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Review

5-Methylcytosine as a marker for the monitoring of DNA methylation

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Abstract

The extent of the DNA methylation of genomic DNA as well as the methylation pattern of many gene-regulatory areas are important aspects with regard to the state of genetic information, especially their expression. There is growing evidence that aberrant methylation is associated with many serious pathological consequences. As genetic research advances, many different approaches have been employed to determine the overall level of DNA methylation in a genome or to reveal the methylation state of particular nucleotide residues, starting from semiquantitative methods up to new and powerful techniques. In this paper, the currently employed techniques are reviewed both from the point of view of their relevance in genomic research and of their analytical application. The methods discussed include approaches based on chromatographic separation (thin-layer chromatography, high-performance liquid chromatography, affinity chromatography), separation in an electric field (capillary electrophoresis, gel electrophoresis in combination with methylation-sensitive restriction enzymes and/or specific sequencing protocols), and some other methodological procedures (mass spectrometry, methyl accepting capacity assay and immunoassays).

Keywords: Reviews; 5-Methylcytosine; DNA

Contents

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1. Introduction

Among the known DNA base modifications, C^5 cytosine methylation has a key role. For the first time, 5-methylcytosine (m^5C) was proved to be present in DNA of *Mycobacterium tuberculosis* by Johnson and Coghill [1], using the m^5C-picrate fraction crystallization. Strong interest and extensive study of DNA methylation state, its appearance, characterisation and function, especially in eukaryotic organisms, dominates a significant part of current biological research [2–4].

5-Methyl-2′-deoxycytidine monophosphate is a product of enzymatic methylation of C^5-carbon position of a cytosine residue in a DNA strand. Enzymes catalysing this reaction, i.e. EC 2.1.1.37 and EC 2.1.1.73, belong to the family of methyltransferases (EC 2.1.1, MTs). These enzymes use *S*-adenosylmethionine (SAM) as a donor of the methyl functional group. 5-Aza-2′-deoxycytidine and 5-azacytidine [5] or *S*-adenosyl-L-homocysteine [6] are widely used inhibitors of those enzymes. Compounds such as sinefungin or A9145C, which intercept SAM synthases, represent other types of inhibitors, indirectly affecting the methylation of DNA [7]. Along with C^5 methylation, N^4-cytosine methylation of unknown function occurs in genomes, introduced by the dual role of adenine N^6/-cytosine N^4-methyltransferase [8].

Several methylation processes can be observed in a cell: de novo cytosine methylation, maintenance methylation during replication of dsDNA, active demethylation during the absence of DNA replication [9,10] and spontaneous demethylation, when maintenance methylation is suppressed. Dinucleotide CpG sequences seem to be primary sites of cytosine C^5 methylation in eukaryotic DNA [11], although it was recently shown [12,4] that methylation of other than CpG sites is also frequent and reaches up to 54.5% at other positions, namely CpA, CpC, CpT and CpNpG. Much is known about the role of DNA methylation in prokaryotic organism; it serves to protect the prokaryotic cell from foreign genetic material. Foreign DNA is marked by methylation and is subsequently recognised and cleaved by the host restriction/modification system [13]. The role of DNA methylation in eukaryotic cells is not fully understood yet, although it is already clear that methylation has mainly two major functions: control of gene expression and protection of the host organism against expression of undesired sequences, like noncoding, repetitive or parasitic ones.

Methylation regulates vital cellular processes, thus any inappropriate methylation/demethylation could cause fatal or lethal consequences, e.g. cancer [14]. Proper knowledge of mechanisms involved in the DNA methylation and its regulation via external stimuli is of great importance for understanding the handling of genetic information and processes related to it. Also the estimation of DNA methylation on the desired relevance level, with corresponding precision, is of great importance.

This review has been written with the purpose of summarising the methodologies which use 5-methyl-2′-deoxycytidine as a marker for DNA methylation monitoring, to discuss their basic principles and modifications, and to compare the utilisation of those approaches and their analytical relevance, along with a short overview of our current knowledge of DNA methylation.

DNA methylation is important, as otherwise the highly unstable base 5-methylcytosine would be

subjected to natural selection and readily converted to thymine by oxidative deamination [15]. In fact, approximately 2–10% of cytosine residues are C^5 methylated in the mammalian genomes [16] and this relatively high proportion is, to a large extent, the consequence of a sophisticated repair system, which protects mammalian cells from the loss of methylcytosine [17] or from unwanted m^5C deamination through hm^5C to thymine [18]. In spite of the repair system, the frequency of dinucleotides which may result from the m^5CpG deamination process on one strand ($m^5CpG \rightarrow TpG$), and its complementary dinucleotide on the second strand (ApC), exhibit higher frequency in genomes than it was statistically expected, because of the hypermutability of m^5CpG [19].

Besides the well documented appearance of DNA methylation within vertebrates [20], this base modification was also observed within other organisms such as *Drosophila melanogaster* [21], which contains about a 50-times lower amount of 5-methylcytosine than mammals [22], or *Trypanozoma cruzi* also with a low content of the modified base [23]. On the contrary, some other eukaryotic organisms, like *Saccharomyces cerevisiae* or *Ceanorhabditis elegans* do not contain modified cytosine in their DNA, which points to the evolutionary significance of DNA methylation [24,25].

In the case of mammals, the significance of DNA methylation is underlined by the fact that there are at least four different DNA methyltransferase enzymes in mammalian cells [26]. Also, mice deficient for some DNA methyltransferases are not viable and die during embryogenesis [27,28]. As was demonstrated in studies of mouse embryonic development, the wave of massive demethylation and de novo methylation occurs predominantly in early embryonic development and the created methylation pattern is subsequently inherited throughout maintenance methylation [29–31].

DNA methylation is a key player in the process of tissue formation—through it gene action can be selectively switched on/off in a given cell [32]. In this sense, DNA methylation provides higher-ordered information compared to the genome itself [33]. The mechanism which selectively methylates corresponding genes in various tissues is not fully understood yet, but it might depend on alternative isoforms of methyltransferase enzymes. It was demonstrated that mRNA coding methyltransferase Dnmt1 is different at its 5′-end. There are alternative exons at 5′-end of the Dnmt1 gene in oocytes, spermatocytes or skeletal muscle [34,35]. The presence of methylated cytosine may directly block the accession of transcription factors to DNA, thus preventing expression of the corresponding gene [36,37]. Alternatively, methylcytosine-binding proteins may inhibit transcription by blocking accession of regulatory elements into corresponding sequence [38].

It is well documented [39,40] that many mammalian genes contain CpG islands at their 5′ends, promoters or 5′untranslated regions, and that these islands are kept unmethylated, thus enabling formation of an alternative chromatin structure, different from the bulk of inactive chromatin [41]. On the contrary, CpG dinucleotides, which are located out of the CpG islands, are methylated and those, which are located in coding regions of the genes, do not influence gene transcription; the methyl group does not interfere with RNA polymerase progression [42]. Not all genes, however, have CpG islands at their regulatory areas [43]. The majority of the genome, approximately 97%, which is full of noncoding sequences and potentially active transposable elements or other intragenomic parasites, requires effective mechanisms for long-term silencing, which is primarily ensured by heavy methylation [44,45]. It represents a very effective mechanism and methylation is able to keep silent areas which are located in the immediate proximity of gene promoters.

CpG islands at active-gene areas are kept unmethylated, but two exceptions exist: imprinted genes and genes, which are subjected to X chromosome inactivation. Inactivation of one of the two X chromosomes in cells of females is required during development, to ensure an equivalent level of expression of X-linked genes in male and female cells [46]. In every cell one of the two X chromosomes is chosen, randomly in somatic cells and preferentially in embryonic cells paternally inherited X chromosome, and the CpG islands in promoters of corresponding genes are methylated and thus the genes are transcriptionally silenced.

Methylation also provides a way for heritable changes in gene expression that occur without a change in the DNA sequence, a phenomenon known

as epigenetics [47,48]. Epigenetics represents a quickly growing field of research, especially in connection with carcinogenesis and aging [49].

Genomic imprinting is a phenomenon related to DNA methylation, which appeared recently [50]. For imprinted genes, the gene action conferred by maternal allele differs from that of the paternal one: in fact one allele is expressed and the other one is silenced. DNA methylation is the only cellular mechanism, which can satisfy all the criteria necessary for efficient imprinting. DNA must be methylated before fertilisation, the gene is basically labelled as an imprinted one, and must be able to confer transcriptional silencing of the gene to confer its stable transmission through mitosis and its reversible passage through the opposite parental germline [51]. Nevertheless, because the mechanism of genomic imprinting is very sophisticated, includes actually more than only methylation [52,53], and is conserved throughout evolution, some mouse genes are imprinted as well, it likely plays an important, but as yet not clearly elucidated role [54,55].

It is also well known that many CpG islands, which are normally methylation free, are heavily methylated in cultured cells [56]. Similar identity of subset of de novo methylated genes in various cell lines suggests, that nonessential genes, i.e. genes which are not necessary for a growth of cells in culture, are suppressed. Therefore this phenomenon needs to be considered when studying methylation in tissue culture cells.

Changes in the methylation pattern often lead to pathological consequences. Methylated cytosines in CpG dinucleotides are primary hotspot for mutations in mammalian genomes [57], affecting many important human genes [58,59]. As it was reported, 35% of mutations within the coding regions of genes occur at CpG dinucleotides, which is a frequency 42-fold higher than would be predicted for random mutations [60]. Methylation in the germline contributes significantly to human genetic diseases [61], with more mutations occurring in paternal germ cells, reflecting probably the heavier DNA methylation in spermatozoons compared to oocytes [62].

Since many genes are directly involved in methylation machinery, several diseases result from their mutations. Examples include the ICF syndrome, manifested by immunodeficiency, mental retardation and facial abnormalities [63], which are associated with mutations in methyltransferase *Dnm*t3b gene [64] or the Rett syndrome, manifested primarily as a female mental retardation [65], which is a consequence of mutations in methylcytosine-binding protein MeCP2 [66].

Abnormal methylation is also closely connected to carcinogenesis [14] and there are multiple mechanisms by which dysregulated methylation contributes to cancer processes. Aberrant promoter hypermethylation of tumour suppressor genes is one of the possibilities [67,68] and in some cases this pathological methylation can be used for early diagnosis [69,70]. On the other hand, global hypomethylation of genomic DNA, which is also connected to carcinogenesis, may activate potential oncogenes, such as H-ras or C-myc [2] and thus directly contribute to the onset of cancer.

Mutations of 5-methylcytosine occur in the coding regions of tumour suppressor genes as well. One of the best studied examples is a p53 tumour suppressor gene, which is expressed in cells when the DNA is damaged and either stops the cells at G1-phase of cell-cycle or drives them to apoptosis [71]. The p53 gene is mutated in about half of all the tumours and a mutation database includes about 4500 mutations [72]. Two hotspots for point mutations, codons 175 and 273, both contain 5-methylcytosine as evidenced by genomic sequencing of human somatic cells [59]. For some kinds of tumours these mutations are predominant, what indicates that 5-methylcytosine is an endogenous cause of cancer in these cases [73]. There are no exogenous mutagens involved in such cases, only 5-methylcytosine [61]. Then we could await other types of mutations in cancers where exogenous factors are clearly present, e.g. in lung cancer because of smoking. It was shown that transversions are predominant in this cancer and 5-methylcytosine plays a marginal role [74,75]. This example clearly illustrates what might be the practical consequences arising from study of 5-methylcytosine in an organism.

Genomic imprinting causes three syndromes, which are based on different gene silencing with DNA methylation. The Prader–Willy and Angelman syndromes, both characterised by mental retardation,

but otherwise having different clinical manifestations [76], result from deletion of the same region at chromosome 15. But while the first is a consequence of lack of expression from paternal copy of chromosome 15, where maternal copy is normally silent, the second is a consequence of missing expression from maternal copy of this chromosome, here the paternal copy is normally silent [77]. The Beckwith–Wiedemann syndrome, which results in overgrowth of affected individuals, is a consequence of genetic disorders of imprinted genes regulating normal growth, which are located at chromosomal region 11p15 [78].

Genomic imprinting also causes a special kind of genetic disease—trinucleotide repeat disorders [79] such as fragile-X syndrome (FX), myotonic dystrophy (DM), Huntington's disease (HD), spinocereberal ataxia type 1 (SCA1), spinal and bulbar muscular atrophy (SBMA) and dentatorubral-pallydoluisian atrophy (DRPLA). They are characterised by unstable trinucleotide repeat expansions, affecting various parts of the corresponding genes. At the same time, all these unstable DNA disorders exhibit some effect of the sex of the disease-transmitting parent, or genomic imprinting. DNA methylation is suspected to play a pivotal role in the aetiology of these pathological processes.

The exact consequences of abnormal DNA methylation were, to a large extent, elucidated for one of the trinucleotide repeat disorders, fragile-X syndrome, which is primarily manifested as a serious mental retardation [80]. In this case the CpGpG sequence motif located in 5′untranslated region of the FMR1 gene is expanded over 200 repeats in cells of affected individuals, comparing to <50 repeats in normal cells, which results in heavy methylation of the whole region, including the adjacent promoter and transcriptional silencing of the FMR1 gene [81]. It subsequently leads to deregulation of mRNA metabolism in a cell, since FMRP protein, which is encoded by FMR1 gene, has a property of selective RNA-binding protein [82]. Recently, Chiurazzi et al. [83] used the demethylating agent 5-aza-2′-deoxycytidine and partially restored FMRP expression in B-lymphoblastoid cell lines derived from fragile-X patients, causing the DNA hypomethylation by blocking the enzyme [5].

Table 1
Regulatory effects of DNA methylation in eukaryotic organisms

Control of gene expression
Regulatory parts of the genes are kept unmethylated, thus enabling transcription
Two exceptions exist
Imprinted genes
Genes subjected to X chromosome inactivation
Protection of organisms from expression of undesired sequences
Heavy methylation prevents their transcription

The state of DNA methylation is also reflected in the content of 5-methyl-2′deoxycytidine and related compounds in urine [84]. The origin of m^5dC is in cellular DNA and might be bound to the activity of the DNA repair system. It was observed that levels of urinary m^5dC are almost 100% higher in patients with leukaemia than in healthy individuals or other cancer type patients, though these results were impeached by Zambonin et al. [85] using LC-ESI MS.

Tables 1 and 2 summarise the regulatory and adverse effects, respectively, of DNA methylation.

2. Routinely used methods for the determination of DNA methylation state

The methylation state of DNA can be evaluated in a different context. There are basically two major branches of DNA methylation monitoring: the first focuses on global methylation level of studied genomes and the second focuses on methylation state of studied regions, mostly CpG islands. The particular purpose within the determination of global or regional methylation state decides about the choice of the proper method, namely with regard to quantification or only qualification of the methylation state. Quantification methods can be used either for total genome composition or, preceded by specific cleavage, to estimate genome composition in chosen regions. Using nearest-neighbour analysis [4], dinucleotide composition can be obtained as limited sequence information.

Generally, determination of the total genome composition is sensitive to presence of RNA, tRNA and rRNA also contain m^5C, and foreign DNA. It is

Table 2
Adverse effects of DNA methylation in eukaryotic organisms

Effect	Consequence
Mutation of 5-methylcytosine in coding regions of the genes	Broken gene activity→ pathological phenotype
Hypermethylation of genes promoters	e.g. Inactivation of tumor-suppressor genes→cancer
Hypomethylation of genomic DNA	e.g. Activation of potential oncogenes→ cancer
Local changes in DNA methylation	Genetic disorders→e.g. fragile X syndrome

recommended, prior to m^5C analysis, to check the tRNA and rRNA content after enzymatic digest by HPLC [86].

2.1. Chromatographic methods

Chromatographic separation techniques provide suitable tools for determination of the m^5C content in the whole genome. They offer not only separation, but also high reproducibility, RSD 0.1–3.0%, sensitivity, and the possibility to use universal and widely accessible instrumentation. Use of microcolumn liquid-chromatography allows also large scale screening analyses. Methylation state can be determined via separation and quantification of related nucleosides or nucleotides. Individual nucleotides are acquired by application of exonuclease III [87]. Nucleosides are released by quantitative hydrolysis of DNA to the respective nucleotides using P1 nuclease, DNAse I or snake venom phosphodiesterase, followed by dephosphorylation by means of alkaline phosphatase treatment [88,89]. Individual bases can be obtained by acidic cleavage of DNA under nondeamination conditions by either 70% $HClO_4$ [90] or 98% formic acid [91].

2.1.1. Thin-layer chromatography (TLC)

As a cheap and fast alternative to column chromatography, TLC was first used for separation and determination of m^5C content [92], but only the introduction of high-performance TLC (HPTLC) allowed the development of quantitative method, which is comparable to other chromatographic methods in its sensitivity [93]. The methods described allow large-scale screening, up to ten samples per plate, with RSD of determination around 3%. The advantages of HPTLC are simple instrumentation, screening scale, low cost and high speed.

Recently, a highly specific and sensitive assay was developed to detect the presence of very low levels of 5-methylcytosine in genomic DNA [22]. In this assay, DNA is degraded enzymatically to nucleosides via primary cleavage of DNA with m^5dC/dC sensitive restriction nucleases, such as MspI. The digest is followed by degradation of nucleotides to individual nucleosides, which are separated by reversed-phase HPLC and fractions containing dC and m^5dC are collected. The samples are then incubated with deoxyribonucleoside kinase (dNK) and [^{32}P]-ATP in order to obtain labelled dCMP and mdCMP. Then, to identify labelled bases exactly, the two-dimensional thin-layer chromatography (2D-TLC) is used, which enables separation of all natural and modified bases. During the labelling process, it was observed that other bases also became labelled; random DNA nicks caused by shearing forces become labelled [94,3]. This procedure was successfully used for detection of 5-methylcytosine in DNA of *Drosophila melanogaster* [22], which contradicted the general opinion that this species does not contain 5-methylcytosine in its genome [95]. This incorrect conclusion was a consequence of low-sensitivity methods, which were previously used for 5-methylcytosine detection in Drosophila. As Drosophlila's genome contains an approximately 50 times lower amount of 5-methylcytosine compared to mammalian genomes, it was under the level of detection for conventional HPLC [22], McrBC cleavage [96] or restriction protection analysis [97].

However, Achwal et al. [21] detected 5-methylcytosine in DNA of Drosophila almost 20 years ago, by using amplified ELISA and photoacoustic spectroscopy.

2.1.2. High-performance liquid chromatography

HPLC is the most commonly used chromatographic method for analysis of the genome-wide methylation. It provides absolute and quantitative information and, because measurements of cytosine and 5-methylcutosine related compounds are made from the same substrate, it requires no internal standard. As a rule, comparison of the simple relative ratios, $(m^5dC/dC)_1$ vs. $(m^5dC/dC)_2$, or expressing the percentage of the m^5dC content as the ratio $m^5dC/(m^5dC+dC)$ is used, with pilot ratios $(m^5dC+dC)/dG$ and dA/dT, which needs to be equal to 1. HPLC is a relatively slow method, separation takes from 15 to 80 min, but it offers unbeatable repeatability of the separation and quantitative determination of separated compounds. In the method optimisation tests of the nucleosides separation, it is suggested to include adenine and 2′-deoxyinosine as standards, because they may coelute with m^5C [98]. The universal HPLC method in gradient mode for separation of nucleosides, its bases and suggested standards was developed by Parra et al. [99].

A number of effective approaches were described for quantifying 5-methylcytosine content in DNA and especially the protocols by Kuo et al. [88], Gehrke et al. [98] and Wagner et al. [92] are widely used. A detailed protocol including advanced sample preparation was provided by Cooney et al. [100]. This method is sensitive enough to quantify small changes in m^5C level, which may occur in organisms during modified diets [101], aging [102] or carcinogenesis [103]. Using HPLC with standard UV detection, 2–10% of the m^5C content can be estimated in 10 μg DNA. Employing ^{32}P postlabelling, as mentioned above, improves the estimation to 0.01% in 1 μg DNA. This approach was also successfully used to prove the existence of low level DNA methylation of unknown purpose in *Aspergillus flavus*, based on the discovery of DNA methyltransferase in *A. flavus* that has homology with other Aspergillus fungi family members [104]. HPLC operating with fluorescence detection, using dansyl chloride as a fluorophore has LOD 11 pmol comparing to 150 pmol for nonderived mdC [91].

The complexity of separation systems, as well as individual properties of HPLC systems, may vary significantly and therefore the system has been generally optimised [87,105].

2.1.3. Affinity chromatography

Affinity chromatography [106] is important part of the recently employed method called identification of CpG islands exhibiting altered methylation patterns (ICEAMP). The proteins are immobilised on the stationary phase and, because of specific interactions of m^5CpG-binding proteins with the methylated CpG sequence, the methylation rich sequences are isolated. Later on, the subtractive hybridisation excludes sequences common for both, studied and normal state DNA, thus leaving only sequences unique for the studied state, e.g. tumour tissue. The results of ICEAMP experiments correspond to the results obtained by parallel bisulphite controls. This method allows comprehensive identification of methylation changes without the necessity of knowing the target sequence region and has no need for the use of MSRE.

2.2. Electromigration methods

Sample separation in an electric field is an often used and a well-established technique. Slab-format gel electrophoresis and also capillary electrophoresis (CE) are heavily used within the DNA research, applied mostly for separation of digested DNA strands.

2.2.1. Capillary electrophoresis

Different modifications of CE, like capillary zone electrophoresis (CZE) or micellar-electrokinetic capillary chromatography (MECC), are frequently used electromigration separation techniques. The determination of m^5C using CE is almost unused [107], although a described MECC separation has limit of detection comparable to HPLC, 350 fmol, and it is possible to determine m^5C in ratio 1/1000 within mdC/dC. However, gel electrophoresis in the slab-format is the most widely used separation technique, although it still suffers from the long time

required for the analysis, low efficiency and difficulties in detection, quantitative analysis and automation. Electromigration methods performed in-solution, especially CE are, on the other hand, very versatile and powerful analytical tools. They are fast and have separation abilities even better than HPLC [108].

The appearance of multichannel CE with mass spectrometric detection gives the potential to become a hot candidate for a future possible screening method [109].

2.2.2. Slab-form gel electrophoresis (GE)

Generally, gel electrophoresis (GE) is a routine separation technique in molecular biology laboratories; it is also widely employed technique for m^5C and/or DNA methylation determination. It is used as a terminal separation method for two basic protocols. The first utilises restriction endonucleases sensitive to m^5C, while the second represents specific sequencing protocols, like bisulphite or hydrazine/permanganate sequencing, which enable exact verification of the methylation state for each cytosine residue in the analysed sequence.

2.2.2.1. Modification-sensitive restriction enzymes. Enzymes which cleave the DNA strand at modified bases are called modification-sensitive restriction enzymes (MSRE). In the context of cytosine modification, the abbreviation is also decoded as methylation-sensitive restriction enzyme. Application of MSRE gives rapid information about the presence of modified bases in the DNA material [4]. It enables probing of both the global methylation level of genomic DNA and of the local methylation pattern as well. There are plenty of available restriction endonucleases with selective cleavage sites, which may serve to distinguish between different types of base modifications, e.g. m^4C and m^5C, or between modified and unmodified bases at the same location in compared materials. The most widely used combination of restriction endonucleases for detection of methylated cytosines are MspI/HpaII isoschizomers [110]. Both enzymes digest CpCpGpG motif in dsDNA, but they recognise different state of the cytosine residue—MspI cleaves both methylated and unmethylated DNA, while HpaII cleaves just the unmethylated CpCpGpG motif. Fig. 1 shows GE separation of digested DNA [Trbušek et al., unpublished data], demonstrating the use of the system for detection of DNA demethylation of genomic DNA in lymphoblastoid cell lines treated with 5-azacytidine; this agent is able to induce DNA hypomethylation by blocking DNA-methyltransferase [5]. It is clear that the major band of genomic DNA is digested by MspI enzyme regardless of DNA-demethylation treatment, while only the band from the treated cells is digested by HpaII. As a control to this experiment, the level of DNA methylation in those samples was also evaluated by HPLC technique. We observed a decrease in 5-methylcytosine content in genomic DNA of about 60% in treated samples compared to untreated ones, which coincided well with the rough results obtained by the restriction enzymes digestion [87].

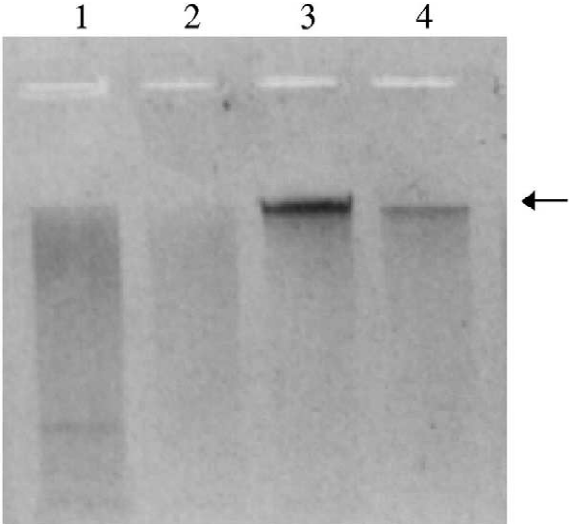

Fig. 1. Use of methylation-sensitive restriction endonuclease for DNA demethylation monitoring. Digestion of genomic DNA (marked by arrow) by MspI endonuclease (which cleaves CCGG motif regardless of its methylation) exhibits no difference between control DNA (lane 1) and DNA isolated from B-lymphoblastoid cell line treated with demethylating agent 5-azacytidine (lane 2). Its methylation-sensitive isoschizomer HpaII digests DNA from an untreated cell line to a much lesser extent (lane 3) compared to DNA from treated cell line (lane 4).

McrBC is another widely used MSRE, which digests ~40–80 base long cleavage sites, bordered with modified cytosines. Higher density of modified cytosines correlates with lower use of this MSRE [4]. McrBC was successfully used to prove that

DNA methylation within the *D. melanogaster* genome is present only in earlier stages of development [111].

Use of methylation-sensitive restriction enzymes for detection of DNA methylation state includes also trinucleotide-repeat disorder fragile-X syndrome. Detection of a methylation state of FMR1-gene promoter combines digestion with restriction enzymes, using methylation-sensitive endonuclease EagI and Southern-hybridisation with probe homologous to the FMR1-gene promoter [112]. Fig. 2 illustrates all the possibilities, which may occur in normal, premutated and fragile-X affected men and women. The methylation state of the promoter thus determines whether the disease appears or not. Although expanded alleles are methylated in almost all cases, there are rare examples of healthy individuals with expanded, but unmethylated FMR1-gene promoter. This example illustrates the critical understanding of the exact molecular mechanisms determining the disease. As was shown in other reports [113,114], methylation state at the EagI restriction site reflects, to a large extent, methylation

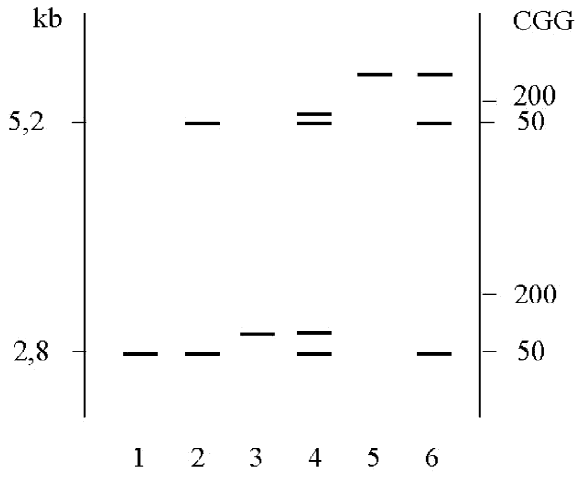

Fig. 2. Southern analysis of FMR1-gene promoter. The DNA is digested with EcoRI+methylation-sensitive EagI restriction enzymes and hybridized to probe homologous to FMR1 promoter. 1, Normal male; 2, normal female; 3, male carrying premutation; 4, female carrying premutation; 5, male carrying full mutation; 6, female carrying full mutation. The figure represents the basic possibilities only. Multiple bands or even smear may occur as a result of mitotic instability of FMR1 allele expansion. Partial methylation may occur in some cases as well.

at numerous other CpG dinucleotides in this region. However, it should be pointed out that this phenomenon, i.e. methylation state at one restriction site reflects methylation of the whole region, is not a rule and must be determined for every situation empirically.

Use of MSRE might theoretically seem to be the fastest way to determine the methylation state of studied cytosine residues. However, use of MSRE for local methylation monitoring requires detailed knowledge of the available restriction sites at the studied region. To successfully carry out the whole procedure, including DNA isolation (the approach requires about 10 μg of pure DNA; with A_{260}/A_{280} ≈1.8), restriction enzyme digestion and especially Southern hybridisation is not as easy as it seems to be, and requires a well-trained molecular biologist. Also ^{32}P isotope is an expensive and dangerous compound. Employing PCR amplification of the studied region, which procedure again requires knowledge of the regional sequence, can surmount these drawbacks. In this case, DNA is digested by methylation-sensitive restriction enzyme, which cleaves the nucleic acid within the subsequently amplified area, than the PCR follows, where the resulting product of expected size appears only if the studied cytosine residue is methylated [115]. The requirement for the amount of DNA is decreased approximately 1000-fold and this approach does not require hybridisation. PCR product is stained with ethidium bromide and run in agarose gel. The problem might be the quantitative evaluation in a case of partial methylation. It would require to employ a competitive PCR, in order to prevent the unchanged ratio between input DNA and the final PCR product.

Methodologically, incomplete digestion could be also a problem of the digestion by methylation-sensitive restriction enzyme, HpaII in this case. It was recommended to use mitochondrial DNA, which is devoid of 5-methylcytosine in mammals, as an internal control of the completeness of the digest [116]. Mitochondrial DNA is co-isolated with genomic DNA, thus controlling every step in DNA isolation and Southern blot, while digestion products of both are visualised on the blot together. After detection of methylation state of the desired sequence, the blot is reprobed with a portion of

mitochondrial genome, which recognises two fragments of 0.43 and 2.30 kbp in complete MspI/HpaII digests. The appearance of any other band indicates incomplete digestion. Thanks to a high abundance of mitochondrial DNA, the signal provided is strong and sensitivity is very high.

A novel method for the rapid detection of abnormal methylation patterns, global and on CpG islands, uses a combination of the restrictive enzymes HpaII and BssIII [117]. The cleavage is followed by AmpliTaq DNA polymerase step for single nucleotide extension using [^{3}H]-dCTP and methyl acceptance assay. The method is susceptible even to a low level of DNA breaks and abasic sites. On the other hand, the method does not involve PCR or DNA methylase reactions and can be applied to nanogram amounts of DNA.

Another utilisation of MSRE comes in combination with restriction landmark genome scanning (RLGS) (see Fig. 3) [118]. This method is suited for wide and simultaneous assessing of the CpG methylation state. Radiolabelled NotI DNA fragments are separated by means of two dimension GE. The specificity of the enzyme used, which cleaves unmethylated sites, allows the basis for this differential methylation analysis.

Amplified fragment length polymorphism (AFLP), as a DNA fingerprinting method, can be also used to identify DNA methylation. AFLP is a multipurpose and commonly used molecular marker method, which can be used for DNA mapping. Comparing it to restriction fragment length polymorphism (RFLP) and PCR-based marker systems, it was proved to be a robust and reproducible marker approach. Together with MSRE and PCR, this technique allows to detect genetic diversity without any previous knowledge about the sequence [119]. It can be also used to study the stability and inheritance of the methylation state [120].

2.2.2.2. Hydrazine and permanganate sequencing. DNA amplification techniques, such as the ligation-mediated polymerase chain reaction (LM-PCR), use a modification of cytosine and 5-methylcytosine residues by means of hydrazine or permanganate for the determination of the DNA methylation (see Fig. 4).

The hydrazine sequencing protocol is based on the fact that cytosine residues are modified by hydrazine and sites containing this modification are subsequently cleaved by piperidine. On the other hand, 5-methylcytosine reacts poorly with hydrazine and thus remains unreacted [121]. After the treatment, LM-PCR is employed, which enables amplification of the studied region by using one gene-specific primer and one linker-primer, which anneals to the linker adopted just by the end of the DNA strand, at its breakage site [122]. Finally, the PCR product is

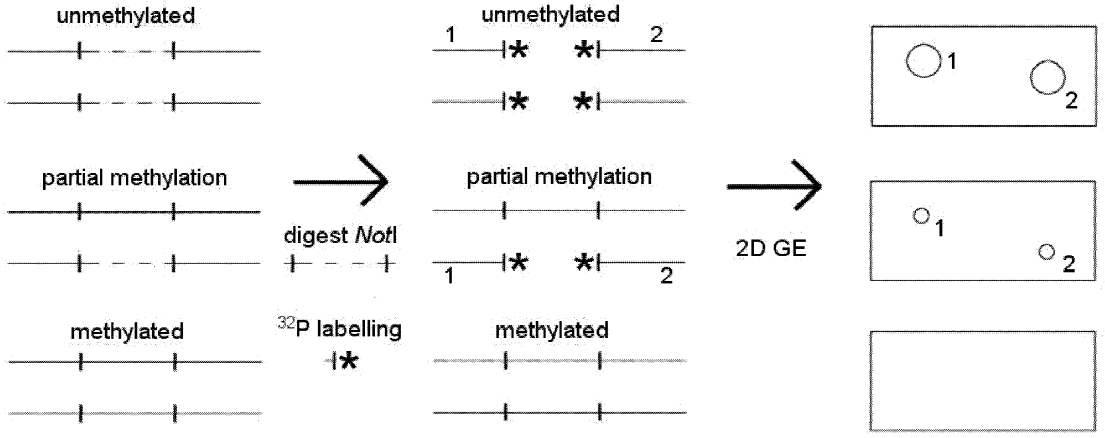

Fig. 3. Use of the restriction landmark genome scanning. The DNA is digested with NotI enzyme and radio-isotopically labelled. Labelled DNA fragments are separated by means of 2-D gel electrophoresis and intensity of the scintillation is detected (pictured as an area of the sample circle). Analysis of methylated DNA results in no detectable scintillation, while higher degree of unmethylated DNA in sample results in higher intensity of scintillation.

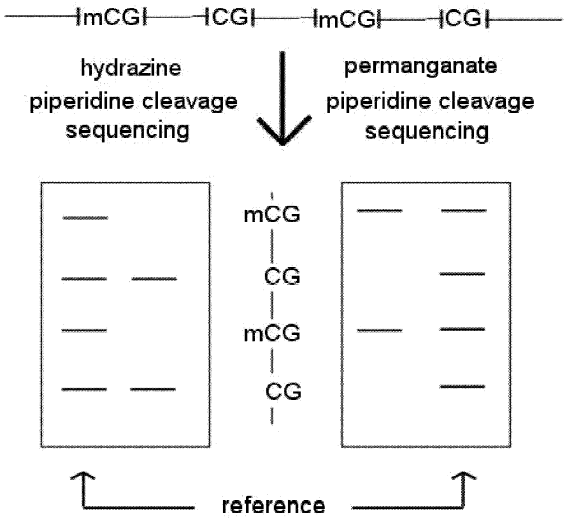

Fig. 4. Hydrazine and/or permanganate sequencing protocol. Single-stranded DNA is subjected to the hydrazine and/or permanganate treatment. Modified DNA is cleaved with piperidine, sequenced and separated using gel electrophoresis. The hydrazine sequencing protocol results in negative detection of methylated sites, while the permanganate protocol results in positive detection of those sites.

sequenced using a nested gene-specific primer. The hydrazine treatment is designed for negative methylation display, bands representing 5-methylcytosine are missing in the sequencing ladder. It suits demethylation studies especially since, in this case, cytosine residues will appear within the studied sequence after successful demethylation of DNA.

Permanganate ($KMnO_4$) modification of DNA provides a positive display of 5-methylcytosine presence [123]. However, the reaction between 5-methylcytosine and permanganate is rather uneven and sequence context-dependent. Thus, this approach should be used mostly as a complementary method to verify the results obtained by alternative approaches.

Recently, Thomassin et al [124] provided a very detailed discussion of both hydrazine and permanganate DNA modifications, including a step-by-step protocol.

A modification to this approach lies in induction of pyrimidine dimers by means of UV irradiation of DNA followed by piperidine cleavage and LM-PCR [4]. TpC and CpC produce those dimers, whereas Tpm^{5}C and Cpm^{5}C do not. So, if there is T or C located before the m^{5}CpG dinucleotide, dimer is not formed. This method is thus able to monitor only a subset of m^{5}C containing sites. Although the method was not explored widely, it seems to be a useful tool for the study of DNA damage caused by UV and its repair mechanism.

2.2.2.3. Bisulphite sequencing. A genomic sequencing protocol that positively displays 5-methylcytosine in individual DNA strands, or bisulphite sequencing, is the most exact methodology known for a detailed monitoring of DNA methylation state (see Fig. 5) [125]. The protocol is based on treatment of genomic DNA with a bisulphite, which results in conversion of cytosine residues to uracil, while 5-methylcytosine remains unchanged. In subsequent PCR amplification, using primers specific for individual DNA strands, all the uracil and thymine residues are amplified as thymines and only 5-methylcytosine is amplified as cytosine. The resulting PCR product can be sequenced directly or may be set cloned into bacteria and the DNA may be sequenced in this manner. The latter provides more detailed information about distribution of methylated residues in individual DNA strands and also, in case that some site is only partially methylated, i.e. the site is methylated in just a proportion of the cells, it enables determination of the proportion of methylated cells. The methodology of bisulphite sequencing is very straightforward and an efficient approach, which, however, is linked to some methodological troubles and discrepancies. Recently, Oakeley [3] has presented a very comprehensive guide of this sequencing protocol, in which paper valuable comments on all the important aspects of this method are provided. Also Thomassin et al. published an article [124], providing valuable comments and a step-by-step protocol.

Recently, modifications of the bisulphite protocol have appeared. To simplify the procedure of PCR assay for detection of m^{5}C, the manual transfer of PCR products for further analysis was substituted by in-tube protocol, involving PCR amplification of bisulphite treated DNA, followed by dsDNA melting analysis using DNA binding dye SYBR Green I (see Fig. 6) [126]. Although the method cannot be used to get information about methylation state of individual

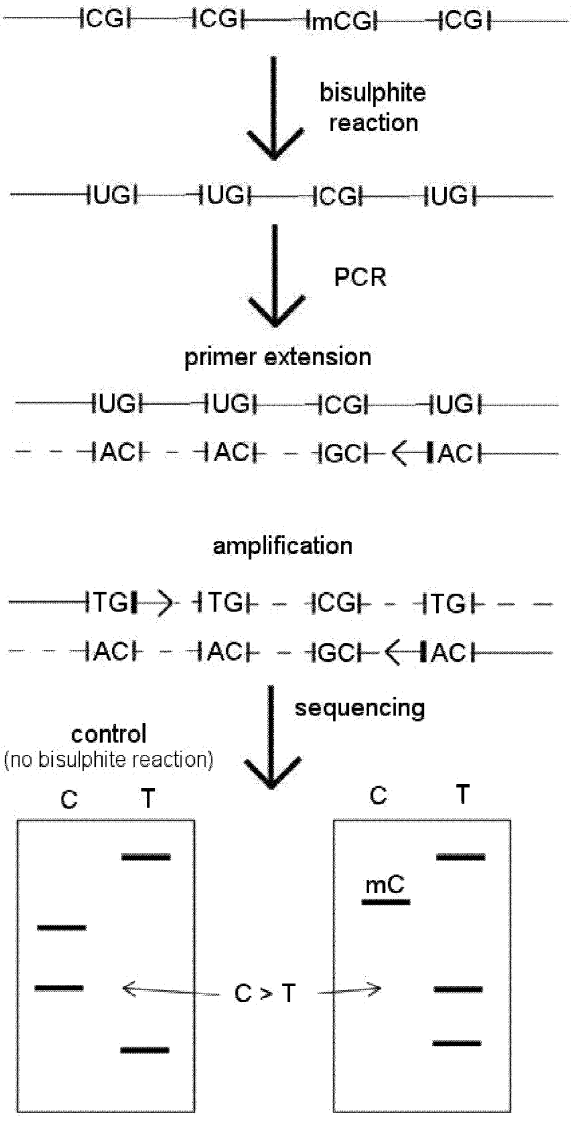

Fig. 5. Bisulphite sequencing protocol. DNA is treated with bisulphite, what results in conversion of cytosines into uracils, while methyl-cytosines remain untouched. Treated sample is sequenced and separated by means of gel electrophoresis. Results show conversion of unmethylated cytosine into thymin (uracil was substituted with thymine during DNA sequencing), methylated cytosine remains at the C lane.

CpGs or individual alleles, it can be used for rapid screening of general methylation level at chosen genes. The method is not suitable for quantitative estimations and for low methylation levels.

Another improvement of the bisulphite protocol

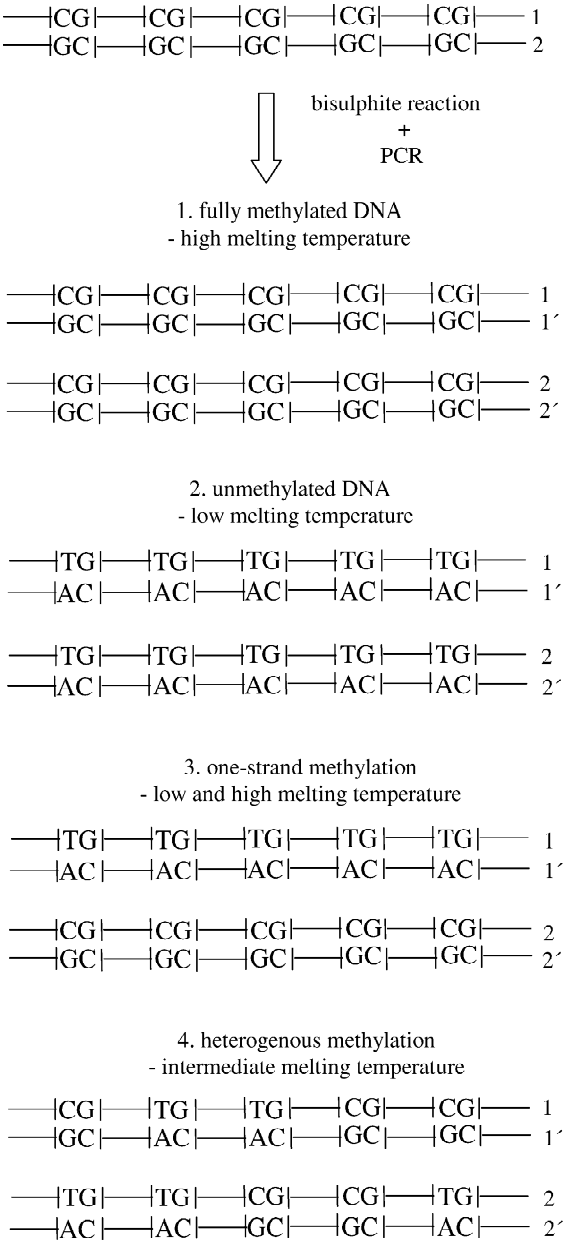

Fig. 6. Detection of the methylation level by means of bisulphite reaction and subsequent DNA melting temperature estimation. Bisulphite-treated DNA is labelled with DNA binding dye SYBR Green I and the melting curves are obtained by monitoring the changes in fluorescence intensity during linear temperature transition from 70 to 98 °C.

introduces alternatives to radioactive labelling in genome-wide methylation analysis [127]. It not only excludes necessity to work with expensive and dangerous radioactive material, but it also allows to analyse DNA methylation state in any sequence context, as opposed to only CpG methylation changes detected by classical radioactive labelling, using SssI methyltransferase. This method is based on the creation of stable fluorescing etheno derivatives of m^5dC by chloroacetaldehyde, while the second reactive base, dA, is removed by acidic depurination. The final fluorescence is thus proportional to the content of m^5dC. The only disadvantages of this approach are the relative slowness and the fact that chloroacetaldehyde is a toxic reagent.

Also other methodologies do include bisulphite sequencing as a methylation indicator, followed by different type of information evaluation, such as COBRA and MS-SNuPE. COBRA, combined bisulphite restriction analysis, combines MSRE with the bisulphite protocol (see Fig. 7) [128]. After the bisulphite reaction and amplification, samples are subjected to MSRE with m^5CpG recognition sites (BstUI, TaqI). As a control, a non-m^5CpG enzyme is used (Hsp92II), indicating incomplete conversion or nonsymmetrical methylation. The reverse experiment, done using Tru9I, provides a positive display of conversion [3]. Another method utilising bisulphite protocol is methylation-sensitive single nucleotide primer extension (MS-SNuPE) (see Fig. 8) [129]. This is a sophisticated approach in which, after the target sequence is found, the bisulphite reaction is conducted and the target sequence is amplified using strand-specific PCR primers. The process proceeds with mixing the sequence with primer, which will hybridise with the modified PCR sequence immediately 5′end from probed cytosine. There are two possible results—either the site was methylated and it is thus untouched by bisulphite reaction or was not methylated and was thus a subject of conversion. Two separate primer extensions are conducted by means of Taq polymerase, one with [^{32}P]-dCTP and the second with [^{32}P]-dTTP. After gel electrophoresis separation, the quantification may be carried out using a scintillation detector. If the cytosine was methylated, the band will be visible in the first primer extension reaction, but not in the second and vice versa. The difficulties of this method lie in the primer design, as it should not contain any CpG or CpNpG sequences. If such a sequence is present, the radioactive label incorporation is no longer linearly proportional to the target cytosine content. It is suggested that the target sequence should contain only A, C and T, while the primer should contain only A, G and T [3].

2.3. Mass spectrometry

Mass spectrometry (MS) is a technique which in principle determines the molecular mass of a compound. Recently, it started to be used as a de novo sequencing tool for obtaining DNA primary structure information [130,131]. Within DNA methylation research, MS methods are mostly unsuitable for quantitative analyses and they are strictly used only as a detection technique for the separation methods.

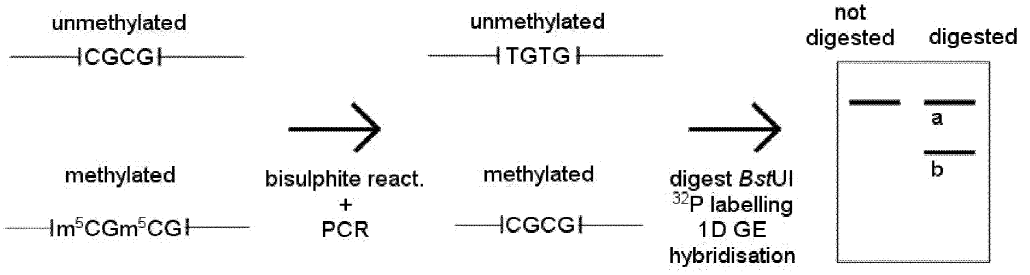

Fig. 7. COBRA—modified bisulphite sequencing protocol. Combination of bisulphite treatment with MSRE is used to quantify the percentage of DNA methylation. Bisulphite modified DNA is digested by MSRE (e.g. BstUI—it cuts methylated/unconverted/DNA), products are separated using gel electrophoresis and visualised by hybridization, using ^{32}P-labelled oligos. Content of methylcytosine in the DNA sample is than calculated as the percentage $m^5C = I_b/(I_b + I_a)$, where I_b and I_a are intensities of the scintillation of bands a and b, where band b represents the methylcytosine content.

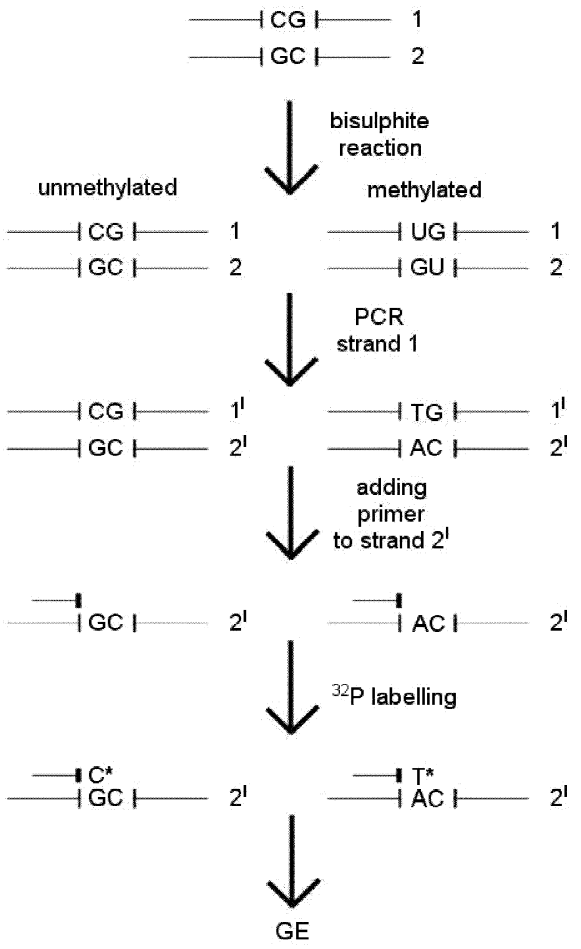

Fig. 8. MS-SNuPE, methylation-sensitive single nucleotide primer extension, combines bisulphite reaction with single nucleotide primer extension. The DNA strand is modified using bisulphite, PCR-amplified and site-specific primers are used to detect positions either modified or unmodified by bisulphite reaction. The final steps of the protocol represent extension of the primer with radioisotope containing single nucleotide and analysis of the modified and labelled primers by means of gel electrophoresis. Intensity of the scintillation belonging to labelled CMP is directly proportional to the content of methylcytosine, $I_{*\mathrm{CMP}}=f$ (c[m^{5}CMP]), while intensity of the scintillation belonging to labelled TMP is indirectly proportional to the content of methylcytosine, $I_{*\mathrm{TMP}}=1/f$ (c[m^{5}CMP]).

2.3.1. Matrix assisted laser/desorption MS

Matrix assisted laser/desorption MS time-of-flight (MALDI TOF) MS was employed in the nucleotide research to identify nucleotide triphosphates [132] or possible mutagen derived arylamid-deoxynucleotides [133]. First attempts were also made to not only identify all the important nucleotides (dAMP, dCMP, m^{5}dCMP, dGMP, dTMP) in the proceeded DNA samples, but also to quantify their amounts (see Fig. 9) [105].

2.3.2. Hyphenated techniques

Separation techniques are often coupled with MS. Such methods, like high-performance separation techniques MS (HPST–MS), are reviewed in several articles [134–136], which cover all the practical aspects of their use in the field of modified nucleotide research. Historically, one of the first hyphenated techniques used was gas chromatography–mass spectrometry (GC–MS), which, refined with use of isotopic dilution, gives detection limits at 30–210 fmol [86].

The most often used method is a combination of HPLC and electrospray ionisation (ESI) MS. HPLC gives good separation and ESI-MS is an ideal detection technique which also provides structural information on the nucleic acid chains. Moreover, employing additional approaches, like ^{32}P labelling or laser induced fluorescence (LIF) with fluorophore tagging, enhances the techniques. Single ion moni-

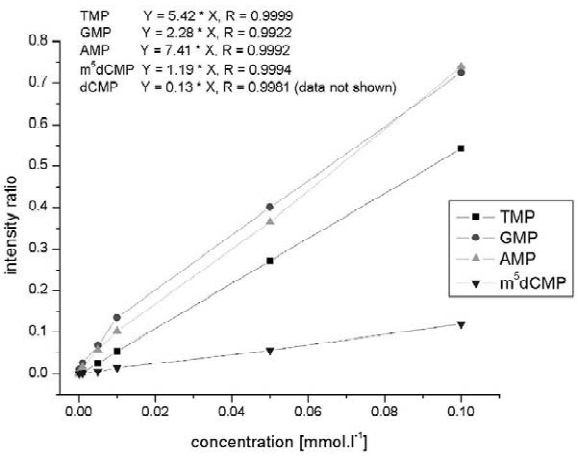

Fig. 9. Quantification of an individual nucleotide content in the model mixture of DNA digest containing TMP, AMP, GMP, dCMP, m^{5}dCMP by means of MALDI TOF MS. Data were acquired in negative mode, matrix: saturated solution of harmane in 0.05 M ammonium citrate dissolved in 50% aqueous methanol. Calibration curves show linearity over the three orders of concentration, from 1.10^{-1} to 1.10^{-4} M.

toring in combination with calibration curves from deuterium labelled analogues was successfully used as a quantification technique within an estimation range of 50 ng/ml to 10 μg/ml, which is the same as for ELISA [137,135].

Taking advantages of both CE and ESI MS, CZE–ESI MS methods were successfully applied to the analysis of modified oligonucleotides obtained from calf thymus DNA, cleaved by nonspecific benzone nuclease, followed by removal of terminal phosphate group by alkali phosphatase [138].

2.4. Methyl accepting capacity assay

A rather simple method for checking the methylation state of DNA is the use of SssI methyltransferase to determine the methyl accepting capacity. Unmethylated CpG sequences are methylated using [^{3}H]-*S*-adenosylmethionine as a donor of methyl functional group. The method is not very sensitive and its repeatability is low on consecutive days. It can be used to quantify small changes in DNA methylation, however experiments need to be done in 1 day, with an error of approximately 5%. Over the course of different days, RSD increases to approximately 30–50%. These errors come primarily from the instability of both enzyme and donor, and at the same time because of the difficulties in estimation of DNA concentration due to the incomplete dissolution of DNA prior to spectroscopic concentration measurement (OD_{260}). It is also suggested, prior to analysis, to cleave the DNA with MSRE without CpG restriction recognition site and to use those fragments as assay substrate [3,139].

2.5. Immunoassays

The use of m^5dC monoclonal antibodies is another way for highly specific mapping of locations of m^5CpG regions. Using antibodies, it is possible to detect m5dC in the ssDNA, mostly after depurination treatment with sulphuric acid, with sensitivity of 1.5 fmol [3,4] and to determine it within the range of 1–40 μ*M* using linear calibration with correlation coefficient of 0.9969 [140]. Its sensitivity makes this approach comparable to HPLC. The crossreactions, mostly to m^5rC and m^5C, being the general disadvantages of immunoassay, are negligible, so they do not interfere the assay [140,141]. Antibodies can also be easily immobilised or modified with fluorophore, making them versatile analytical tool [141,84]. To normalise the data given by the analysis, it is suggested to also acquire fluorescence data of the total DNA content in the sample, after the antibody fluorescence has been recorded. Ethidium bromide reaction causes fluorescence proportional to the amount of DNA and such a value can be used to normalise the values of the fluorescence intensity coming from labelled antibody [3].

Monoclonal antibodies in combination with secondary antibody–fluorochrome conjugates were successfully used for in situ monitoring of the DNA methylation [142], permitting also a quantitative evaluation [143].

3. Critical notes to the used analytical approaches with respect to biological relevance of the analytical data

Any analytical approach has its limitations coming from the principles it is based on. It is quite important be mindful of the particular aims of an analysis and in accordance with them to correctly choose a suitable method, thus to minimise the influence of the mentioned limitations on the interpretation of data and their evaluation.

Generally, the methods of molecular biology are time consuming and laborious. The determination of the methylation state arises in all cases from several steps. Each is different and has a different level of difficulty, but some of them are already part of laboratory routine, such as DNA isolation, PCR or gel electrophoresis. It is often not useful to try to spare tens of minutes using HPTLC instead of HPLC, while the previous procedure was overnight DNA digest. But it is worth choosing HPTLC while running screening experiments, because one plate allows several runs to be done in parallel instead of series of runs on HPLC. On the other hand, just to choose less resource consuming method may improve the routine and will lower the demands. The appropriate choice of experimental conditions along with the optimisation of the analytical method may result in significant lowering of the resources needed [87].

Quite often, a once developed method can be overlooked in an overwhelming number of relevant articles. Good example is comparison of HPLC analysis of nucleosides by Cheng et al. [144] and Kakutani et al. [145]. A choice of slightly different mobile phase (10 *M* H_4PO_3, pH 5.6, 2.5% methanol vs. 50 *M* NaH_2PO_4, pH 4.0, 2.5% methanol) and a bigger column (150×4.6 mm vs. 100×4.6 mm) results in analysis time-wise twice as long, i.e. 60 instead of 30 min, which is a reasonable difference in resource utilisation.

Another problem in the proper choice of method is

Table 3
Experimental methods listed in Section 2, using 5-methylcytosine as a marker for DNA methylation monitoring, are summarised in relation to the possible aims of the study; additional information, such as the minimal amount of DNA necessary for analysis or the main disadvantages, is included

Method	Detection	Sensitivity (m^5C LOD)	DNA amount needed	DNA form	Repeatability (%)	Limitations	Major advantages
HPTLC	Scintillation	20 fmol	5 μg	dNMP	4.0–6.0	Other dNMP are labelled too	Parallel runs
HPLC	Optical —UV Scintillation Fluorescence MS	400 fmol	<1 μg	N, dN, dNMP	Validated 0.3–3.0		Repeatability Sensitivity
CE	Optical —UV Scintillation, Fluorescence MS	350 fmol	<1 μg	N, dN, dNMP	Validated 3.0–6.0		Rapid, parallel runs
MS		100 fmol	<1 μg	N, dN, dNMP	10–20	Repeatability	Rapid
Isotope dilution	ESI	5 fmol	1 μg	N, dN, dNMP	~10	Isotopic labelling	
GC–MS							
HPLC–MS	ESI	30 fmol	<1 μg	N, dN, dNMP	3		
Immunoassay	Fluorescence Scintillation	1.5 fmol		ssDNA, in situ	25–50	Crossreactions	Specificity
Methylation capacity assay	Scintillation	N/A		ssDNA	5, 50	Unstable reagents, DNA concentration estimation	Simplicity
MSRE	GE, Southern blot hybridisation	N/A	>5 μg 10 ng (PCR)	ss, dsDNA	4.0–10.0	Incomplete cleavage, CpNpG, m^5Cpm^5C	Site specific
Hydrazine/ permanganate sequencing	GE	250 fmol	1–2 μg	ssDNA		Permanganate: context sensitive	Complementary (hydrazine + permanganate)
Bisulphite sequencing	GE	2.5 fmol	10 ng	ssDNA		Incomplete reactions, DNA denaturation, slow	Sensitive, easy, (selective cell line, individual cell)
Bisulphite sequencing + chloro-acetaldehyde	Fluorescence	175 fmol	10 μg	ssDNA	6.0–10.0	Time consuming, toxic reagents	No extensive purification
COBRA	GE	125 fmol	1 μg	ssDNA	~5–10	Cleavage site sensitive	Fast, sensitive
MS-SNuPE	GE	500 fmol	5 ng	ssDNA	~5–10	Sensitive to rich CpG regions	No MSRE
AFLP	GE	N/A	250 ng	ssDNA			

the fact that not all the methods presented in literature were correctly evaluated from the analytical point of view—by means of their repeatability and reproducibility, and if applicable, by means of relative standard deviation of m^5C determination, limits of detection, sensitivity and resolution, i.e. they were not validated. Unsuitable presentation of results may lead to a serious waste of the resources while trying to apply such a method in praxis.

In Table 3, the methodologies discussed in the article are summarised.

4. Conclusions

Monitoring of DNA methylation attracts the continuing attention of the research community. This fact is clearly reflected in the number of methods and approaches developed. There are enough methods in the methylation analysis arsenal to map m^5C content and distribution, although, generally, there is a necessity to complement them to obtain the correct results. The necessity of balanced and verified data in such an important field can be achieved by selection of a competent experimental approach and analytical method. Known limitations of the methods should allow scientists to choose analytical methods to prove the findings within DNA methylation research, avoiding the complications which can be brought about by particular methods. The chosen approach will deeply influence the results, as the available methods allow mostly only a limited picture of the methylation state. The overview of the methods, which use m^5C as a marker for monitoring of the DNA methylation state, is supposed to help the researches in their efforts.

Although it might appear that the field of methylation state analysis is already complete, there are still several directions which attract the attention of researchers to develop new methods and approaches. Such novel techniques are required within in situ methylation monitoring, e.g. dsDNA in chromatin structure, within the introduction of rapid automated screening methods for medical purposes or more straightforward and simpler methods accompanied by sequence information.

Methylomics is a continuously growing and expanding branch of biological nuclear research, which combines the disciplines of analytical biochemistry, molecular cell biology and genetics, being thus a bridge across applications and fundamental research of high importance.

5. Nomenclature

N	base
rN	ribonucleoside
dN	deoxyribonucleoside
dNMP	nucleotide monophosphate
dNTP	nucleotide triphosphate
N1pN2	sequence of dN_1MP dN_2MP
m^5, m^4	C^5 and C^4 carbon methylation, respectively
hm5C	5-hydroxymethyl cytosine
PCR	polymerase chain reaction
LM-PCR	ligation-mediated polymerase chain reaction
GE	gel electrophoresis
SAM	*S*-adenosylmethionine
MT	methyltransferase
ssDNA	single strand DNA
dsDNA	double strand DNA
MSRE	methylation/modification-sensitive restriction enzymes
AFLP	amplified fragment length polymorphism
RFLP	restriction fragment length polymorphism
ICEAMP	identification of CpG islands exhibiting altered methylation patterns
MS SNuPE	methylation-sensitive single nucleotide primer extension
COBRA	combined bisulphite restriction analysis
RLGS	restriction landmark genome scanning

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