the methylation patterns of individual alleles.

2.3.4 Methylation-specific PCR

Methylation-specific PCR (MSP) is sensitive and specific for methylation of virtually any block of CpG sites in a CpG island (28). Primers should be designed to distinguish methylated from unmethylated DNA in bisulfite-modified DNA, taking advantage of the sequence differences resulting from bisulfite modification. Unmodified DNA or DNA incompletely reacted with bisulfite can also be distinguished, since marked sequence differences exist between these DNAs. The frequency of CG sites in CpG islands renders this technique uniquely useful and extremely sensitive for such regions. Since the two strands of DNA are no longer complementary after bisulfite treatment, primer can be designed for either modified strand. The fragment of DNA to be amplified is intentionally small, to allow the assessment of methylation patterns in a limited region and to facilitate the application of this technique to samples, such as paraffin blocks, where amplification of larger fragments is not possible.

2.3.5 HRM or MS-MCA

A new more reliable method for promoter methylation analyses in clinical samples is described by Tomasz K. Wojdacz et al. (55). The new approach is based on high resolution melting (HRM) which was originally developed for SNP genotyping (56). In 2003, Worm et al. first described a new in-tube PCR assay for the detection of aberrant DNA methylation that uses a thermal cycler integrated with a fluorometer and exploits differences in melting temperature (Tm) between methylated and unmethylated alleles after bisulfite treatment (57). They also called this method methylation-specific melting curve analysis (MS-MCA).

HRM relies upon on the precise monitoring of the change of fluorescence as a DNA duplex melts. Like many real-time PCR techniques, HRM utilizes the ability of certain dyes to fluoresce when intercalated with double-stranded DNA. Two advances have made high resolution melting possible. The first is the introduction of intercalating dyes that do not inhibit PCR reactions at the concentrations necessary for them to fully saturate the target DNA duplexes. The second is the
development of instrumentation that is able to monitor the changes of fluorescence with high accuracy. By comparing the melting profiles of unknown samples with the profiles of fully methylated and unmethylated references amplified after bisulfite modification, it is possible to detect methylation with high sensitivity and moreover estimate the extent of methylation of the screened samples. But the disadvantage is it is still impossible to know which CpG is methylated at the same time.

2.4 Quantitative analysis
Based on qualitative analysis, some new methods for quantitative methylation detection have been developed. Compared to qualitative analysis, they can not only detect methylation at multiple CpG sites but also give the accurate amount of methylation or the percentage of methylation. But these PCR-based methods interrogate the methylation status only at CpG sites that are complementary to the primers. Therefore, the predominant methylation pattern in a sample is not reflected in the results of such experiments, and bisulfite sequencing for genomic is necessary to provide a complete picture of the heterogeneous methylation patterns.

2.4.1 Ms-SnuPE
Methylation-sensitive single nucleotide primer extension (Ms-SnuPE) is a rapid quantitative method for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA followed by single nucleotide primer extension (58). After bisulfite treatment, quantitation of the ratio of methylated versus unmethylated cytosine (C versus T) at the original CpG sites can be easily determined by incubating the gel-isolated PCR product, primer(s), and Taq polymerase with either [32P]dCTP or [32P]dTTP followed by denaturing polyacrylamide gel electrophoresis and phosphorimage analysis. Opposite strand Ms-SNuPE primers can also be designed which would incorporate either [32P]dATP or [32P]dGTP to assess methylation status depending on which CpG site is analyzed.
Ms-SNuPE can be used for the rapid detection and quantitation of methylation differences in genomic DNA. Furthermore, the amount of methylation at multiple CpG sites can be analyzed in a single reaction by using a multiplex oligonucleotide strategy and without the use of restriction enzymes. The Ms-SNuPE technique should prove to be extremely useful for detecting and quantitating abnormal methylation changes in cancer cells when only small amounts of DNA such as micro-dissected material are available.

2.4.2 COBRA
Xiong et al. developed a quantitative technique called combined bisulfite restriction analysis (COBRA) to determine DNA methylation levels at specific gene loci in small amounts of genomic DNA (59). Methylation-dependent sequence differences are introduced into the genomic DNA by the standard sodium bisulfite treatment and then PCR amplification. This combination of bisulfite treatment and PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine. This sequence conversion can lead to the methylation dependent creation of new restriction enzyme sites or it can lead to the methylation-dependent retention of pre-existing sites such as BstUI (CGCG). The primers used in the PCR reaction do not contain CpG dinucleotides so that the
amplification step does not discriminate between templates according to their original methylation status. Therefore, in the mixed population of resulting PCR fragments, the fraction that has retained restriction site that contains a CpG(s) should be a direct reflection of the percentage DNA methylation at that site in the original genomic DNA. The most accurate and reliable method to quantitate the relative amounts of digested and undigested PCR products was to perform an unlabeled PCR reaction, followed by a purification step to ensure subsequent complete cutting, then restriction digestion, polyacrylamide gel electrophoresis, electroblotting, oligo hybridization and phosphorimager quantitation. This hybridization strategy allows flexibility provided by the choice of probe position, relative to the restriction sites. Results obtained with different probes can be used to corroborate DNA methylation values obtained for a particular CpG site.

COBRA combines the powerful features of ease of use, quantitative accuracy, and compatibility. But there is the same problem with the methods based on the use of MSREs, such as MSRE-Southern and Hpa II PCR. They suffer the limitation that they provide information only about CpGs within the cleavage sites of the specific enzymes.

### 2.4.3 QMSP

An advancement of conventional MSP is real-time quantitative MSP (QMSP) (60), which permits reliable quantification of methylated DNA. It is more sensitive than conventional PCR and can detect methylation in samples with substantial contamination of normal DNA (1:10,000). Furthermore, it is a high-throughput technique, which could make QMSP suitable for implementation in nationwide screening programs.

After sodium bisulfite conversion, genomic DNA is amplified by fluorescence-based, real-time quantitative PCR. In brief, bisulfite-converted genomic DNA is amplified using locus-specific PCR primers flanking an oligonucleotide probe with a 5’ fluorescent reporter dye (6FAM) and a 3’ quencher dye (TAMRA). The 5’ to 3’ nuclease activity of Taq DNA polymerase can be detected by various instruments, including the Roche/Boehringer Mannheim LightCycler, the PE Biosystem ABI Prism 7700/7900 and 5700 GeneAmp Sequence Detection Systems. After crossing a fluorescence detection threshold, the PCR amplification results in a fluorescent signal proportional to the amount of PCR product generated. Initial template quantity can be derived from the cycle number at which the fluorescent signal crosses a threshold in the exponential phase of the PCR reaction. There are some other chemistry substances for template detection including SYBR green dye intercalation as well as hybridization probes, hydrolysis probes and molecular beacons.

QMSP is not only highly specific, sensitive and reproducible, but that it also can rapidly detect biologically relevant information in patient samples. QMSP is a PCR-based method that requires only minute amounts of DNA of modest quality, making it compatible with small biopsies and paraffin-embedded tissues. So QMSP has a good future as a screening tool in clinical application. We suppose to look for the genes that are methylated in cancers and unmethylated in normals. However some genes are also methylated in normals, the methylation level is lower if compared to cancers. Then how to solve this problem seems very important. Alternatively a cut-off level can be chosen. There are still different opinions about how to choose the cut-off level, such as
above the highest normal or above the lowest cancer.

2.5 Complex informational analysis

Detection and characterization of aberrantly methylated alleles of specific genes in neoplastic tissues have previously relied upon two different methodological principles. The first of these principles is to determine the methylation status of single CpG sites by using either methylation-sensitive restriction enzymes or MSP. The major disadvantage of this approach is that partial methylation involving only sites outside recognition or primer binding sequences will not be detected. The second principle is to collectively amplify methylated and unmethylated alleles from a sample of bisulfite-treated DNA followed by assessment of the methylation status of all CpG sites within the amplified region by sequence analysis. Although powerful, detection of methylated alleles in a tumor sample by bisulfite genomic sequencing may be complicated by the contamination of samples with nonneoplastic cells. Furthermore, the exact methylation profile of individual alleles can only be determined through the inclusion of an extensive and laborious subcloning step. But complex informational analysis can give both methylation and unmethylation information of all CpG sites within the amplified region by sequence analysis.

2.5.1 MS-DGGE

To study in detail the patterns of cytosine methylation at the CpG island of single gene in tumor, Aggerholm et al. devised a novel method that is based on (a) treatment of genomic DNA with sodium bisulfite, (b) amplification of both methylated and unmethylated sequences by PCR, and (c) resolution of differentially methylated sequences by denaturing gradient gel electrophoresis (DGGE) (61). Following bisulfite treatment, methylated and unmethylated sequences are predicted to differ in thermal stability due to their different GC contents and can be physically separated by electrophoresis in a gel containing an increasing gradient of chemical denaturants. The produced gel provides a detailed visual display of the methylation status in complex cell populations and a simple means for isolating clonotypic epigenotypes.

This method combines nondiscriminatory amplification of methylated and unmethylated sequences using bisulfite-treated DNA as template and resolution of differentially methylated alleles by DGGE. The principle is that an increase in DNA methylation, i.e., an increase in the numbers of CpG sites involved, is associated with an increase in GC content and, hence, in thermal stability.

2.5.2 MS-SSCP

Maekawa et al. introduced a procedure combining bisulfite treatment and PCR-single-strand conformation poly-morphism (SSCP) (62). Primers are designed annealing to the converted DNA sequences which have no CpG sites in the corresponding region of the original DNA, amplifying both unmethylated and methylated DNA. Marked sequence differences exist between amplified products from unmethylated and methylated DNA, and could be distinguished by mutation screening methods, i.e., SSCP. Moreover, Maekawa et al. has succeeded in achieving an exact quantification of polymorphic DNA sequences and of expression levels of genes encoding homologous sequences, by SSCP in combining an automated fluorescence-based DNA sequencer with a data processing computer. Bisulfite-
Different techniques for methylation detection

PCR-SSCP (BiPS) is easily applied to the methylation assay of any sequence. Of course, it can be used without radioactive materials. Silver staining or other staining methods, or an automated sequencer with fluorescence labeling can be applied for the signal detection. One of the great advantages of SSCP is that alleles with different methylation patterns are physically separated in the gel and may be extracted for subsequent sequence analysis. This provides a simple means for identifying individual epigenotypes in a pool of DNA without time-consuming cloning analysis. The limitation of MS-SSCP is that probably not all methylation changes are resolved. First, conventional SSCP analysis has a specificity of <70%. Second, in unmethylated bisulfite-treated DNA, the number of bases is reduced from 4 to 3, which significantly reduces the ability of the single stranded molecules to form secondary structures. Conversion of non-CpG cytosines may also reduce or entirely abolish internal base pairing of methylated sequences. Until more systematic studies have clarified this issue, MS-SSCP may not be considered as a generally applicable method for resolving gene specific methylation changes.

2.5.3 MS-DHPLC

Baumer et al. described a new procedure for the analysis of the methylation status of imprinted genes based on methylation-specific PCR followed by denaturing high performance liquid chromatography (MSP/DHPLC) (63). The MSP/DHPLC method is based on PCR amplification of gene segments which show parent-of-origin specific methylation. Genomic DNA is subjected to an in vitro bisulfite treatment prior to PCR amplifications using primers specific for modified DNA. Both alleles are theoretically amplified with equal efficiency and are represented by identically sized PCR products; they differ, however, at a number of positions within the amplified DNA segment. The DHPLC analysis allows a very efficient resolution of the two populations of PCR products. The method offers a rapid and very reliable alternative to conventional methods used for such purposes such as Southern blots and methylation specific PCR (allele-specific MSP).

3. TO FIND AND APPLY DNA METHYLATION AS A DIAGNOSTIC TOOL

This overview describes current key techniques for methylation detection and also the most important advantages and disadvantages. Choice of methodology will not only depend on the available equipment and expertise, but also depend on the quality and quantity of samples. Choosing a technique mainly depends on the aims of methylation study. Therefore the techniques in this review were classified due to applications (summarized in table 2). As a diagnostic tool, mostly the gene-specific methylation analysis is used to determine whether or not aberrantly methylated alleles are present in the sample. Every laboratory will be able to study DNA methylation, using even limited amounts of material from old archives. So until now it is still the most popular technique to detect methylation all over the world. Compared to conventional MSP, real-time quantitative MSP (Q MSP) is more sensitive and can detect methylation in samples with substantial contamination of normal DNA (1:10.000). It is also a method that permits reliable quantification of methylated DNA. Because of using locus-specific PCR primers flanking an oligonucleotide probe, it is more specific than MSP. Furthermore, it is a
high-throughput technique, which could make QMSP suitable for implementation in nation-wide screening programs. The quantitative nature of the assay enables choosing a cut-off at a certain level.

In 2003, Harden et al. first developed QMSP as a diagnostic tool in prostate adenocarcinoma (64). He also reported the combination of histology and GSTP1 QMSP to detect 79% prostate adenocarcinoma, 15% improvement over histology alone. The results suggested that the addition of GSTP1 QMSP to routine histologic analysis of paraffin-embedded biopsy samples is likely to improve the sensitivity of diagnostic needle biopsies. In another study by the same group, with QMSP and empirically defined cutoff values, the combined use of GSTP1 and APC demonstrated a theoretical sensitivity of 98.3% for prostate carcinoma, with 100% specificity (65). Later, several other studies have reported using QMSP in other tumor tissues. Combining the 2 methylation markers, p16INK4a and RARB2, yielded a sensitivity of 69% and a specificity of 87% for the diagnosis of pulmonary malignancy (66). For six genes (Cyclin D2, FOXE1, NPTX2, ppENK, p16, and TFP12), pancreatic juice methylation was quantified using QMSP with a cutoff of >1% methylated DNA, that reached a sensitivity of 82% and a specificity of 100% (67). Another study demonstrated the novel finding of tumor-associated epigenetic markers in bone marrow aspirates/blood and their potential role as targets for molecular detection. QMSP was performed using a selected tumor-related gene panel for RAR-ss2, MGMT, RASSF1A, and APC (68). Tumor-associated hypermethylated DNA was identified in 7 (21%) of 33 bone marrow aspirates and 9 (27%) serum samples.

In addition, our group would like to use QMSP as a diagnostic tool in cervical cancer screening. So we can make a cut-off above the level of the highest methylated normal cervical sample or the highest methylated LSIL sample. Using QMSP in cervical scrapings and paired fresh frozen tissue, we previously demonstrated 32 of 48 (67%) SCC cases to be methylated above the highest methylated normal sample (i.e. “hypermethylated”) for any of the gene promoters APC, DAPK, GSTP1 and MGMT (69). In another study by our group, six (CALCA, DAPK, ESR1, TIMP3, APC and RAR-β2) out of 12 gene promoters analyzed were significantly hypermethylated in cervical cancer scrapings compared to scrapings from histologically normal cervices. Sensitivity of QMSP analysis with a four-gene panel (DAPK, CALCA, ESR1 and APC) was 89%, which was equivalent to Hr-HPV analysis and conventional cytomorphology, but with a higher specificity (100%) (70).

Promoter hypermethylation analysis holds most potential as a new diagnostic test for cervical cancer. Table 3 summarized previous results of genes that are methylated in cervical samples. In comparison to MSP, QMSP has two major theoretical advantages: 1) clear-cut results after the definition of CIN II/III and cervical cut-off levels; 2) QMSP is amenable to high throughput analysis. Further improvement of sensitivity for QMSP can be accomplished by discovering new genes methylated specifically in cervical cancer. High throughput screening techniques such as expression-microarrays, to find cancer-specific down-regulated genes in combination with algorithms that predict which genes are most likely to be methylation-sensitive, can be used for new marker discovery. With combination with new genes that are specific methylated in cervical cancer, we can increase the sensitivity of QMSP, however the specificity will not decrease.
When a gene panel will be discovered with sensitivity and specificity that exceed conventional cytology and equals the combination of cytology and HPV DNA analysis, QMSP can be used to replace the current screening techniques. Although current results for hypermethylation analysis are promising, more studies including larger patient populations, preferably randomized clinical trials, need to be performed.
Different techniques for methylation detection


Different techniques for methylation detection


Different techniques for methylation detection


Table 1: Overview of cancer-specific DNA methylation markers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Tumor type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P14&lt;sup&gt;ARF&lt;/sup&gt;</td>
<td>MDM2 inhibitor</td>
<td>Bladder, colon, oral, stomach, thyroid</td>
<td>(71-75)</td>
</tr>
<tr>
<td>p15&lt;sup&gt;INK4b&lt;/sup&gt;</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td>Stomach, liver, lung, leukaemia</td>
<td>(76-79)</td>
</tr>
<tr>
<td>p16&lt;sup&gt;INK4a&lt;/sup&gt;</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td>Bladder, breast, colon, head and neck, liver, lung, oesophageal, oral, stomach, thyroid</td>
<td>(72;73;76;77;79-92)</td>
</tr>
<tr>
<td>APC</td>
<td>Inhibitor of β-catenin</td>
<td>Colon, oral, oesophageal, lung, endometrial, cervical</td>
<td>(69;70;93-97)</td>
</tr>
<tr>
<td>BRCA1</td>
<td>DNA repair, transcription</td>
<td>Breast, ovary</td>
<td>(98;99)</td>
</tr>
<tr>
<td>CDH1</td>
<td>E cadherin, cell adhesion</td>
<td>Bladder, breast, stomach, leukaemia</td>
<td>(77;83;100;101)</td>
</tr>
<tr>
<td>DAPK 1</td>
<td>Pro-apoptotic</td>
<td>Bladder, head and neck, stomach, cervical</td>
<td>(69;70;77;83;84;102)</td>
</tr>
<tr>
<td>RARB</td>
<td>Retinoic acid receptor-β</td>
<td>Bladder, breast, cervix</td>
<td>(70;83;103;104)</td>
</tr>
<tr>
<td>MGMT</td>
<td>DNA repair of 06–alkyl-guanine</td>
<td>Head and neck, lung, cervix</td>
<td>(69;84;88)</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
<td>Breast</td>
<td>(70;105)</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
<td>Breast</td>
<td>(105)</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Conjugation to glutathione</td>
<td>Stomach, lung, prostate, cervix</td>
<td>(69;77;84;106)</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase-2</td>
<td>Colon</td>
<td>(107)</td>
</tr>
<tr>
<td>GATA4, GATA5</td>
<td>Transcription factor</td>
<td>Colon</td>
<td>(108)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Ras effector homologue</td>
<td>Lung</td>
<td>(109)</td>
</tr>
<tr>
<td>MLH1</td>
<td>DNA mismatch repair</td>
<td>Colon</td>
<td>(110)</td>
</tr>
<tr>
<td>TWIST1</td>
<td>acrocephalosyndactly 3; Saethre-Chotzen syndrome</td>
<td>Breast, cervix</td>
<td>(103;111)</td>
</tr>
<tr>
<td>CCNA1</td>
<td>cell-division cycle</td>
<td>Bladder, head and neck, oral, cervix</td>
<td>(112-116)</td>
</tr>
</tbody>
</table>
Table 2: Techniques described in this review

<table>
<thead>
<tr>
<th>Applications</th>
<th>Technique</th>
<th>Treatment of DNA</th>
<th>PCR-based</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global methylation analysis</td>
<td>HPLC</td>
<td>Enzymatic hydrolysis</td>
<td>No (30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPCE</td>
<td>Enzymatic hydrolysis</td>
<td>No (31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLC</td>
<td>Enzyme digestion</td>
<td>No (32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SssI methyl acceptance assay</td>
<td>None</td>
<td>No (34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloracetaldehyde reaction</td>
<td>Bisulfite treatment</td>
<td>No (36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immunological techniques</td>
<td>Denaturation/depurination</td>
<td>No (37;38)</td>
<td></td>
</tr>
<tr>
<td>Marker discovery</td>
<td>MS-AP-PCR</td>
<td>Enzyme digestion</td>
<td>Yes (43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCA-RDA</td>
<td>Enzyme digestion</td>
<td>Yes (46)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMH with CpG-island array</td>
<td>Enzyme digestion/ Bisulfite treatment</td>
<td>Yes (39;117;118)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RLGS</td>
<td>Enzyme digestion</td>
<td>No (47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment DAC+/- TSA microarray</td>
<td>Demethylation</td>
<td>No (41;119)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSO</td>
<td>Bisulfite treatment</td>
<td>No (49;120)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIMS</td>
<td>Enzyme digestion</td>
<td>Yes (50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Illumina</td>
<td>Bisulfite treatment</td>
<td>Yes (40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methyl-Dip</td>
<td>Sonication</td>
<td>Yes (42)</td>
<td></td>
</tr>
<tr>
<td>Qualitative analysis</td>
<td>MSRE-Southern</td>
<td>Enzyme digestion</td>
<td>No (53)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HpaI PCR</td>
<td>Enzyme digestion</td>
<td>Yes (54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bisulfite genomic sequencing</td>
<td>Bisulfite treatment</td>
<td>Yes (27)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSP</td>
<td>Bisulfite treatment</td>
<td>Yes (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HRM or MS-MCA</td>
<td>Bisulfite treatment</td>
<td>Yes (55-57)</td>
<td></td>
</tr>
<tr>
<td>Quantitative analysis</td>
<td>MS-SnuPE</td>
<td>Bisulfite treatment</td>
<td>Yes (58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>COBRA</td>
<td>Bisulfite treatment</td>
<td>Yes (59)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylight or QMSP</td>
<td>Bisulfite treatment</td>
<td>Yes (60)</td>
<td></td>
</tr>
<tr>
<td>Complex informational analysis</td>
<td>MS-DGGE</td>
<td>Bisulfite treatment</td>
<td>Yes (61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MS-SSCA</td>
<td>Bisulfite treatment</td>
<td>Yes (62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MS-DHPLC</td>
<td>Bisulfite treatment</td>
<td>Yes (63)</td>
<td></td>
</tr>
</tbody>
</table>

HPLC - high performance liquid chromatographic; HPCE - high-performance capillary electrophoresis; TLC - thin-layer chromatography; MS-AP-PCR - methylation-sensitive arbitrarily primed PCR; MCA-RDA - methylated CpG island amplification representational difference analysis; DMH - differential methylation hybridization; RLGS - restriction landmark genomic scanning; MSO - methylation-specific microarray; AIMS - amplification of inter-methylated site; Methyl-Dip - methyl DNA immunoprecipitation; MSRE - methylation-sensitive restriction endonuclease; MSP - methylation specific PCR; MS-HRM - Methylation-sensitive high resolution melting analysis; MS-MCA - methylation-specific melting curve analysis; MS-SnuPE - methylation-sensitive single nucleotide primer extension; COBRA - combined bisulfite restriction analysis; QMSP - real-time quantitative MSP; MS-DGGE - methylation-specific denaturing gradient gel electrophoresis; MS-SSCA - methylation-specific single-strand conformation analysis; MS-DHPLC - methylation-specific denaturing high-performance liquid chromatography
Table 3: Overview of literature: Previous results of genes in cervical samples.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Samples</th>
<th>Method</th>
<th>Results of normal cervix</th>
<th>Results of ASCUS</th>
<th>Results of LSIL</th>
<th>Results of HSIL</th>
<th>Results of SCC</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPK</td>
<td>Tampon collected cervical specimen</td>
<td>QMSP</td>
<td>N=10 1/13 (8%)*</td>
<td>n.p.</td>
<td>N=10 1/13 (8%)*</td>
<td>7/31 (23%)</td>
<td>9/11 (82%)</td>
<td>(102)</td>
</tr>
<tr>
<td>RAR-β2</td>
<td>Fresh frozen tissue</td>
<td>MSP</td>
<td>4/30 (13%)</td>
<td>n.p.</td>
<td>None of 7</td>
<td>5/17 (29%)</td>
<td>5/19 (26%)</td>
<td>(104)</td>
</tr>
<tr>
<td>DAPK, RAR-β</td>
<td>Cervical scrapings</td>
<td>Nested MSP</td>
<td>None of 11 (both genes)</td>
<td>n.p.</td>
<td>None of 17 (both genes)</td>
<td>7/11 (64%) 1/11 (9%)</td>
<td>n.p.</td>
<td>(121)</td>
</tr>
<tr>
<td>DAPK, RAR-β, TWIST1</td>
<td>Cervical scrapings</td>
<td>MSP</td>
<td>9/181 (5%)*</td>
<td>9/181 (5%)*</td>
<td>9/181 (5%)*</td>
<td>13/23 (57%)</td>
<td>68/92 (74%)</td>
<td>(111)</td>
</tr>
<tr>
<td>CCNA1</td>
<td>Fresh biopsies and scrapings**</td>
<td>Duplex MSP</td>
<td>None of 25</td>
<td>n.p.</td>
<td>None of 13</td>
<td>4/11 (36%)</td>
<td>28/30 (93%)</td>
<td>(112)</td>
</tr>
<tr>
<td>TSLC1</td>
<td>Paraffin embedded tissue</td>
<td>Sequencing (BSP)</td>
<td>None of 9</td>
<td>n.p.</td>
<td>None of 12</td>
<td>7/20 (35%)</td>
<td>30/52 (58%)</td>
<td>(122)</td>
</tr>
<tr>
<td>SPARC, TFPI2</td>
<td>Cervical scrapings</td>
<td>QMSP***</td>
<td>3/21 (14%)*</td>
<td>n.p.</td>
<td>n.p.</td>
<td>n.p.</td>
<td>20/22 (91%)</td>
<td>(123)</td>
</tr>
<tr>
<td>APC, DAPK, MGMT, GSTP1</td>
<td>Cervical scrapings</td>
<td>QMSP***</td>
<td>5/41 (12%)*</td>
<td>n.p.</td>
<td>n.p.</td>
<td>n.p.</td>
<td>35/48 (73%)</td>
<td>(69)</td>
</tr>
<tr>
<td>APC, CALCA, DAPK, ESR1</td>
<td>Cervical scrapings</td>
<td>QMSP***</td>
<td>None of 19</td>
<td>n.p.</td>
<td>n.p.</td>
<td>n.p.</td>
<td>25/28 (89%)</td>
<td>(70)</td>
</tr>
<tr>
<td>DAPK1, IGSF4, SPARC, TFPI2</td>
<td>Cervical scrapings</td>
<td>QMSP***</td>
<td>8/30 (26.7%)*</td>
<td>8/30 (26.7%)*</td>
<td>4/30 (13.3%)*</td>
<td>25/39 (64.1%)</td>
<td>n.p.</td>
<td>(124)</td>
</tr>
</tbody>
</table>

n.p = not performed, * Results were combined for this study, ** Fresh biopsies for cancers and controls, scrapings for LSIL/HSIL, *** A cut-off level was chosen for this study.