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Review article

Detection of DNA methylation in eucaryotic cells

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Abstract: The methods of molecular biology allow for analyzing the methylation pattern in the whole genome and in particular genes. We differentiate methylated sequences from unmethylated ones by means of cutting the genomic template with methylation-sensitive restriction enzymes or by sodium bisulfite DNA modification. Chemical modification precedes most quantitative and qualitative PCR techniques: MS-PCR, MS-nested PCR, Real-Time PCR, QAMA, HeavyMethyl, MS-HRM. Restriction enzymes, on the other hand, may be used together with PCR or hybridisation methods (Southern blot and microarrays). PCRs are conducted with primers specific for methylated and unmethylated sequences and sometimes, similarly to hybridisation techniques, with specifically labeled probes or dyes intercalating to double-stranded nucleic acids. The most advanced methylation detection techniques (MALDI-TOF MS and HPLC) significantly reduce the amount of biological material used for tests, but they require specialist equipment.

Key words: MS-PCR - MS-nested PCR - Real-Time PCR - QAMA - HeavyMethyl - MS-HRM - MALDI-TOF MS - HPLC - microarrays

Introduction

Changes in the methylation pattern may appear in epithelial cells even a couple of years before cancer. That is why using them as biomarkers creates a real chance for an early diagnosis and an adequate risk evaluation of a neoplastic diseases. It has been recently suggested that the examination of DNA methylation pattern might be useful for lung cancer detection. A five-year survival of patients with prostate, breast and colorectal cancer, which are detected by means screen tests, is 4-6 times higher than the survival of patients with lung cancer, for which there are no early detection methods [1].

DNA methylation as an epigenic marker

Molecular biology techniques allow for analyzing the methylation pattern in the whole genome and in particular genes. The methods which describe methylation in the whole genome usually require large amounts of DNA and, for this reason, they are useless for biomarkers analysis. They can be successfully applied while searching for new tumor suppressor genes and to monitor global methylation changes. Some promising results have been obtained from testing single genes extracted from a small amount of biological material [1].

The so-far described methods of methylation examination can be classified according to the following criteria:

1. the scope of the analyzed biological material (genome, particular genes),
2. the type of technique used,
   a. cutting with methylation-sensitive restriction enzymes (combined with hybridisation or PCR),
   b. hybridisation methods,
      - Southern blot,
      - microarrays,
   c. PCR (qualitative and quantitative),
   d. others.

Examination of single gene methylation

Southern blot

The first methods of methylation pattern analysis were Southern blot and cutting with restriction enzymes combined with PCR. Southern hybridisation bases on

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Table 1. Gene-specific DNA methylation assays described in this review arranged by their application with respect to genome coverage.

Application	Technique	Specimen treatment	DNA amount	Reference
Qualitative analysis of single CpGs	Southern blot	Enzyme digestion	≥ 5μg	[2]
	Methylation-sensitive enzymes and PCR	Enzyme digestion	1 μg	[3,4]
	MS-PCR	Bisulfite conversion	5μg	[2,8]
	MS-nested PCR	Bisulfite conversion	5μg	[9]
Quantitative analysis of single CpGs	MALDI-TOF MS	Bisulfite conversion	≤ 250 ng	[1,10]
	MethyLight	Bisulfite conversion	≤ 250 ng	[12]
	QAMA	Bisulfite conversion	≤ 250 ng	[1,13]
	HeavyMethyl	Bisulfite conversion	30 pg	[7]
	MS-HRM	Bisulfite conversion	1μl	[14]
	MB-PCR	Recombinant proteins	160 pg	[15]
	Bisulfite PCR and dNMP analysis	Bisulfite conversion	100-500 ng	[6]
	MDFS	Bisulfite conversion	3 ng	[17]
	COMPARE-MS	Enzyme digestion	4-100 ng	[18]
Analysis of genome-wide methylation content	aPRIMES	Enzyme digestion	500 ng	[1,20]
	HPLC	Total hydrolysis	< 1 μg	[1,21]
	RLGS	Enzyme digestion	>1 μg	[1,22]

the fact that methylation-sensitive restriction enzymes are not able to cut methylated sequences of CpG islands. This method provides an assessment of the overall methylation status of CpG islands. What limits this method is that a large amount of high-molecular-weight DNA (5 μg or more) has to be used and it is possible to detect methylation occurring in at least several percent of alleles (Table 1). What is more, the information can be obtained only from those CpG sites where there are cutting areas recognized by the applied restriction enzymes [2].

Cutting with restriction enzymes and PCR

The restrictive analysis combined with PCR appeared to be a more sensitive method than Southern blot. In 1989, Judith Singer-Sam *et al.* [3,4] while examining mouse DNA, designed the PCR method with the application of primers flanking the sites digested with the restriction enzyme *Hpa*II (Tab.1). If the DNA is treated with *Hpa*II prior to PCR, only methylated templates are amplified (protected against cutting). All unmethylated DNA must be cut, otherwise also the templates, which have not been fully digested, will amplify giving false-positive results. This method, similarly to Southern blot, enables CpG methylation monitoring only in the sequences which contain methylation-sensitive restriction sites and is useful to assay samples with a considerable percentage of methylated alleles. Because of that, it can be applied to analyze genomic imprinting and the X chromosome inactivation. An additional limitation of this method are the difficulties in differentiating completely cut unmethylated DNA from a low quantity of methylated alleles. That is why it is not used to detect the hypermethylation of tumor suppressor genes in a small amount of biological material, or when the methylated alleles constitute a small fraction of the DNA [3,4].

Bisulfite method

Restriction enzymes, which differentiate methylated sequences from unmethylated ones, may be applied only to the sequences, which contain the sites they are cutting. A biochemical DNA modification by means of sodium bisulfite (the bisulfite method) has been invented in order to correctly identify all methylated and unmethylated CpG islands present in the genome. It precedes most quantitative and qualitative PCR techniques adopted for methylation analysis.

Sodium bisulfite deaminates cytosine located on single-stranded DNA. 5,6-dihydrocytosine-6-sodium sulphonate, which forms in acidic pH, is an indirect product of this reaction. The change of environment to

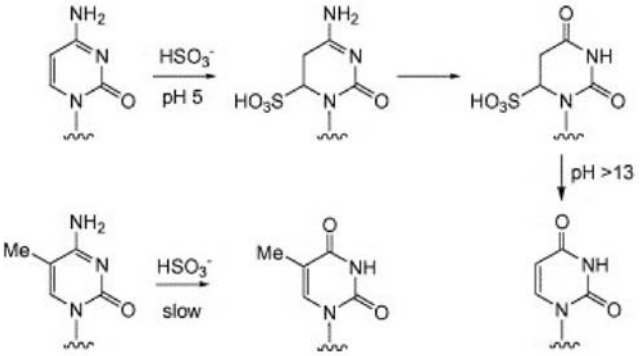

Fig. 1. The reactions of DNA bases with sodium bisulfite. Sodium bisulfite causes the deamination of a cytosine residue in a single-stranded DNA through formation of a 5,6-dihydrocytosine-6-sulfonate intermediate at acidic pH. The deaminated bisulfite adduct is converted into a uracil residue through elimination of bisulfite at alkaline pH. 5-Methylcytosine also yields thymine with sodium bisulfite, but the reaction rate for the bisulfite adduct formation is much slower [5].

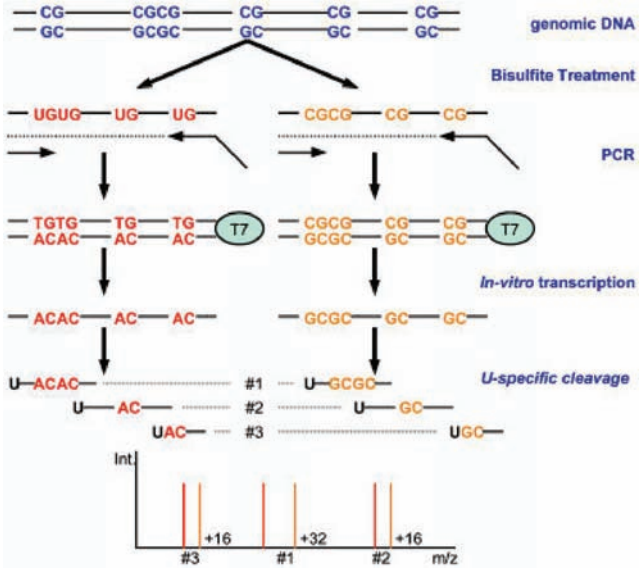

Fig. 2. Analysis of methylation by base-specific cleavage and MALDI-TOF MS. Treated bisulfite is PCR amplified by using primers located outside of the CpG islands with one primer tagged with a T7 promoter sequence. The PCR product is transcribed into a RNA transcript and cleaved base specifically. The cleavage products are analyzed by MALDI-TOF MS, and a characteristic mass signal pattern is obtained. In the case shown here, the PCR product is transcribed from the reverse strand and cleaved U specifically. A methylated template (here indicated in yellow) carries a conserved cytosine, and, hence, the reverse transcript of the PCR product contains CG sequences. In an unmethylated template (indicated in red), the cytosine is converted to uracil. The reverse transcript of the PCR product therefore contains adenosines in the respective positions. The sequence changes from G to A yield 16-Da mass shifts. Cleavage product 1 has two methylation sites. Mass signals of the cleavage product will differ by 32 Da when both CpG sites are either methylated or nonmethylated. For cleavage products 2 and 3, mass shifts of 16 Da will be observed, because each contains only one methylation site. The spectrum can be analyzed for the presence/absence of mass signals to determine which CpGs in the template sequence are methylated, and the ratio of the peak areas of corresponding mass signals can be used to estimate the relative methylation. This assay enables the analysis of mixtures without cloning the PCR products [10].

alkaline one causes the degradation of sodium bisulfite and the transformation of indirect product into uracil. 5-methylcytosine may also undergo such a reaction (deamination to thymine). However, in this case, the process of indirect product creation, is very slow and the time of reaction inhibits the formation of the final product (Fig. 1) [5].

A long incubation with sodium bisulfite (≥16 hours) damages about 60% of purine bases and phosphodiester bindings in the DNA molecule and destroys pyrimidine bases. The change of pH into alkaline generates the creation of purine-free and pyrimidine-free sites by means of breaking N-glycoside bonds. The number of these sites increases when the reaction with sodium bisulfite is longer and they are more often the effect of depyrimidination than depurination [5]. Long incubation, high temperature and high molar concentration of sodium bisulfite degrades even 84-96% DNA while using less aggressive media may limit cytosine conversion [2,6].

Various amplification protocols, which were drafted before 2001, recommended using 50-500 ng DNA treated with sodium bisulfite, later studies reduced this amount to 30 pg [6,7]. Some biotechnological companies producing ready-made kits for efficient DNA modification and purification of as little as 1 ng DNA. DNA fragmentation is prevented during bisulfite conversion reaction by a DNA Protect Buffer. However, the composition of this reagent remains confidential.

Qualitative PCR methods

MS-PCR. MS-PCR (*Methylation Specific PCR*) was described in 1996. First tests covered the genes *p-16*, *p-15*, *VHL* and *Ecad*. DNA, previously treated with sodium bisulfite, was amplified with two pairs of primers: for the methylated and the unmethylated sequence, and then, it was cut with the restriction enzyme *Bst*UI. A third pair of primers, prolonged only with those DNA molecules, which did not undergo modification, was used as a control of chemical modification efficiency [2,8].

The advantages of this method include a short time of analysis, the possibility of obtaining results from small amounts of DNA (5 μg), specificity provided by primers and a significant sensitivity - methylation is detected even when only 0,1% of alleles are methylated (Tab. 1). The drawbacks of this method, similarly to other PCR techniques, are connected with the possibility of contamination of the analyzed sample and obtaining false-positive results [2,8].

MS-nested-PCR. MS-nested-PCR is a modified version of MS-PCR. This method involves a double-stage PCR. During the first stage, the applied primers recognized templates modified by sodium bisulfite, but they do not differentiate methylated sequences from unmethylated ones. Reaction products are diluted and amplified with two pairs of internal primers, one of them is specific for the methylated sequence and the other - for the unmethylated one. The nested-PCR reaction increases the method's sensitivity to 10^{-5}, which made it possible to detect DNA methylation in saliva samples collected from patients with small cell lung cancer (Tab. 1) [9].

Quantitative PCR methods

Matrix-assisted laser desorption/ionization time-to-flight mass spectrometry (MALDI-TOF MS). Using PCR in combination with mass spectrometry, methylation pattern of normal and neoplastic lung tissues was determined for 47 genes in 48 patients and the samples were classified according to their histopathology. The modification with sodium bisulfite was followed by the amplification of the IGF2/H19 region (situated on the chromosome 11p15.5) with the application of primers containing a fragment of T7 promoter. The 416 bp long product, including 26 CpG sites, was transcribed *in vitro*, creating a mosaic DNA/RNA structure and cut with RNaseA in the way depending on U or C bases. Mass signals transmitted by DNA molecule was analyzed in MALDI-TOF MS. The relation between the methylated and unmethylated DNA was established by comparing the intensity of signals (Fig. 2) [10].

The MALDI-TOF MS method detects 5% methylation without the need to conduct MS-PCR or clone PCR products. It is also applied while looking for single nucleotide polymorphisms (SNP). It has been found that in a 500 bp long template, 98% of all possible SNP homozygotes and heterozygotes are identified [11]. The method can be applied to analyze samples coming from different sources, *e.g.* paraffin blocks or fragments collected by means of laser microdissection. This technique, however, requires a lot of work and expensive equipment such as mass spectrometer [1,10].

Real-Time PCR (MethyLight). The MethyLight technique bases on a fluorescent analysis of PCR products. DNA, modified by sodium bisulfite, is amplified with primers specific for a given sequence, which limit oligonucleotide probes with a fluorescent reporter dye at the 5'end (6-carboxyfluorescein-6-FAM) and a quencher at the 3'end (TAMRA). 5' $\rightarrow$ 3'nuclease activity of *Taq* DNA polymerase cuts the probe and frees the reporter whose fluorescence is detected by a laser detector, *e.g.* ABI Prism 7700 Sequence Detection System. Having crossed the threshold point, the fluorescence is proportional to the number of copies of the amplified sequence [12].

There are three ways to differentiate between methylated and unmethylated template sequences: design methylation-specific primers containing or not CpG dinucleotides, design fluorescent labeled probe with one or several CpG sites, design both the above primers and probes (Fig. 3) [12].

MethyLight not only makes it possible to differentiate between methylated and unmethylated sequences, but also defines the specific methylation pattern. It allows for testing small samples of biological material with a low quality of DNA (degraded DNA) and to obtain comparable results (Table 1) [1].

Quantitative analysis of methylated alleles (QAMA). QAMA is a newer version of Real-Time PCR, which applies TaqMan probes based on groove binder (MGB) technology. There are two types of probes: specific for methylated and unmethylated sequences, which is achieved by means of using different fluorescent dyes (VIC and FAM) [1,13].

The method is single stage, it ensures a high efficiency of methylation evaluation and the assessment of methylated and unmethylated sequences during one reaction, it shortens significantly the time of analysis and diminishes the probability of contamination. Mutations and polymorphous sequences may hinder probes binding. The probes recognize only completely methylated or unmethylated sequences, which makes it impossible to analyze partially methylated areas [1,13].

HeavyMethyl. HeavyMethyl detects methylated sequences in samples with a low concentration of DNA. Oligonucleotide blockers, which are not lengthened, bind to sodium bisulfite modified DNA template in a methylation-dependent way, and the binding sites correspond to 3' sites of primer binding. Reactions with blockers makes primer binding and amplicon synthesis impossible whereas the detachment of blockers exposes primer binding sites and enables the reaction (Fig. 4) [7].

The high sensitivity and specificity of this method makes it appropriate for clinical analyses, such as the analyses of cell-free DNA in serum and other body fluids. It is estimated that it is about 10 to 50 ng/ml of blood in healthy persons. This method may be adopted both for quantitative and qualitative methods, and, thanks to specific primers and blockers, it enables the detection of even 30 pg of methylated DNA. In comparison to conventional MS-PCR, it is more complicated and requires a more accurate optimization (Table 1) [1,7].

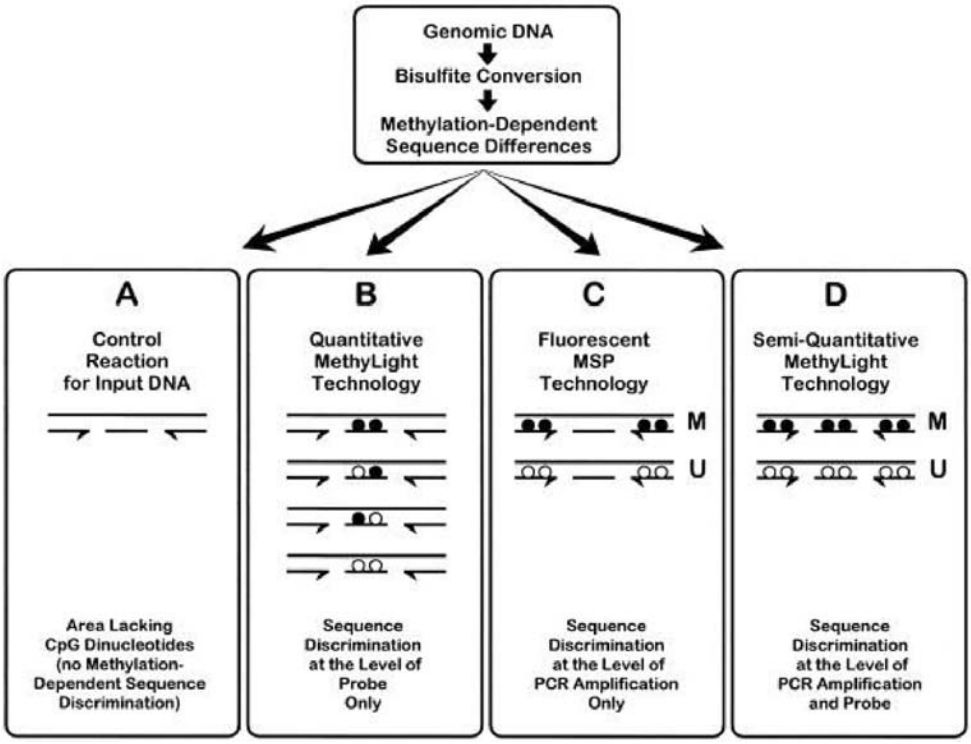

Fig. 3. Schematic of the theoretical basis of MethyLight technology. Genomic DNA is first chemically modified by sodium bisulfite. This generates methylation-dependent sequence differences at CpG dinucleotides by converting unmethylated cytosine residues (locations indicated by white circles) to uracil, while methylated cytosine residues (locations indicated by black circles) are retained as cytosine. Fluorescence-based PCR is then performed with primers that either overlap CpG methylation sites or that do not overlap any CpG dinucleotides. Sequence discrimination can occur either at the level of the PCR amplification process or at the level of the probe hybridization process, or both. Sequence discrimination at the PCR amplification level requires the primers and probe (application D), or just the primers (application C), to overlap potential methylation sites (CpG dinucleotides). Only two [fully methylated (M) and fully unmethylated (U)] of the many theoretical methylation permutations are shown. The MethyLight assay can also be designed such that sequence discrimination does not occur at the PCR amplification level. If neither the primers nor the probe overlap sites of CpG dinucleotides (application A), then no methylation-dependent sequence discrimination occurs at the PCR amplification or probe hybridization level. This reaction represents amplification of the converted genomic DNA without bias to methylation status, which can serve as a control for the amount of input DNA. When just the probe overlaps methylation sites (application B), then sequence discrimination can occur through probe hybridization. The design of separate probes for each sequence variant resulting from different methylation patterns (22 = 4 probes in the case of two CpGs, as illustrated) can potentially serve as a quantitative version of the MethyLight technology [12].

Methylation-sensitive high resolution melting (MS-HRM). The HRM method was invented in order to genotype SNP polymorphisms. The monitoring of fluorescence changes occurring during the melting of DNA double helix base on the ability of some dyes to intercalate double-stranded nucleic acids.

A modified version - MS-HRM allows for the assessment of methylation level by means of comparing the melting profiles of unknown samples with the profiles of completely methylated and unmethylated control, and detects even 0.1% methylation. What complicates the method is the necessity of using dyes, which do not inhibit PCR reactions, in the concentration necessary for a complete DNA saturation, as well as the necessity of having special equipment to measure the melting [14].

Methyl-binding (MB)-PCR. In the (MB)-PCR method we use recombined proteins, which are coated onto the walls of PCR tubes and selectively bind methylated fragments of a genomic DNA mixture. Methylation degree of specific fragments is assessed in the same tube using gene-specific PCR (Real-Time PCR). This method does not require a modification with sodium bisulfite or using restriction enzymes, and it allows for a sensitive methylation detection in 160 pg -10 ng genomic DNA (Table 1) [15].

Bisulfite-PCR and dNMP analysis. The method combines three stages: modification with sodium bisulfite, PCR and the analysis of deoxyribonucleoside monophosphates ($dNMP_s$). PCR products, hydrolyzed to dNMP monomers, are quantitatively analyzed by means of capillary electrophoresis. The relation of cytosines to thymines, which is determined during electrophoresis, reflects the relation of methylcytosines to cytosines in the analyzed sequence. Cloning and sequencing of PCR products is not required, and

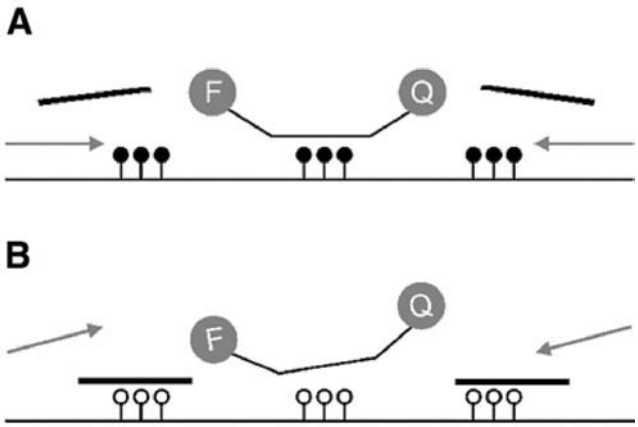

Fig. 4. Principle of HeavyMethyl. (A) When the DNA is methylated, the blocker oligonucleotides (solid black) do not bind, leaving the primer- binding site accessible for the primers (gray arrows) to bind and amplify the target. The amplification is detected with a methylation specific oligonucleotide probe [solid black, labeled with fluorescent dye (F) and quencher (Q) in a 5'-exonuclease assay, used here as an example for a real-time detection method]. (B) When the DNA is unmethylated, the blocker oligonucleotides bind, blocking the access of the primers to their binding sites. No PCR product is generated [7].

the final results may be obtained within a couple of hours (electrophoresis lasts only 1.5 hours) [16].

Methylation-dependent fragment separation (MDFS). Capillary electrophoresis is also used in the MDFS method. Electrophoresis is conducted after sodium bisulfite modification of genomic DNA and amplification with specific primers labeled with fluorescent dye (6-FAM). The migration speed of polymorphic sequences is different, which allows for the identification of methylated and unmethylated DNA (Fig. 5) [17].

Numerous methylation analysis methods such as, for instance, hybrydization techniques, base on the usage of primers or probes designed for completely methylated or unmethylated target sequences. As a result, there are no products or they are unspecific when methylation is only partial. The MDFS method limits the creation of unspecific products and the high resolution of electrophoresis enables the detection of differences concerning single C/T nucleotides [17].

Combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS). In the COMPARE-MS method, genomic DNA, which has been digested with methylation-sensitive restriction enzymes, is precipitated with the application of polypeptides immobilized on a magnetic solid matrix and containing domains binding methylated DNA. The next stage is Real-Time PCR. Thanks to combining restriction enzyme digestion with Real-Time PCR, the risk of obtaining false-positive results is minimalized. A very high sensitivity of this method enables methylation detection when the concentration of unmethylated sequences is 1000-10000 times higher than that of methylated ones, and the specificity reaches even 100% [18].

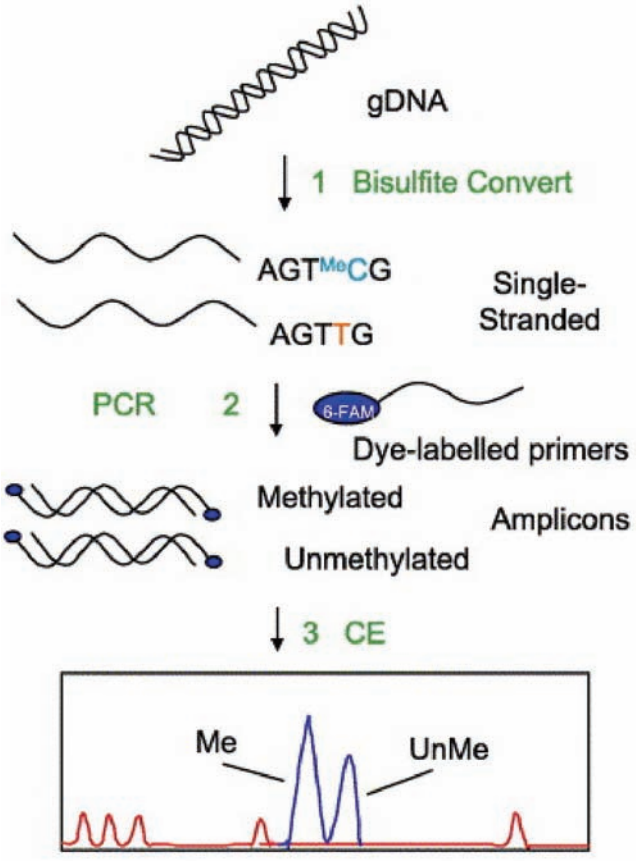

Fig. 5. The schematic representation of the workflow for methylation-dependent fragment separation (MDFS) has three steps. In step 1, following bisulfite conversion, methylated (Me) gDNA differs from unmethylated (UnMe) gDNA by the presence of multiple 5mC versus U bases. In step 2, a region of interest is PCR amplified using a single set of FAM dye-labeled primers that amplify the gDNA regardless of the methylation status. In step 3, the presence of the multiple polymorphisms (C vs. T) leads to differential migration times during fragment analysis by CE so that an amplicon from fully methylated gDNA is readily resolved from an amplicon from fully unmethylated gDNA [17].

Genomic methylation testing

PCR methods allow for a simultaneous analysis of single genes or a small number of genes. The recent research focuses on the development of techniques enabling the analysis of a large number of genes during one reaction. These conditions are fulfilled by, among others, microarrays, high-performance liquid chromatography (HPLC) and restriction landmark genomic scanning (RLGS).

Microarrays

Single-stranded DNA fragments are immobilized during the formation of microarrays on positively charged nylon membrane and hybrydization is conducted in appropriate conditions. If oligonucleotide probes, which are specific for methylated and unmethylated sequences, are attached to the membrane, we add sodium bisulfite modified PCR products to the hybridization solution. When genomic DNA or PCR products are immobilized, both pre-modified with sodium bisulfite and cut with restriction enzymes, and oligonucleotide probes are in the solution. Hybrydization signals are most often detected by means of chemiluminescence (Fig. 6) [19].

Microarrays allow for a simultaneous analysis of numerous CpG sites. It is necessary to conduct control tests and make a standard curve in order to eliminate probe errors resulting from inappropriate template recognition and to exclude cross reactions [1].

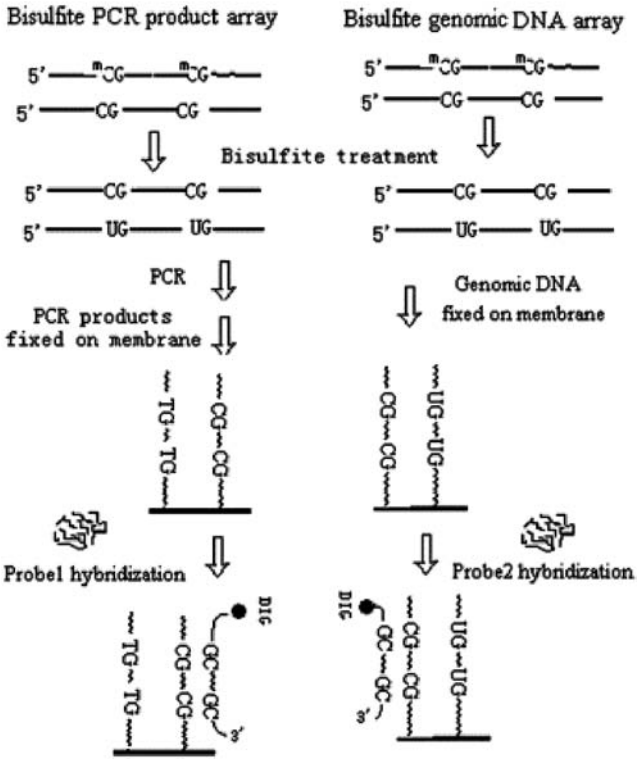

Fig. 6. Schematic outline for the analysis of DNA methylation of two array methods based on membrane hybridization and chemiluminescent detection. Two procedures (bisulfite PCR product array and bisulfite genomic DNA array) are introduced in the outlines. On the left procedure (the bisulfite PCR product array), the genomic DNA was treated with bisulfite to convert unmethylated cytosine to uracil but to conserve methylated cytosine. The region of interest was then amplified by PCR in sequencing primers, converting originally unmethylated CpG dinucleotide to TpG while conserving originally methylated CpG. PCR products were then fixed on nylon membrane and hybridized with 17-bp oligonucleotide probe 1 labeled with digoxigenin (DIG). On the right procedure (the bisulfite genomic DNA array), bisulfite-modified genomic DNA was directly spotted on the nylon membrane and hybridized with probe 2. Hybridization signals were visualized through chemiluminescent detection [19].

Array-based profiling of reference-independent methylation status (aPRIMES)

In a new hybridization method - Array-PRIMES, genomic DNA is digested with restriction enzyme *Mse*I and digestion products are joined with linkers. A half of ligation products is digested by methylation-sensitive restriction enzymes - *Hpa*II and *Bst*UI, and the other half is cut in a methylation-dependent way by *Mcr*BC endonuclease. Digestion products undergo amplification with linker-specific primers. Methylation-sensitive digestion results in the lack of unmethylated PCR products while methylation-specific digestion results in the lack of methylated products. In this way methylated sequences are differentiated from unmethylated ones and then, they are labeled with different fluorescent dyes: Cyc3 or Cyc5, combined with dCTP, and incubated with specific probes. DNA fragments compete with one another to bind with the appropriate probe (Fig. 7) [20].

Array-Primes is a modification of DMH (differential methylation hybridization), where no restriction enzyme *Mcr*BC is used [1].

Both methods, thanks to using different restriction enzymes and hybridization requiring competition between DNA fragments, allow for an accurate description of methylation status of thousands of sequences during one reaction [20]. The specificity of these methods depends on the efficiency of digestion with endonucleases, and an incomplete digestion may yield false-positive results.

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) has been adjusted to DNA methylation measurement levels. The first stage of this method consists of a complete hydrolysis of genomic DNA. The process of hydrolysis is started by nuclease P1 or snake venom phosphodiesterase, and continued by alkaline phosphatase leading to the formation of deoxyribonucleosides. Free nucleosides are introduced onto the column with, silica-polymer solid deposit, where the analyzed mixture is separated [1].

The mobile phase traverses the column under pressure depending on the altitude of the deposit and the forced velocity. The components which have been separated on the column get to the detector. The appearance of a component of the separated mixture in the mobile phase changes light transmission (increases or decreases absorption). This change is registered by a photo-element in the form of a chromatographic sig-

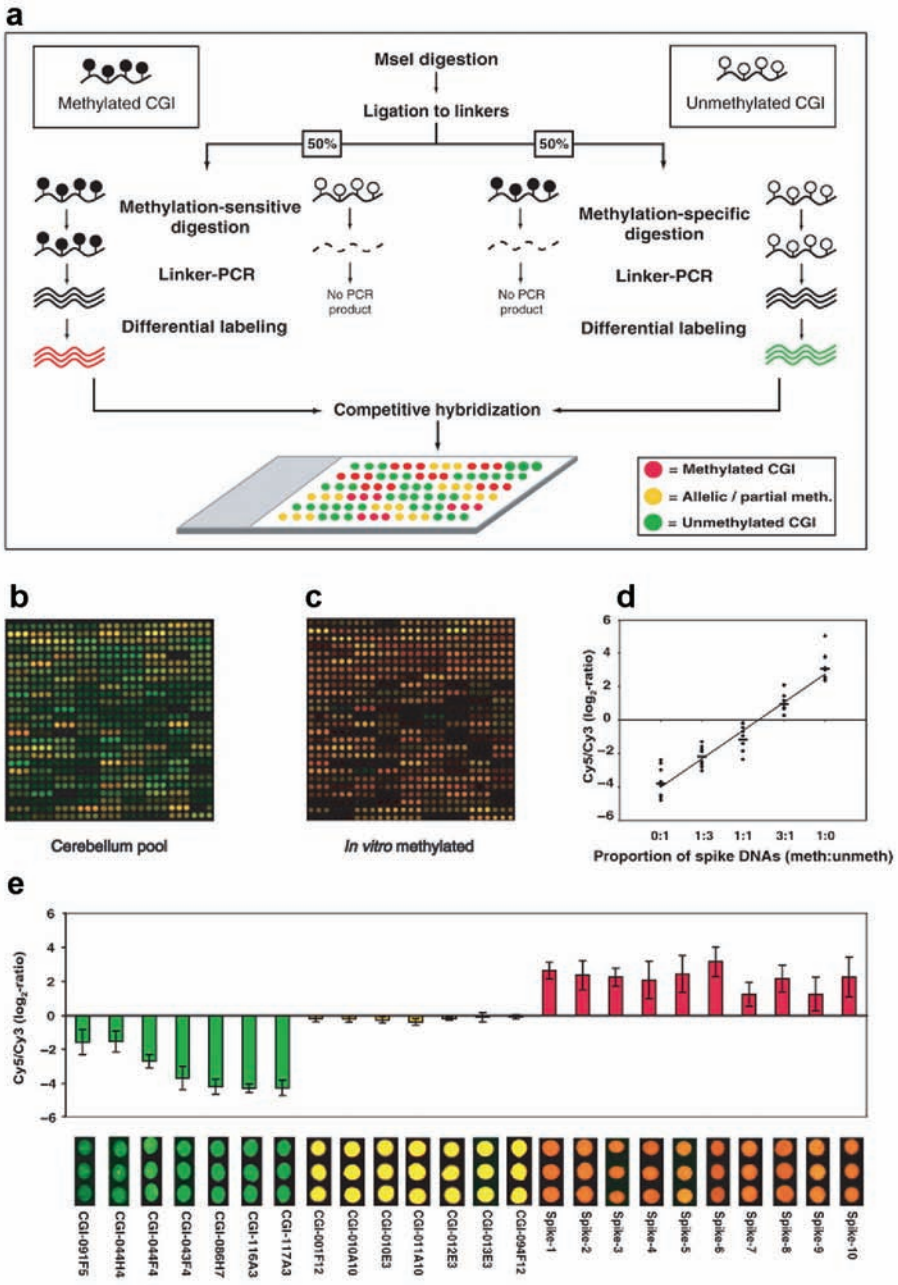

Fig. 7. Flowchart for the array-PRIMES procedure and performance of the method. (a) Flowchart and overview of expected results for methylated and unmethylated CGIs. (b and c) Performance of aPRIMES on CGI microarrays. All clones are spotted in triplicate. A representative block is shown for (b) normal cerebellum (pool of five unaffected donors) and (c) in vitro methylated tumor DNA. Red spots indicate methylated clones, green spots indicate unmethylated clones, yellow spots indicate mixed or allelic methylation, and can also be caused by a lack of appropriate restriction sites. (d) Quantification of methylation of spike CGIs using different proportions of methylated and unmethylated spike DNA spiked into genomic DNA of one tumor. Spots indicate the normalized ratios of all eight spike clones used in each of five experiments; short horizontal lines represent the median ratio of eight clones. The regression line is based on the medians depicted in the diagram. (e) Performance of internal control clones. Median values and median absolute deviations from the median (MADs) of 20 unselected aPRIMES experiments (upper part) and spot data from one representative hybridization (lower part) are shown. Methylated clones are highlighted in red and are expected to result in positive ratios; mitochondrial clones with methylation-sensitive restriction sites are highlighted in green and are to have negative ratios. Mitochondrial clones without methylation-sensitive restriction sites are depicted in yellow and are to be balanced [20].

nal. Qualitative analysis is conducted by means of comparing the retention time of the model and the analyzed sample. The qualitative analysis bases on the proportionality between the size of registered signals (surface under the peak) for the analyzed and model substance [21].

A higher specificity and sensitivity of this method is achieved by its combination with mass spectrometry. The sample is concentrated on the chromatography column and the losses connected with its transportation are avoided. What is more, the combination of both techniques enables a simultaneous analysis of complicated mixtures and a small usage of eluents due to the specificity of applied chromatography columns [1].

The efficiency of the separation of analytes may be limited by the type of applied stationary and mobile phases, pH and temperature fluctuations in the mobile phase and the contamination of the sample with RNA [1].

Restriction landmark genomic scanning (RLGS)

Unlike chromatographic techniques informing about the total amount of methylcytosines in the analyzed area of the genome, RLGS focuses on the methylation of particular CpG islands. High-molecular-weight DNA is digested with methylation-sensitive restriction enzyme *Not*I and labeled at the ends by [α-^{32}P]dGTP

and [α-^{32}P]dCTP, and then it is again digested with another endonuclease - *Eco*RV. The obtained fragments are separated in 0,8 % agarose gel and cut in gel with a third restriction enzyme - *Hinf*I. When the digestion is completed, there is the second electrophoretic separation in 5% polyacrylamide gel, which is then dried and X-irradiated for 5 - 10 days. Labeled radioactive fragments show differences dependent on the state of methylation [22].

This method enables a simultaneous analysis of up to 2000 loci and makes it possible to detect new hypermethylated sequences in the genome. It can be used while searching for new genes undergoing imprinting and methylated genes in different types of human carcinoma. What limits this method is the necessity of using high-molecular-weight DNA (more than 1 μl) and the fact that methylation can only be measured on these CpG islands, which contain non-polymorphous restriction sites recognized by the applied endonucleases. Because of this, RLGS is useless for the analysis of samples containing small amounts of DNA and the obtained results must be confirmed with other methods [1].

Summary

The application of molecular biology techniques requires overcoming certain limitations. The methods of methylation testing must be characterized by a great sensitivity, because body fluids, which are the most common and easily available material for analysis, contain minimal amounts of neoplastic DNA. They must also be specific enough to guarantee the differentiation between methylation pattern of neoplastic cells and the changes occurring in normotypical cells.

There are numerous technologies which are useful for the analysis of methylation pattern, but non of them is universal. While choosing the method, one must consider the type, amount and quality of the analyzed biological material, the possessed laboratory and specialist equipment. The right choice of procedure will minimize the risk of contamination and ensure repeatable results.

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