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REVIEWS

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DNA Methylation Profiling in Cancer: From Single Nucleotides Towards the Methylome

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Abstract—The genomic DNA methylation pattern (methylome) is a cell epigenetic program that controls the expression of genetic information. The methylation pattern substantially changes in early carcinogenesis. A detailed survey of the methylcytosine distribution in the genome in norm and pathology is of immense importance for a better understanding of the etiology of cancer and its early diagnosis. The techniques available make it possible to simultaneously examine many samples (high-throughput analysis) and to examine large genome loci or even the total methylome (large-scale analysis). The review considers the main trends in the development of new approaches to DNA methylation and describes the techniques most commonly used in the field, their application, and results. Emphasis is placed on the use of various DNA microarrays (oligonucleotide microarrays, BAC arrays, etc.) as a method of choice for epigenetic analysis of tumors. Alternative sequence-based techniques of methylation analysis are discussed. The use of large-scale analysis to identify new epigenetic markers and to develop an epigenetic classification of neoplasms is considered.

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Genetically identical cells of multicellular organisms are specialized via realizing different parts of the total genetic program. Possessing the total genetic program involves a potential risk of an unauthorized access to prohibited information [1]. The cell that has gained such an access can evade natural controls and give the origin to a tumor.

As early as in 1970, Vanyushin, Belozersky, and colleagues [2] assumed that DNA methylation is a natural mechanism regulating the expression of eukaryotic genes. The product of DNA methylation, 5-methylcytosine ($5^{m}C$, "fifth base"), was already known at that time [3]. Modification predominantly affects cytosines of CG dinucleotides in mammals. Vanyushin and colleagues [2, 4] were among the first to demonstrate the tissue specificity of methylation, as well as changes in the total methylation level and the methylcytosine distribution through DNA in cancer cells [5]. The characteristic distribution of methylated and nonmethylated CG dinucleotides in the genome is known as the genomic DNA methylation pattern, or methylome. Since the DNA methylation pattern is reproduced upon DNA replication [6–8] and is associated with the level of gene transcription [9–11], it was assumed that genomic DNA methylation carries hereditary information, termed epigenetic. Epigenetic information includes the mitotically and/or meiotically inherited changes in gene functions that cannot be explained by the modification of DNA nucleotide sequences [12]. Yet the principal possibility of epigenetic regulation was skeptically received, and the progress in studying DNA methylation was delayed for a long time.

Now, analysis of DNA methylation is one of the most intensely developing fields of molecular biology [13]. DNA methylation is critical for normal embryo development [14]. Many studies testify to substantial changes in the DNA methylation pattern in tumors. As experimental findings are accumulating, the concept of carcinogenesis is reviewed [15]. Mutations are no longer regarded as the only lesions capable of inducing cell malignant transformation. A second hit [16] can be provided by hypermethylation of a tumor suppressor gene [10, 11, 17–23] or a loss of imprinting [24]. The DNA methylation pattern changes during aging, which allows a new interpretation for the well-known age-related increase in the risk of tumorigenesis. The fate of the cell that has acquired an oncogenic mutation seems to strongly depend on the existing epigenetic alterations [25], which arise in early transfor-

mation, when the cell still lacks the malignant phenotype [26–30]. According to P. and L. Medavars, mutations trigger carcinogenesis, while the epigenome status plays a predisposing role [31]. In the functional context, epigenetic and genetic alterations are similarly important for carcinogenesis and are closely associated with each other. Abnormal methylation of genes involved in a carcinogenesis-related signaling pathway can (!) predispose to an accumulation of mutations in genes of the same pathway [15].

The CG dinucleotides potentially subject to methylation are nonuniformly spread through the genome. Some of them cluster to form CpG islands [30], which are GC-rich DNA regions that vary in size from several tens of base pairs to several kilobases and occur predominantly in 5' regions (promoters, first exons) of most human genes [32]. The other CG dinucleotides are scattered through the genome. The majority of promoter CpG islands are nonmethylated in normal differentiated cells [33], while scattered CG dinucleotides are methylated. An opposite pattern is observed in cancer, which is characterized by local hypermethylation of CpG islands and global hypomethylation of other regions of the genome [34]. De novo methylation of CpG islands in tumor cells is accompanied by transcriptional suppression of neighbor genes [10, 11, 17–23, 35–42]. Epigenetic gene silencing is reversible and the genes involved can be reactivated by demethylating agents [30, 43–45]. DNA hypomethylation is associated with genome instability and further tumor progression [46–48].

The mechanisms of the above substantial changes in methylome are poorly understood. The methylation pattern has been described in detail for no more than 0.1% of the genome [49]. It is clear that methylome analysis in tumor cells is of immense importance for a better understanding of the etiology of neoplasms. DNA methylation profiling can be employed in the early diagnosis of cancer, a search for prognostic epigenetic markers, and cancer prevention [26, 28, 30, 42, 50–52]. This brings to the forefront the technical aspect of DNA methylation profiling.

It is necessary, first, to distinguish C and 5^mC and, second, to map them in the genome. Third, we think it expedient to perform a large-scale analysis at an as high as possible resolution without restriction to an individual gene or a locus.

Recent years have brought great progress in the development of new techniques addressing the methylome. It is possible now to simultaneously probe the methylation status for thousands of CG dinucleotides in the genome [53]. The methods of large-scale analysis continue to improve at a high rate.

Here we consider the technical aspect of DNA methylation profiling in cancer. We discuss the principles of the main DNA methylation profiling methods, the logic of their further improvement, and potential results, which can be expected to provide further insight into the mechanisms of carcinogenesis and to allow more efficient diagnosis and therapy of cancer.

It is expedient to briefly consider the early approaches to discrimination between C and 5^mC, which underlie, to a certain extent, all modern techniques of large-scale analysis of the methylome.

GENERAL PRINCIPLES OF DISCRIMINATION BETWEEN C AND 5^mC

Two main approaches are used to distinguish C and 5^mC. One takes advantage of the fact that methylation-sensitive restriction endonucleases selectively cleave DNA in the recognition site containing C but not 5^mC [54, 55]. This approach is a priori limited to the recognition sites of a restriction enzyme. Yet a combination of several enzymes makes it possible to examine a substantial number of CG dinucleotides in the genome [49]. Digestion with methylation-sensitive restriction enzymes is employed in the largest-scale methylome analysis techniques.

The other approach to discrimination between C and 5^mC is reducing the epigenetic to a genetic difference with subsequent molecular genotyping. Frommer et al. [56] have proposed DNA modification with sodium bisulfite for this purpose. In the presence of HSO_3^-, nonmethylated cytosine is selectively deaminated to yield uracil, whereas 5^mC does not enter into the reaction. When the target locus is PCR-amplified, the amplicon contains thymine in place of uracil so that cytosine methylation profiling is reduced to identifying T and C. This problem can be solved by any molecular genotyping technique (PCR, single-nucleotide primer extension (SNuPE) [57], sequencing [56], etc.). Bisulfite-based DNA methylation profiling has allowed a breakthrough in epigenetic studies [28], making every CG dinucleotide accessible for analysis.

A third approach to discrimination between C and 5^mC is based on 5^mC immunoprecipitation and is considered below, in the section focusing on large-scale analysis.

FROM INDIVIDUAL NUCLEOTIDES…

The standard experimental approach implies a preliminary selection of a target gene or locus for methylation profiling. Genes can be selected by function (e.g., proapoptotic genes) or transcriptional silencing. The choice is usually based on the assumption that hypermethylation of the corresponding CpG island is involved in the mechanisms deregulating gene expression in a tumor. Several simple procedures address the methylation pattern of CpG islands. The most common one is methylation-sensitive restriction enzyme

analysis with subsequent PCR (MSe-PCR) [58], methylation-specific PCR (MSp-PCR) [59], and combined bisulfite–restriction enzyme analysis (COBRA) [60].

MSe-PCR reports methylation for one or a few CG dinucleotides contained in the recognition site(s) of methylation-sensitive restriction enzymes (e.g., *Bst*UI, *Not*II, and *Hpa*II) [39]. Nonmethylated DNA is cleaved and PCR with primers flanking the recognition site(s) is impossible in the ideal case. The presence or absence of the amplification product reflects the methylation status of the target cytosines.

MSp-PCR is based on selective amplification of methylated and nonmethylated DNAs modified with bisulfite. Two or more target CG dinucleotides are in the regions of primer annealing to native DNA. PCR is carried out separately with two pairs of primers, one corresponding to the methylated DNA template (which contains CG after bisulfite modification) and the other corresponding to the nonmethylated template (which contains UG in the same position). Amplification is strongly specific under optimal conditions. The methylation status of the target CG dinucleotides is established depending on the primer pair that allows amplification.

Both MSe-PCR and MSp-PCR fail to report the extent of cytosine methylation, which can vary. In contrast, COBRA is a quantitative assay. The essence of COBRA is that the recognition site for a restriction enzyme is preserved or eliminated after bisulfite modification of DNA, depending on the methylation status of the target CG dinucleotide. The preservation of the site (i.e., cleavage of the PCR product) suggests cytosine methylation.

The techniques are simple and readily available and, consequently, continue to be widely used. It is obvious, however, that their informativeness is low: only one or a few CG dinucleotides are examined and their methylation status is used to suggest the methylation status for the total CpG island. Yet even neighbor cytosines can differ in methylation [61] and selective analysis of one or a few of them can yield false positive or false negative results.

Bisulfite sequencing was developed to comprehensively examine individual loci at a high resolution [56]. Although addressing individual loci, bisulfite sequencing is now the most informative technique, reporting the methylation status for every CG dinucleotide. DNA is modified with bisulfite and amplified in PCR, and the resulting amplicons are sequenced according to Sanger [62]. C/T polymorphism is directly detectable on the sequencing diagram. Since PCR products are examined to infer the extent of methylation for each 5^mC, significant results are obtained only when methylated and nonmethylated DNAs are amplified with the same efficiencies [63]. To achieve this, the primers should lack CG dinucleotides or, when impossible, have a single-nucleotide substitution at the position corresponding to 5^mC. This condition is built in the MethPrimer program, which serves to design the primers for bisulfite sequencing, as well as for MSe-PCR, and is publicly available at www.urogene.org/methprimer/index1.html [64].

Bisulfite modification of DNA is involved in many techniques employed in both local and large-scale epigenetic analyses. To avoid false positive results (incomplete C⟶U conversion), it is necessary to use an optimized protocol (e.g., [65]) or commercial kits. Systematic analysis of the artifacts arising in bisulfite DNA modification suggests several causes for incomplete C⟶U conversion: incomplete denaturation of the DNA sample, its contamination with protein or RNA, and a high density of CG dinucleotides in the target locus [65]. Hence, complete C⟶U conversion in bisulfite-modified DNA should be verified, for instance, by bisulfite sequencing (Fig. 1a). Commercial kits for bisulfite modification of genomic DNA (Qiagen, BioCat, etc.) ensure a high reproducibility of the results and considerably reduce the experiment time (from ~20 to 4–5 h).

It is clear that the PCR products can be directly sequenced (Fig. 1) or preliminarily cloned in a vector. The second variant yields unequivocal results and is preferred in most cases. However, since the extent of DNA methylation may vary among cells, it is necessary to sequence many random clones (e.g., see [21]). It is for this reason that the analysis is usually limited to a few subjects: tumor cell lines or several clinical specimens.

In the case of a direct sequencing of the PCR products, double peaks of cytosine and thymine are detectable on the sequencing diagram when the extent of CG dinucleotide methylation varies (Fig. 1c). In the ideal case, the ratio between peak amplitudes (or, more correctly, peak areas) reflects the frequency of a particular CG dinucleotide in the sample examined [66]. Yet the nucleotide sequence is greatly simplified after bisulfite modification (e.g., tetranucleotides CTTT, CCTT, CCCT, CCCC, CTCT, and TTTC are indistinguishable after modification and are identified as TTTT) and completely lacks cytidines (guanidines) apart from those methylated in the original sample. This requires additional normalization of the fluorescent signals for quantitative analysis. Epigenomics AG is developing a bioinformatic solution of this problem [67] and direct bisulfite sequencing of the PCR products has been chosen for the pilot project of the Human Epigenome Consortium [68]. Although Sanger bisulfite sequencing is a gold standard for methylation profiling [49], this method is too time-consuming and laborious to be widely employed in routine large-scale analysis of the methylome in tumors.

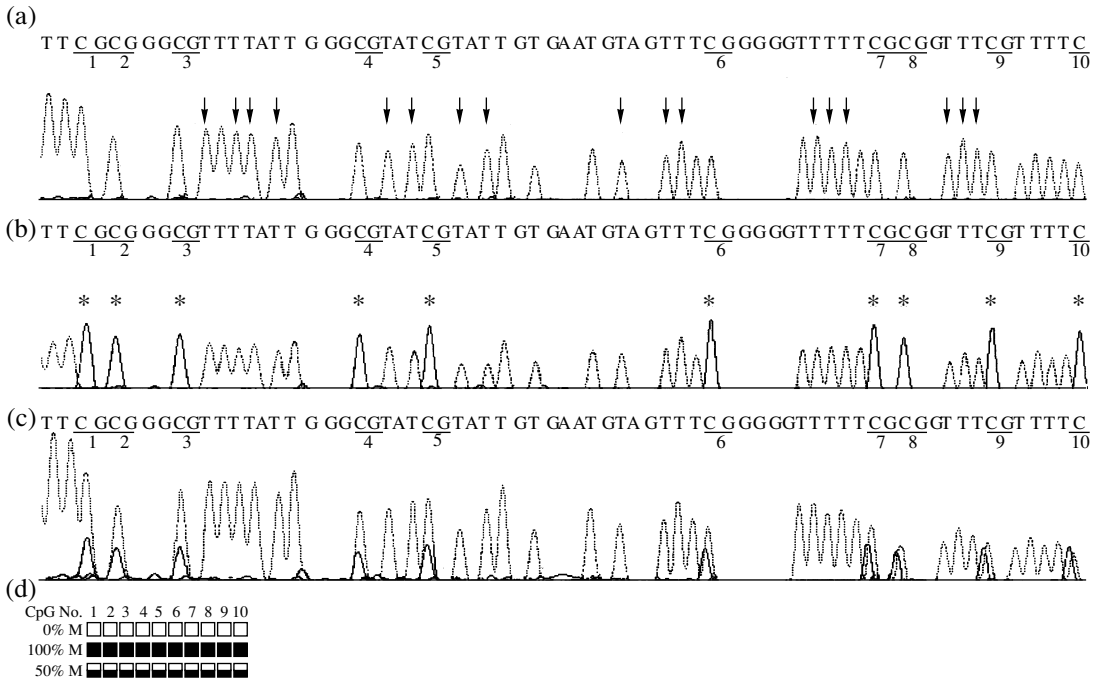

Fig. 1. DNA methylation profiling by direct bisulfite sequencing. The sequencing diagrams were obtained for the promoter CpG island of *wif1*. The gene product inhibits the Wnt signaling pathway, which is abnormally activated in some tumors (e.g., in colorectal carcinoma [22]). Only T and C peaks are shown (dashed and solid lines, respectively). The nucleotide sequence at the top of each diagram corresponds to bisulfite-modified DNA apart from cytosines (shown nonconverted to thymines) of CG dinucleotides (underlined and numbered). The principles of bisulfite DNA modification are described in the text. An optimized protocol [65] ensures complete C⟶T conversion (the corresponding thymines are indicated with arrows). Cytosines occurring in modified DNA correspond exclusively to 5^{m}C in native DNA (asterisks). (a) Peripheral blood DNA of a healthy donor is nonmethylated. (b) The same DNA incubated with *Sss*I methyltransferase is completely methylated. (c) An equimolar mixture of completely methylated and nonmethylated DNAs (serves to verify the equal efficiency of amplification for the methylated and nonmethylated alleles). (d) Bisulfite sequencing data are often represented in the form of a methylation map with symbols (squares, circles, etc.) showing individual CG dinucleotides and the extent of symbol filling corresponding to the extent of cytosine methylation.

It is clear that bisulfite sequencing yields far more informative results as compared with the above methods. Yet all techniques of local epigenetic analysis have common drawbacks: their throughput is low and quantitative interpretation of the results is problematic. Quantitative analysis is important, in particular, for monitoring DNA demethylation during chemotherapy with demethylating agents such as 5-aza-2'-deoxycytidine, or decitabine [45]. The drawbacks have been overcome in more recent modifications of MSp-PCR and bisulfite sequencing.

MethyLight is a technique that combines MSp-PCR and real-time PCR [69]. Bisulfite pyrosequencing, proposed by Biotage (Sweden), is gradually becoming a standard in qualitative analysis. The method is based on fluorescence detection of pyrophosphate released during DNA synthesis [70] and allows real-time nucleotide sequence analysis for several tens of samples [71]. Its drawback is a short reading length (about 100 bp vs. 700 bp in Sanger sequencing).

To reduce the cost of analysis, MALDI-TOF mass spectrometry was used in place of fluorescence detection. The extent of cytosine methylation was quantitated for individual cytosines by a combination of MALDI-TOF mass spectrometry with a primer extension technique [72] or a hybridization of bisulfite-modified DNA with peptide nucleic acid probes. The techniques allow simultaneous analysis of several hundreds of samples.

The methods of local DNA methylation analysis are further improved to increase their throughput without changing the general principle, examination of a selected locus. Such methods are appealing for clinical diagnosis with the use of epigenetic markers. For instance, methylation of a single CG dinucleotide in the first intron of *zap70* correlates with the level of *zap70* transcription [74] and is regarded as a main prognostic marker in B-cell chronic lymphocytic leukemia [75]. Methylation profiling of the CpG islands in a limited number of genes can be used for the epigenetic diagnosis of known tumors and the identification of new ones, as convincingly demonstrated in some works [76, 77]. As a molecular marker, methylation of particular CG dinucleotides is preferable in clinical analysis owing to a greater DNA stability.

Genomic DNA for epigenetic analysis can be isolated not only from biopsy material but also from various biological fluids: blood plasma and serum, urine, saliva, sputum, etc. [78, 79]. Data on the genes whose hypermethylation in biological fluids is regarded as a tumor marker have been reviewed in [28].

Commercial kits for epigenetic analysis of some tumor markers are already available, though only for research purposes. For instance, Biotage employs bisulfite pyrosequencing for studying the promoters of *p16* (*cdkn2a*), *hmlh1*, and *mgmt.* The methylation status of *Alu* and LINE-1 repeats has been proposed as a marker of global DNA hypomethylation in tumor cells [80, 81]. These repeats are usually methylated in normal tissues and demethylated in neoplasms, which is detectable by pyrosequencing and MethyLight. Available methods to monitor global DNA hypomethylation are important for chemotherapy with demethylating drugs [45] and clinical studies of new such drugs.

Regardless of the method employed, analysis is complicated by the heterogeneity of tumor specimens, which always contain normal cells as well. This problem is especially pressing when PCR is used to amplify DNA. In the case of blood malignancies, transformed cells can be isolated from peripheral blood, for instance, by using magnetic particles with immobilized antibodies against cell surface antigens or by fluorescence-activating cell sorting (FACS). The heterogeneity of solid tumors can be reduced to a minimum by using laser microdissection [82].

The techniques of local epigenetic analysis can be used beyond clinical laboratories as well. For instance, MSp-PCR is convenient for a rapid preliminary screening of candidate genes in a set of specimens. The scheme selection of candidate genes $\longrightarrow$ preliminary MSp-PCR analysis (COBRA, etc.) $\longrightarrow$ detailed analysis has been used in many works and has made it possible to demonstrate the epigenetic inactivation of tumor suppressor genes in various neoplasms (e.g., *apc* in colorectal carcinoma [83]). It is obvious, however, that further insight into the regularities and mechanisms of methylome alterations in carcinogenesis is rather difficult to gain as long as candidate genes are selected and analysis is restricted to a few CG dinucleotides.

…TOWARDS LARGE-SCALE METHYLOME ANALYSIS

Since the above one experiment–one or a few loci strategy is apparently insufficient, development of new analytical procedures is under way. A natural idea is to expand the scale of analysis and to simultaneously characterize many loci or, in the ideal case, the total methylome. This new strategy obviates the need to select the loci to be examined.

One of the first techniques of large-scale analysis is restriction landmark genome scanning (RLGS), addressing the total genome. The technique combines digestion of genomic DNA with a methylation-sensitive restriction enzyme (usually, *Not*I) and separation of radiolabeled products by two-dimensional electrophoresis [84, 85]. A difference in spot intensity on the autoradiographs of tumor and control DNAs suggests different extents of methylation for the corresponding CG dinucleotides, which are mapped by sequencing the differing fragment. RLGS has been used to study the global changes in DNA methylation for several malignancies. For instance, a substantial number of CpG islands differentially methylated in transformed and normal B cells were detected with two restriction enzymes in chronic lymphocytic leukemia [86]. Thorough analysis of some candidate genes revealed new epigenetic markers; e.g., the methylation status of *twist2* proved to correlate with the prognosis in lymphocytic leukemia [87]. Yet RLGS has a low resolution and a low throughput, needs a large amount of DNA, and is laborious, thus being of a limited utility.

Highly efficient nonmethylated genomic sites coincidence cloning (NGSCC) has been developed to identify the nonmethylated cytosines in extended polygenic loci of more than 1 Mb [88]. The gist is comparing the methylation pattern of a particular locus with the identical cloned (i.e., completely nonmethylated) nucleotide sequence. PCR amplification substantially reduces the DNA amount necessary for analysis. NGSCC was used to map the nonmethylated cytosines in a 1-Mb locus of chromosome 19 and revealed DNA sites differentially methylated in seminoma and normal testicular parenchyma [89]. The use of NGSCC is limited because the PCR products must be sequenced in order to map the nonmethylated cytosines.

The main progress in techniques for large-scale analysis is associated with DNA microarrays [90], which are designed to simultaneously address many CG dinucleotides [53]. The development of oligonucleotide microarray technology has already allowed large-scale analysis of gene expression [91] and simultaneous examination of tens of thousands of single-nucleotide polymorphisms [92].

Particular solutions (hereafter referred to as platforms), as well as the procedures of analysis and signal detection, greatly vary. Methylation analysis with DNA microarrays is usually based on the standard principles: bisulfite modification and digestion with restriction enzymes are utilized in the majority of techniques. Microarrays only provide a high throughput and a new approach to $5^{m}C$ mapping. Platforms are developed at a high rate and new ones can be expected in the nearest future.

Oligonucleotide Microarrays for Analysis of Bisulfite-Modified DNA

Analysis of bisulfite-modified DNA consists in hybridization to oligonucleotide microarrays. The resolution and the quality of results are similar to those of bisulfite sequencing. We think that the oligonucleotide microarrays currently used to analyze bisulfite-modified DNA provide for a transition to real large-scale methylation profiling. The progress consists in increasing the number of target CG dinucleotides and the scale of analysis is now comparable with that of pyrosequencing. Yet the method has sufficient potential to allow a transition from quantity to quality.

Oligonucleotide microarrays are usually obtained by immobilizing presynthesized oligonucleotide on a glass slide (spotted arrays) [93–95] or synthesizing oligonucleotides in situ [92, 96, 97]. Illumina has suggested an alternative principle of microarrays: synthetic oligonucleotides are immobilized on glass beads, which are spread on the surface of an optic fiber chip (BeadArray technology) [98, 99].

A protocol for hybridization analysis of bisulfite-modified DNA has been developed in our lab with the use of in situ synthesized oligonucleotide microarrays (Febit Biotech AG, Germany) [96]. The Geniom One instrument utilizes a digital micromirror device for UV-activated in situ oligonucleotide synthesis [100], which makes it possible to easily change the microarray composition as required for the experiment. A microarray is a system of microchannels in a glass plate; oligonucleotides are synthesized on the microchannel surface. Parallel hybridization with several tens of thousands of oligonucleotide probes is now available with such microarrays.

A sample is usually a mixture of biotinylated fragments PCR-amplified from bisulfite-modified DNA. Each CG dinucleotide is examined using a pair of oligonucleotide probes (17–25 nt) [96, 101, 102]. The probes of a pair are fully complementary to either a methylated (M probe) or a nonmethylated (U probe) sequence. The target CG dinucleotide corresponds to the central nucleotide of a probe. This ensures the selective binding of the probe with the target, since hybridization of a target to a probe differing by one central nucleotide is unlikely (the principle of the method is shown in Fig. 2). After hybridization, the microarray is incubated with a streptavidin–R-phycoerythrin conjugate to amplify the fluorescent signals, which are recorded using a digital camera. The signal intensities from the probes of a pair reflect the relative contents of methylated and nonmethylated cytosines in the given position. The ratio of the signal intensity from the M probe to the total signal intensity from the two probes of a pair characterizes the extent of methylation of the target cytosine and is known as the methylation index $I_M/(I_M + I_U)$, where I_M and I_U are the intensities of the fluorescent signals from the M and U probes, respectively (Fig. 2c). To simultaneously analyze the methylation status of many cytosines in various samples, it is necessary that the microarray contain the corresponding number of oligonucleotide probe pairs.

We studied the extent of DNA methylation with two approaches [103, 104]. One included a preliminary selection of the microarray oligonucleotide probes that distinguish DNAs with different extents of methylation (0, 50, and 100%). This procedure is necessary because the efficiency of a probe is poorly predictable, depending on the possible formation of secondary structures and nonspecific hybridization. A typical diagram of the methylation index computed for CG dinucleotides of DNA specimens with the known methylation extents is shown in Fig. 2d. It is seen that the experimental estimates agree well with the actual extent of DNA methylation.

Alternatively, it is possible to directly compare the DNA methylation indices for tumor and control DNAs without preliminarily selecting the probes. This qualitative analysis rapidly detects the changes in DNA methylation and time-consuming probe selection is avoided.

The same experimental platform is used in the arrayed primer extension (APEX) with DNA polymerase [105, 106]. In this case, oligonucleotide probes for the microarray are synthesized in the 5' $\longrightarrow$ 3' direction and, consequently, can be extended by one nucleotide after hybridization with the target [107].

Efficient as it is, the technique has some drawbacks to be overcome. The drawbacks are related to the properties of bisulfite-modified DNA. Since nucleotide sequences are greatly simplified, cross-hybridization is far more likely than in tests for single nucleotide polymorphisms, which substantially reduces the scale of analysis. Moreover, the high density of CG dinucleotides in CpG islands requires probes containing two, three, or even more cytosines. Synthesis of oligonucleotides for all possible combinations of methylated and nonmethylated states significantly reduces the number of cytosines accessible for hybridization analysis.

To increase the scale of DNA methylation profiling, it is necessary to use standard commercial platforms with a high oligonucleotide density (Affymetrix, NimbleGen, Agilent, and Illumina) [68]. The principles of large-scale analysis with these and other platforms are briefly considered below.

Restriction Enzyme Analysis on DNA Microarrays

In RLGS, differentially methylated CG dinucleotides are mapped using two-dimensional electrophoresis with subsequent sequencing of the corresponding restriction fragments. Hybridization with

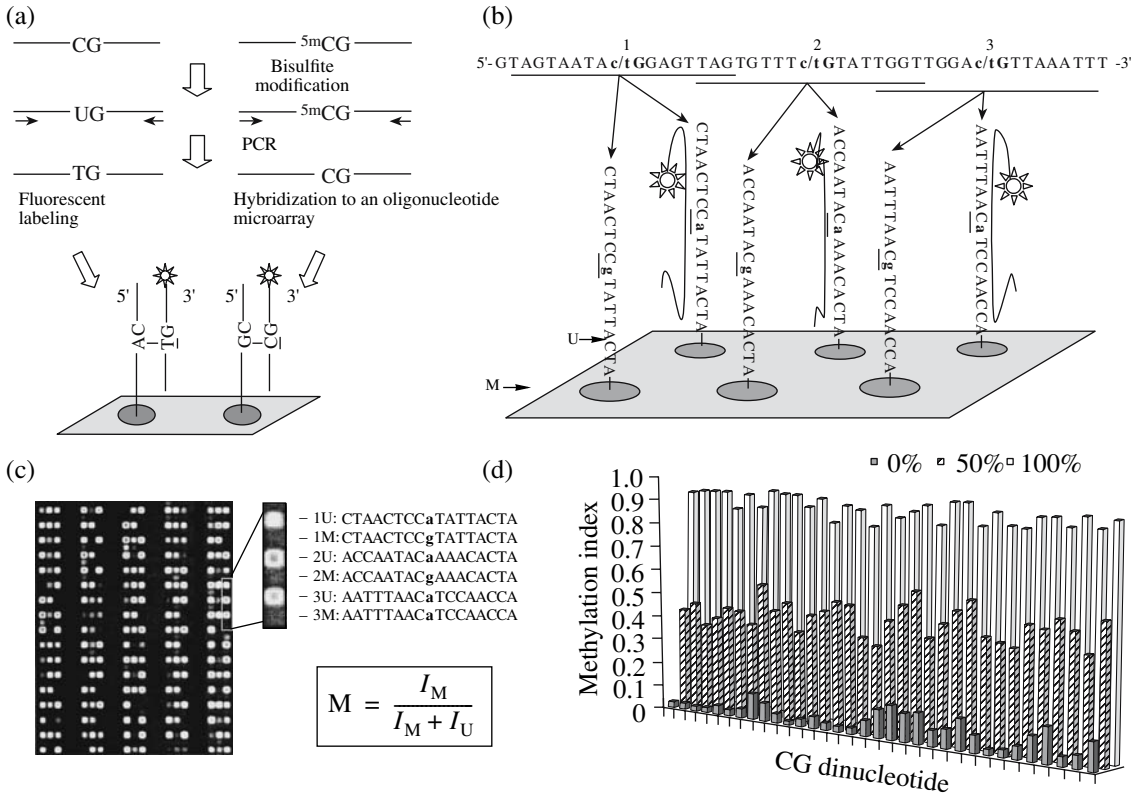

Fig. 2. DNA methylation profiling by hybridization to oligonucleotide microarrays. (a) Principal scheme of the experiment. When genomic DNA is treated with sodium bisulfite, cytosine is deaminated to yield uracil, while methylcytosine does not enter into the reaction. The DNA region of interest is amplified in PCR and the product is biotinylated and hybridized to an oligonucleotide microarray. The methylation status of a CG dinucleotide is determined using a pair of probes. One probe (on the left) is complementary to the nonmethylated target region and the other (on the right) is complementary to the methylated target region. One of the probes specifically binds to the target under optimal conditions of hybridization and washing, allowing discrimination between 5^{m}C and C. (b) Methylation profiling of a DNA fragment. The fragment harbors three CG dinucleotides (shown in bold and numbered at the top of the sequence). After bisulfite modification, cytosine or thymine (small letters) occur in place of C in the fragment. The methylation status of each cytosine is determined using a pair of 17-mer probes immobilized in an oligonucleotide microarray (shown at the bottom). M and U are the probes (see text for detail). Each probe is complementary to the region underlined in the fragment sequence; the target cytosine (underlined) is in the center of the probe. Hybridization of the biotinylated target fragment with the probes is shown. (c) Analysis of the fluorescent signals. An oligonucleotide microarray is hybridized with biotinylated target DNA and incubated with phycoerythrin. The microarray fragment described in Fig. 2b is magnified. It is seen from the fluorescent signals that all three CG dinucleotides are nonmethylated (completely nonmethylated DNA was used as a target). M, methylation index (see text for detail). (d) Discrimination of CG dinucleotides differing in the extent of methylation. The diagram shows the methylation indices computed for DNA with the known extent of methylation (0, 50, or 100%). The PCR products used for hybridization correspond to the promoter regions of *calca, cdh1, dapk1, grm7*, and *cdkn2b* (see text for details).

DNA microarrays is far more efficient in this case. Several techniques have been developed for this purpose, differing in sample preparation and the platform employed (oligonucleotide microarrays, immobilized PCR products, etc.).

BAC arrays, which contain immobilized BAC clones, provide a convenient platform for total genome analysis of hypermethylation of CpG islands, which often occur in BAC nucleotide sequences. Tissue-specific methylation was examined using a microarray of 2413 unique BAC clones, covering the genome with an average interval of about 1 Mb [108]. The control and test DNA samples were digested with *Eco*RV and a methylation-sensitive restriction enzyme (*Not*I or *Bss*HII), the fragments were labeled with the fluorescent dyes Cy3 and Cy5, and comparative hybridization was performed. As a result, differentially methylated loci were observed in DNAs of astrocytes, keratinocytes, and peripheral blood cells.

Comparative restriction enzyme analysis of DNA methylation in two specimens can yield false positive results when a restriction site harbors a polymorphism. This problem is overcome using methylation-sensitive *Hpa*II along with its methylation-insensitive isoschizomer *Msp*I. Comparative hybridization is carried out with *Hpa*II and *Msp*I fragments of one genomic DNA specimen. Before hybridization, the methylated or nonmethylated genome fraction is usu-

Basic techniques of DNA methylation profiling

Technique	Method to discriminate C and 5^mC	Principle	Reference
MSe-PCR	Methylation-sensitive restriction	The methylation status of the CG dinucleotide in the restriction site determines whether DNA is intact after digestion and the fragment containing the given C is amplified	[58]
MSp-PCR	Bisulfite DNA modification	Target CG dinucleotides are in the primer annealing sites. Two different primer pairs allow selective amplification of methylated and nonmethylated DNAs	[59]
COBRA		After bisulfite modification, the restriction site is preserved or eliminated depending on the methylation status of its CG dinucleotide, which is tested by restriction enzyme digestion and subsequent PCR	[60]
Bisulfite sequencing		Bisulfite-modified DNA is sequenced according to Sanger	[56]
Primer extension		Target CG dinucleotide is immediately downstream of the primer annealing site. Primer extension by one nucleotide reports C or T, suggesting 5^mC or C	[57]
Bisulfite pyrosequencing		Bisulfite-modified DNA is pyrosequenced	[70, 71]
MethyLight		MSp-PCR variant with real-time PCR	[69]
RLGS	Methylation-sensitive restriction	Radiolabeled genomic DNA fragments from several specimens are separated by two-dimensional electrophoresis and the intensities of spots on autoradiographs are compared	[83, 84]
NGSCC		An extended genomic DNA locus and the identical cloned fragment are digested with a methylation-sensitive restriction enzyme, ligated with adaptors, hybridized, and PCR-amplified	[87]
APEX	Bisulfite DNA modification	Extension of oligonucleotide probes immobilized on a chip	[104, 105]
Hybridization on oligonucleotide microarrays		DNA is PCR-amplified and hybridized to an oligonucleotide microarray containing a pair of probes for each CG dinucleotide	[95]
	Methylation-sensitive restriction	HELP and MIAMI: comparative hybridization of the *Hpa*II (methylation-sensitive) and *Msp*I (methylation-insensitive) digestion products of one DNA specimen	[108, 109]
	5^mC precipitation	Comparative hybridization of the enriched methylated fractions from several genomic DNA specimens	[110]
Hybridization on BAC microarrays	Methylation-sensitive restriction	Comparative hybridization of several genomic DNA specimens after digestion with a methylation-sensitive restriction enzyme	[107]
MSDK		Genomic 5^mC are mapped by SAGE after digestion with a methylation-sensitive restriction enzyme	[112]

ally enriched via amplification of the restriction fragments with ligated adapters, which substantially reduces the DNA amount necessary for analysis (to less than 500 ng) [49]. For instance, *Hpa*II tiny fragment enrichment by ligation-mediated PCR (HELP) [109] allowed DNA methylation profiling for mouse DNA regions of a total size of 6.2 Mb with a resolution of several hundreds of base pairs. Comparative hybridization of amplified *Hpa*II and *Msp*I fragments of the same genomic DNA specimen was performed using 50-mer oligonucleotide microarrays. The microarray has ten unique oligonucleotide probes for each fragment. In total, 13,000 probes make it possible to identify 1339 amplified restriction fragments. The same idea underlies the microarray-based integrated analysis of methylation by isoschizomers (MIAMI) [110], which was used to examine the promoter region of 8091 genes in cultured lung cancer cells.

Apart from PCR amplification, methylated DNA immunoprecipitation (Me-DIP) with antibodies against 5^mC [111] is used to enrich the methylated genome fraction. Subsequent comparative analysis of the DNA methylation profile in various samples can be carried out with any of the above platforms. A drawback of the method is that a large DNA amount (more than 8 μg) is necessary for analysis [49].

Restriction enzyme analysis has a far lower resolution as compared with bisulfite DNA modification, but its scale is far larger. Thus, oligonucleotide tiling arrays are already available for the *Arabidopsis thaliana* and *Escherichia coli* complete genomes; an array addressing methylation of the human genome is expected for the nearest future.

All microarray-based techniques of DNA methylation profiling have their advantages and drawbacks. The critical parameters of sample preparation for large-scale DNA methylation profiling and a comparison of various platforms have been described in [49]. It is expedient to enrich the nonmethylated genome fraction, because its analysis is far more informative and sensitive than analysis of the methylated fraction. The results of restriction enzyme analysis with microarrays are highly reproducible ($R^2 = 0.997$).

The choice of a particular platform depends on the objective of a study. For instance, when the objective is to monitor the changes in the total methylome rather than to examine an individual locus (even an extended one), the optimal combination of a scale and a resolution is achieved by hybridization to genome microarrays with methylation-sensitive restriction or Me-DIP.

Microarrays Only?

Large-scale methylation profiling of genomic DNA is now associated mostly with DNA microarray technology. New technological platforms and experimental approaches are developed with enthusiasm because one experiment can yield ample information, unconceivable before. Yet this technology has its limitations.

As methods of DNA sequencing are improved, the line-up may change. Based on Sanger sequencing, methylation profiling is now performed by methylation-specific digital karyotyping (MSDK), in which 5^mC is mapped via serial analysis of gene expression (SAGE) [112] after genomic DNA digestion with methylation-sensitive restriction endonuclease [113]. This method of global analysis has revealed substantial changes in DNA methylation pattern not only in breast cancer cells but also in stromal and myoepithelial cells, testifying again that the microenvironment of a tumor is important for its progression.

Further development of bisulfite pyrosequencing may allow a breakthrough in large-scale methylation profiling. A new high-throughput instrument for DNA pyrosequencing has already been designed (454 experimental sequencing machine, Roche) [114]. Several millions of amplified genomic DNA fragments are sequenced in parallel owing to their distribution through picowells of a fiber optic chip. Real-time sequencing proceeds at a rate of 25 Mb per 4-h run. For instance, the *Mycoplasma genitalium* genome (580,069 bp) has been sequenced de novo in one run with an accuracy of 99.96%. A similar technology designed to sequence bisulfite-modified DNA with the same or a higher throughput can be expected to allow large-scale analysis at a 1-bp resolution. To achieve this, it is necessary to eliminate some problems, for instance, those related to the simplification of nucleotide sequences after bisulfite modification or the presence of numerous repeats in DNA.

Alternative technologies are sought to allow high-throughput DNA sequencing. We think that the greatest promise is held by nanopore DNA sequencing, where single-stranded DNA is drawn through a nanopore [115, 116]. As a nucleotide passes through the nanopore, the instrument records a physical parameter, specific to each of the four bases. A parameter allowing a 1-nt resolution is the distribution of the current that arises in nanoelectrodes surrounding the nanopore. A mathematical model has been constructed on the basis of the behavior of a single-stranded molecule in solution and has predicted the possibility of high-rate accurate sequencing [116]. The throughput of one nanopore is theoretically sufficient for sequencing the human or a comparable genome in 7 h.

When this idea is realized, it will be possible for the first time to obviate synthesis of the complementary strand in DNA sequencing, which will reduce the cost of sequencing by several orders of magnitude. With such a technique, the DNA methylation pattern may be established via direct discrimination between C and 5^mC without bisulfite modification.

CONCLUSIONS

DNA methylation profiling techniques developed from the detection of individual 5^mC to genome-wide quantitative analysis in recent years. The intense search for new platforms is determined by the immense importance of DNA methylation for gene expression regulation, carcinogenesis, and some hereditary disorders. Notwithstanding the great progress in the field, the understanding of the role of DNA methylation is still comparable with the tip of the iceberg. The best-studied issue is the role of hypermethylation of promoter CpG islands in transcriptional gene silencing associated with carcinogenesis. The answers to many other questions remain under water. The main questions are why and how the methylome is substantially changed in pathology. It is unclear why CpG islands are normally nonmethylated in most cases and what factors cause their hypermethylation in tumors. Why are tumor suppressor genes subject to methylation? How significant is the position of the CpG island relative to the coding gene region? What is the role of DNA hypomethylation?

To answer these and many other questions, it is necessary to obtain far more ample experimental data

and, consequently, to develop new techniques, improving the scale, resolution, and throughput of analysis. It should be noted that DNA methylation is only a part of the epigenetic information of the cell and is associated with the chromatin state [117–120]. Hence, large-scale methylation profiling must be accompanied by studies of chromatin modification. A collation of epigenetic data with the results of large-scale gene expression profiling and proteome analysis in tumors will provide further insight into the fine mechanisms of carcinogenesis, which is of immense importance for diagnosis and the targeted therapy of cancer.

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REFERENCES

1. Weinberg R.A. 2007. *The Biology of Cancer*. N.Y.: Garland Science.
2. Vanyushin B.F., Tkacheva S.G., Belozersky A.N. 1970. Rare bases in animal DNA. *Nature*. **225**, 948–949.
3. Hotchkiss R.D. 1948. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J. Biol. Chem*. **168**, 315–332.
4. Vanyushin B.F., Mazin A.L., Vasiliev V.K., Belozersky A.N. 1973. The content of 5-methylcytosine in animal DNA: The species and tissue specificity. *Biochim. Biophys. Acta*. **299**, 397–403.
5. Burthseva N.N., Azizov Yu.M., Itkin B.Z., Vanyushin B.F. 1977. Changes in cattle lymphocyte DNA methylation in chronic lympholeukosis. *Biokhimiya*. **42**, 1690–1696.
6. Wigler M., Levy D., Perucho M. 1981. The somatic replication of DNA methylation. *Cell*. **24**, 33–40.
7. Stein R., Gruenbaum Y., Pollack Y., Razin A., Cedar H. 1982. Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc. Natl. Acad. Sci. USA*. **79**, 61–65.
8. Bestor T.H., Laudano A.P., Mattaliano R., Ingram V.M. 1988. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells: The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol*. **203**, 971–983.
9. Baylin S.B., Hoppener J.W., de Bustros A., Steenbergh P.H., Lips C.J., Nelkin B.D. 1986. DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res*. **46**, 2917–2922.
10. Greger V., Passarge E., Hopping W., Messmer E., Horsthemke B. 1989. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet*. **83**, 155–158.
11. Sakai T., Toguchida J., Ohtani N., Yandell D.W., Rapaport J.M., Dryja T.P. 1991. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am. J. Hum. Genet*. **48**, 880–888.
12. Russo V.E.A., Martienssen R.A., Riggs A.D. 1996. *Epigenetic Mechanisms of Gene Regulation*. N.Y.: Cold Spring Harbor Lab. Press.
13. Moskalyov E.A. 2004. *Proc. Conf. "Philosophical Problems in Biology and Medicine,"* Voronezh, pp. 21–23.
14. Okano M., Bell D., Haber D., Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*. **99**, 247–257.
15. Baylin S.B., Ohm J.E. 2006. Epigenetic gene silencing in cancer: A mechanism for early oncogenic pathway addiction? *Nature Rev*. **6**, 107–116.
16. Knudson A.G. 2001. Two genetic hits (more or less) to cancer. *Nature Rev. Cancer*. **1**, 157–162.
17. Liu T., Raval A., Chen S.-S., Matkovic J.J., Byrd J.C., Plass C. 2006. CpG island methylation and expression of the secreted Frizzled-related protein gene family in chronic lymphocytic leukemia. *Cancer Res*. **66**, 653–658.
18. Calvisi D.F., Ladu S., Gorden A., Farina M., Corner E.A., Lee J.-S., Factor V.M., Thorgeirsson S.S. 2006. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. *Gastroenterology*. **130**, 1117–1128.
19. Aguilera O., Fraga M.F., Ballestar E., Paz M.F., Herranz M., Espada J., Garcia J.M., Munoz A., Esteller M., Gonzalez-Sancho J.M. 2006. Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene*. **25**, 4116–4121.
20. Chim C., Fung T., Cheung W., Liang R., Kwong Y. 2004. *SOCS1* and *SHP1* hypermethylation in multiple myeloma: Implications for epigenetic activation of the Jak/STAT pathway. *Blood*. **103**, 4630–4635.
21. He B., You L., Uematsu K., Zang K., Xu Z., Lee A.Y., Costello J.F., McCormick F., Jablons D.M. 2003. *SOCS-3* is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. *Proc. Natl. Acad. Sci. USA*. **100**, 14,133–14,138.
22. Esteller M., Sparks A., Toyota M., Sanchez-Cespedes M., Capella G., Peinado M.A., Gonzalez S., Tarafa G., Sidransky D., Meltzer S.J., Baylin S.B., Herman J.G. 2000. Analysis of *adenomatous polyposis coli* promoter hypermethylation in human cancer. *Cancer Res*. **60**, 4366–4371.
23. Mertens D., Wolf S., Tschuch C., Mund C., Kienle D., Ohl S., Schroeter P., Lyko F., Dohner, Stilgenbauer S., Lichter P. 2006. Allelic silencing at the tumor-suppressor locus 13q14.3 suggests an epigenetic tumor-suppressor mechanism. *Proc. Natl. Acad. Sci. USA*. **103**, 7741–7746.
24. Cui H., Horon I.L., Ohlsson R., Hamilton S.R., Feinberg A.P. 1998. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nature Med*. **4**, 1276–1280.
25. Feinberg A.P. 2004. The epigenetics of cancer etiology. *Semin. Cancer Biol*. **14**, 427–432.
26. Holst C.R., Nuovo G.J., Esteller M., Chew K., Baylin S.B., Herman J.G, Tlsty T.D. 2003. Methylation of p16(INK4a) promoters occurs in vivo in histologically

normal human mammary epithelia. *Cancer Res*. **63**, 1596–1601.

27. Pepe M.S., Etzioni R., Feng Z., Potter J.D., Thompson M.L., Thornquist M., Winget M., Yasui Y. 2001. Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst*. **93**, 1054–1061.
28. Laird P.W. 2003. Early detection: The power and the promise of DNA methylation markers. *Nature Rev. Cancer*. **3**, 253–266.
29. Crawford Y.G., Gauthier M.L., Joubel A., Mantei K., Kozakiewicz K., Afshari C.A., Tlsty T.D. 2004. Histologically normal human mammary epithelia with silenced *p16* (*INK4a*) overexpress *COX-2*, promoting a premalignant program. *Cancer Cell*. **5**, 263–273.
30. Yoo C., Jones P.A. 2006. Epigenetic therapy of cancer: Past, present, and future. *Nat. Rev. Drug Discov*. **5**, 37–50.
31. Vanyushin B.F. 2005. Enzymatic DNA methylation: Epigenetic control over genetic functions of the cell. *Biokhimiya*. **70**, 598–611.
32. International Human Genome Sequencing Consortium. 2001. Initial sequencing and analysis of the human genome. *Nature*. **409**, 860–921.
33. Weber M., Hellmann I., Stadler M.B., Ramos L., Paabo S., Rebhan M., Schubeler D. 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature Genet*. **39**, 457–466.
34. Feinberg A.P., Vogelstein B. 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*. **301**, 89–92.
35. Babenko O.V., Zemlyakova V.V., Saakyan S.V., Brovkina A.F., Strelnikov V.V., Nemtsova M.V., Zaletaev B.V. 2002. *RB1* and *CDKN2A* functional defects resulting in retinoblastoma. *Mol. Biol*. **36**, 777–783.
36. Zemlyakova V.V., Zhevlova, A.I., Strelnikov, V.V., Lyubchenko L.N., Vishnevskaya Ya.B., Tretyakova V.A., Zaletaev D.V., Nemtsova M.V. 2003. Abnormal methylation of several tumor suppressor genes in sporadic breast cancer. *Mol. Biol.* **37**, 696–703.
37. Ivanova T., Petrenko A., Gritsko T., Vinokourova S., Eshilev E., Kobzeva V., Kisseljov F., Kisseljova N. 2002. Methylation and silencing of the retinoic acid receptor-β 2 gene in cervical cancer. *BMC Cancer*. **2**, 4.
38. Ivanova T., Vinokurova S., Petrenko A., Eshilev E., Solovyova N., Kisseljov F., Kisseljova N. 2004. Frequent hypermethylation of 5' flanking region of *TIMP-2* gene in cervical cancer. *Int. J. Cancer*. **108**, 882–886.
39. Loginov V.I., Malyukova A.V., Seryogin Yu.A., Hodyrev D.S, Kazubskaya T.P., Ermilova V.D., Garkavtseva R.F., Kisselev L.L, Zabarovsky E.R., Braga E.A. 2004. Methylation level of the *RASSF1A* gene in epithelial tumors of the kidney, mammary gland, and ovaries. *Mol. Biol*. **38**, 654–667
40. Zemlyakova V.V., Strelnikov V.V., Zborovskaya I.B., Balukova O.V., Maiorova E.V., Vasil'ev E.V., Zaletaev D.V., Nemtsova M.V. 2004. Comparison of aberrant methylation of CpG islands in the *p16*/*CDKN2A* and *p14*/*ARF* promoters in non-small cell lung cancer and acute lymphoblastic leukemia, *Mol. Biol*. **38**, 966–972.
41. Malyukova A.V., Loginov V.I., Hodyrev D.S., Kadyrova E.P., Pronina I.V., Ivanova T.A., Kisseljov, F.L., Zabarovsky E.R., Kisselyova N.P., Braga E.A. 2004. Methylation of the putative suppressor gene *RASSF1* in primary cervical tumors. *Mol. Biol*. **38**, 1005–1013.
42. Kekeeva T., Zhevlova A., Podistov Yu., Solov'eva Yu.V., Zaletaev D.V., Nemtsova M.V. 2006. Abnormal methylation of tumor suppressor genes and microsatellite instability in cervical precancer. *Mol. Biol*. **40**, 224–230.
43. Plumb J.A., Steele N., Finn P.W., Brown R. 2004. Epigenetic approaches to cancer therapy. *Biochem. Soc. Trans*. **32**, 1095–1097.
44. Fang M.Z., Wang Y., Ai N., Hou Z., Sun Y., Lu H., Welsh W., Yang C.S. 2003. Tea polyphenol (–)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res*. **63**, 7563–7570.
45. Issa J.-Pj., Gharibyan V., Cortes J., Jelinek J., Morris G., Verstovsek S., Talpaz M., Garcia-Manero G., Kantarjian H.M. 2005. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. *J. Clin. Oncol*. **23**, 3948–3956.
46. Eden A., Gaudet F., Waghmare A., Jaenisch R. 2003. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science*. **300**, 455.
47. Suter C.M., Martin D.I., Ward R.L. 2004. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *Int. J. Colorect. Dis*. **19**, 95–101.
48. Kisseljova N.P., Kisseljov F.L. 2005. DNA demethylation and carcinogenesis. *Biokhimiya*. **70**, 900–911.
49. Schumacher A., Kapranov P., Kaminsky Z. 2006. Microarray-based DNA methylation profiling: Technology and applications. *Nucleic Acids Res*. **34**, 528–542.
50. Kawakami K., Brabender J., Lord R.V., Groshen S., Greenwald B.D., Krasna M.J., Yin J., Fleisher A.S., Abraham J.M., Beer D.G., Sidransky D., Huss H.T., Demeester T.R., Eads C., Laird P.W., Ilson D.H., Kelsen D.P., Harpole D., Moore M.B., Danenberg K.D., Danenberg P.V., Meltzer S.J. 2000. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. *J. Natl. Cancer Inst*. **92**, 1805–1811.
51. Zaletaev D., Nemtsova M., Strelnikov V., Babenko O.V., Vasil'ev E.V., Zemlyakova V.V., Zhevlova A.I., Drozd O.V. 2004. Diagnostics of epigenetic alterations in hereditary and oncological disorders. *Mol. Biol*. **38**, 213–223.
52. Cui H., Cruz-Correa M., Giardiello F.M., Hutcheon D.F., Kafonek D.R., Brandenburg S., Wu Y., He X., Powe N.R., Feinberg A.P. 2003. Loss of IGF2 imprinting: A potential marker of colorectal cancer risk. *Science*. **299**, 1753–1755.
53. Hoheisel J.D. 2006. Microarray technology: Beyond transcript profiling and genotype analysis. *Nature Rev. Genet*. **7**, 200–210.
54. Bird A.P., Southern E.M. 1978. Use of restriction enzymes to study eukaryotic DNA methylation: 1. The methylation pattern in ribosomal DNA from *Xenopus laevis*. *J. Mol. Biol*. **118**, 27–47.

55. Waalwijk C., Flavell R.A. 1978. DNA methylation at a CCGG sequence in the large intron of the rabbit β-globin gene: Tissue-specific variations. *Nucleic Acids Res*. **5**, 4631–4634.

56. Frommer M., McDonald L., Millar D., Collis C., Watt F., Grigg G.M., Molloy P.L., Paul C.L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA*. **89**, 1827–1831.

57. Gonzalgo M.L., Jones P.A. 2002. Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). *Methods*. **27**, 128–133.

58. Singer-Sam J., LeBon J.M., Tanguay R.L., Riggs A.D. 1980. A quantitative *Hpa*II-PCR assay to measure methylation of DNA from a small number of cells. *Nucleic Acids Res*. **18**, 687.

59. Herman J.G., Graff J.R., Myohanen S., Nelkin B.D., Baylin S.B. 1996. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*. **93**, 9821–9826.

60. Xiong Zh., Laird P.W. 1997. COBRA: A sensitive and quantitative DNA methylation assay. *Nucleic Acids Res*. **25**, 2532–2534.

61. Dupont J.M., Tost J., Jammes H., Gut I.G. 2004. De novo quantitative bisulfite sequencing using the Pyrosequencing technology. *Anal. Biochem*. **333**, 119–127.

62. Sanger F., Nicklein S., Coulson A.R. 1977. DNA sequencing with chain-terminator inhibitors. *Proc. Natl. Acad. Sci. USA*. **74**, 5463–5467.

63. Warnecke P., Stirzaker C., Melki J., Millar D., Paul C., Clark S. 1997. Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucleic Acids Res*. **25**, 4422–4426.

64. Li L.C., Dahiya R. 2002. MethPrimer: Designing primers for methylation PCRs. *Bioinformatics*. **18**, 1427–1431.

65. Warnecke P.M., Stirzaker C., Song J., Grunau C., Melki J.R., Clark S.J. 2002. Identification and resolution of artifacts in bisulfite sequencing. *Methods*. **27**, 101–107.

66. Melki J.R., Vincent P.C., Brown R.D., Clark S.J. 2000. Hypermethylation of E-cadherin in leukemia. *Blood*. **95**, 3208–3213.

67. Lewin J., Schmitt A.O., Adorjan P., Hildmann T., Piepenbrock C. 2004. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. *Bioinformatics*. **20**, 3005–3012.

68. Callinan P.A., Feinberg A.P. 2006. The emerging science of epigenomics. *Hum. Mol. Genet*. **15**, R95–R101.

69. Trinh B.N., Long T.I., Laird P.W. 2001. DNA methylation analysis by Methylight technology. *Methods*. **25**, 456–462.

70. Ronaghi M., Uhlen M., Nyren P. 1998. A sequencing method based on real-time pyrophosphate. *Science*. **281**, 363–365.

71. Tost J., Dunker J., Gut I.G. 2003. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *BioTechniques*. **35**, 152–156.

72. Tost J., Schatz P., Schuster M., Berlin K., Gut I.G. 2003. Analysis and accurate quantification of CpG methylation by MALDI mass spectrometry. *Nucleic Acids Res*. **31**, e50.

73. Schatz P., Distler J., Berlin K., Schuster M. 2006. Novel method for high throughput DNA methylation marker evaluation using PNA–probe library hybridization and MALDI-TOF detection. *Nucleic Acids Res*. **34**, e59.

74. Corcoran M., Parker A., Orchard J., Davis Z., Wirtz M., Schmitz O.J., Oscier D. 2005. *ZAP-70* methylation status is associated with *ZAP-70* expression status in chronic lymphocytic leukemia. *Haematologica*. **90**, 1078–1088.

75. Crespo M., Bosch F., Villamor N., Bellosillo B., Colomer D., Rozman M., Marce S., Lopez-Guillermo A., Campo E., Montserrat E. 2003. *ZAP-70* expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N. Engl. J. Med*. **348**, 1764–1775.

76. Adorján P., Distler J., Lipscher E., Model F., Müller J., Pelet C., Braun A., Florl A.R., Gutig D., Grabs G., Howe A., Kursar M., Lesche R., Leu E., Lewin A., Maier S., Müller V., Otto T., Scholz C., Schulz W.A., Seifert H.-H., Schwope I., Ziebarth H., Berlin K., Piepenbrock C., Olek A. 2002. Tumor class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Res*. **30**, e21.

77. Guo J., Burger M., Nimmrich I., Maier S., Becker E., Genc B., Duff D., Rahmatpanah F., Chitma-Matsiga R., Shi H., Berlin K., Huang T.H.-M., Caldwell C.W. 2005. Differential DNA methylation of gene promoters in small B-cell lymphomas. *Am. J. Clin. Pathol*. **124**, 430–439.

78. Zou H.Z., Yu B.M., Wang Z.W., Sun J.Y., Cang H., Gao F., Li D.H., Zhao R., Feng G.G., Yi J. 2002. Detection of aberrant p16 methylation in the serum of colorectal cancer patients. *Clin. Cancer Res*. **8**, 188–191.

79. Grady W.M., Rajput A., Lutterbaugh J.D., Markowitz S.D. 2001. Detection of aberrantly methylated *hMLH1* promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res*. **61**, 900–902.

80. Weisenberger D.J., Campan M., Long T.I., Kim M., Woods C., Fiala E., Ehrlich M., Laird P.W. 2005. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res*. **33**, 6823–6836.

81. Yang A.S., Estecio M.R., Doshi K., Kondo Y., Tajara E.H., Issa J.-Pj. 2004. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res*. **32**, e38.

82. Kitahara O., Furukawa Y., Tanaka T., Kihara C., Ono K., Yanagawa R., Nita M.E., Takagi T., Nakamura Y., Tsunoda T. 2001. Alterations of gene expression during colorectal carcinogenesis revealead by c DNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res*. **61**, 3544–3549.

83. Esteller M., Sparks A., Toyota M., Sanchez-Cespedes M., Capella G., Peinado M.A., Gonzalez S., Tarafa G., Sidransky D., Meltzer S.J., Baylin S.B., Herman J.G. 2000. Analysis of *adenomatous polyposis coli* promoter hypermethylation in human cancer. *Cancer Res*. **60**, 4366–4371.

84. Costello J.F., Fruhwald M.C., Smiraglia D.J., Rush L.J., Robertson G.P., Gao X., Wright F.A., Feramisco J.D., Peltomaki P., Lang J.C., Schuller D.E., Yu L., Bloomfield C.D., Caligiuri M.A., Yates A., Nishikawa R., Su Huang H., Petrelli N.J., Zhang X., O'Dorisio M.S., Held W.A., Cavenee W.K., Plass C. 2000. Aberrant CpG-island methylation has non-random and tumor-type-specific patterns. *Nature Genet*. **24**, 132–138.

85. Costello J.F., Smiraglia D.J., Plass C. 2002. Restriction landmark genome scanning. *Methods*. **27**, 144–149.

86. Rush L., Raval A., Funchain P., Johnson A., Smith L. 2004. Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. *Cancer Res*. **64**, 2424–2433.

87. Raval A., Lucas D.M., Matkovic J.J., Bennett K.L., Liyanarachchi S., Young D.C., Rassenti L., Kipps T.J., Grever M.R., Byrd J.C., Plass C. 2005. TWIST2 demonstrates differential methylation in immunoglobulin variable heavy chain mutated and unmutated chronic lymphocytic leukemia. *J. Clin. Oncol*. **23**, 3877–3885.

88. Azhikina T., Gainetdinov I., Skvortsova Y., Batrak A., Dmitrieva N., Sverdlov E. 2004. Non-methylated Genomic Sites Coincidence Cloning (NGSCC): An approach to large scale analysis of hypomethylated CpG patterns at predetermined genomic loci. *Mol. Genet. Genomics*. **271**, 22–32.

89. Azhikina T.L., Sverdlov E.D. 2005. Study of tissue-specific CpG-methylation of DNA in extended genomic loci. *Biokhimiya*. **70**, 722-730.

90. Barsky V., Kolchinsky A., Lysov Yu., Mirzabekov A. 2002. Biological microchips with hydrogel-immobilized nucleic acids, proteins, and other compounds: Properties and applications in genomics. *Mol. Biol*. **36**, 563-584.

91. Syagailo Y.V., Kusnezow W., Hoheisel J.D. 2006. Microarray-based expression profiling: From technological basics to diagnostic perspectives. In: *Apoptosis and Cancer Therapy.* Eds. Debatin K.M., Fulda S. Weinheim: Wiley-VHC, pp. 60–85.

92. Matsuzaki H., Loi H., Dong S., Tsai Ya-Yu, Fang J., Law J., Di X., Liu W.-M., Yang G., Liu G., Huang J., Kennedy G.C., Ryder T.B., Marcus G.A., Walsh P.S., Shriver M.D., Puck J.M., Jones K.W., Mei R. 2004. Parallel genotyping of over 10,000 SNPs using a one-primer assay on a high-density oligonucleotide array. *Genome Res*. **14**, 414–425.

93. Shi H., Maier S., Nimmrich I., Yan P.S., Caldwell C.W., Olek A., Huang T.H.-M. 2003. Oligonucleotide-based microarray for DNA methylation analysis: Principles and applications. *J. Cell. Biochem*. **88**, 138–143.

94. Gitan R.S., Shi H., Chen C.-M., Yan P.S., Huang T.H.-M. 2002. Methylation-specific oligonucleotide microarray: A new potential for high-throughput methylation analysis. *Genome Res*. **12**, 158–164.

95. Hou P., Ji M., Li S., He N., Lu Zh. 2004. High-throughput method for detecting DNA methylation. *J. Biochem. Biophys. Meth*. **60**, 139–150.

96. Mund C., Beier V., Bewerunge P., Dahms M., Lyko F., Hoheisel J.D. 2005. Array-based analysis of genomic DNA methylation patterns of the tumor suppressor gene *p16*INK4A promoter in colon carcinoma cell lines. *Nucleic Acids Res*. **33**, e73.

97. Balog R.P., de Souza Y.P., Tang H.M., DeMasellis G.M., Gao B., Avila A., Gaban D.J., Mittelman D., Minna J.D., Luebke K.J., Garner H.R. 2002. Parallel assessment of CpG methylation by two-color hybridization with oligonucleotide arrays. *Anal. Biochem*. **309**, 301–310.

98. Barker D.L., Theriault G., Che D., Dickinson T., Shen R., Kain R. 2003. Self-assembled random arrays: High-performance imaging and genomics applications on a high-density microarray platform. *Soc. Photo-Optical Instrumentation Engineers*. **4966**, 1–11.

99. Bibikova M., Lin Zh., Zhou L., Chudin E., Garcia E.W., Wu B., Doucet D., Thomas N.J., Wang Y., Vollmer E., Goldmann T., Seifart C., Jiang W., Barker D.L., Chee M.S., Floros J., Fan J.-B. 2006. High-throughput DNA methylation profiling using universal bead arrays. *Genome Res*. **16**, 383–393.

100. Baum M., Bielau S., Rittner N., Schmid K., Eggelbusch K., Dahms M., Schlauersbach A., Tahedl H., Beier M., Güimil R., Scheffler M., Hermann C., Fun J.-M., Wixmerten A., Rebscher H., Hönig M., Andreae C., Büchner D., Moschel E., Glathe A., Jäger E., Thom M., Greil A., Bestvater F., Obermeier F., Burgmaier J., Thome K., Weichert S., Hein S., Binnewies T., Foitzik V., Müller M., Stähler C.F., Stähler P.F. 2003. Validation of a novel, fully integrated and fexible microarray benchtop facility for gene expression profiling. *Nucleic Acids Res*. **31**, e151.

101. Beier V., Hoheisel J. 2004. Charakterisierung von DNA-Methylierungsmustern mit Hilfe von Oligonukleotid-Microarrays. *Bioforum*. **5**, 31–33.

102. Beier V., Mund C., Hoheisel J.D. 2007. Monitoring methylation changes in cancer. In: *Advances in Biochemical Engineering/Biotechnology: Analytics of Protein-DNA Interactions*. Ed. Seitz H. Heidelberg: Springer Verlag.

103. Moskalyov E.A., Beier V., Eprintsev A.T., Hoheisel J.D. 2006. *Statusseminar Chiptechnologien: Entwicklung, Forschung und Routine. Abstract Book*. Frankfurt am Main, p. 59.

104. Moskalyov A.A., Beier V., Hoheisel J.D., Eprintsev A.T. 2006. Biology: Science of the 21st century. *Abstr. 10th Pushchino Young Scientists Conf.* Pushchino, p. 32.

105. Kurg A., Tonisson N., Tolett J., Georgiou I., Shumaker L., Metspalu A. 2000. Arrayed primer extension: Solid phase four-color DNA resequencing and mutation detection technology. *Genetic Testing*. **4**, 1–7.

106. Tonisson N., Zernant J., Kurg A., Pavel H., Slavin G., Roomere H., Meiel A., Hainaut P., Metspalu A. 2002. Evaluating the arrayed primer extension resequencing assay of *TP53* tumor suppressor gene. *Proc. Natl. Acad. Sci. USA*. **99**, 5503–5508.

107. Beier M., Hoheisel J.D. 2002. Analysis of DNA-microarrays produced by inverse in situ oligonucleotide synthesis. *J. Biotechnol*. **94**, 15–22.

108. Ching T.-T., Maunakea A.K., Jun P., Hong C., Zardo G., Pinkel D., Albertson D.G., Fridlyand J., Mao J.-H., Shchors K., Weiss W.A., Costello J.F. 2005. Epigenome analysis using BAC microarrays identify evolutionary conservation of tissue-specific methylation of *SHANK3*. *Nature Genet*. **37**, 645–651.

109. Khulan B.,Thompson R.F.,Ye K., Fazzari M.J., Suzuki M., Stasiek E., Figueroa M.E., Glass J.L., Chen Q., Montagna C., Hatchwell E., Selzer R.R., Richmond T.A., Green R.D., Melnick A., Greally1 J.M. 2006. Comparative isoschizomer profiling of cytosine methylation: The HELP assay. *Genome Res*. **16**, 1046–1055.

110. Hatada I., Fukasawa M., Kimura M., Morita S., Yamada K., Yoshikawa T., Yamanaka S., Endo C., Sakurada A., Sato M., Kondo T., Horii A., Ushijima T., Sasaki H. 2006. Genome-wide profiling of promoter methylation in human. *Oncogene*. **25**, 3059–3064.

111. Wilson I.M., Davies J.J., Weber M., Brown C.J., Alvarez C.E., MacAulay C., Schubeler D., Lam W.L. 2006. Epigenomics: Mapping the methylome. *Cell Cycle*. **5**, 155–158.

112. Velculescu V.E., Zhang L., Vogelstein B., Kinzler K.W. 1995. Serial analysis of gene expression. *Science*. **270**, 484–487.

113. Hu M., Yao J., Cai L., Bachman K.E., van den Brule F., Velculescu V., Polyak K. 2005. Distinct epigenetic changes in the stromal cells of breast cancers. *Nature Genet*. **37**, 899–905.

114. Margulies M., Egholm M., Altman W.E., Attiya S., Bader J.S., Bemben L.A., Berka J., Braverman M.S., Chen Y.-J., Chen Z., Dewell S.B., Du L., Fierro J.M., Gomes X.V., Godwin B.C., He W., Helgesen S., Ho C.H., Irzyk G.P., Jando S.C., Alenquer M.L.I., Jarvie T.P., Jirage K.B., Kim J.-B., Knight J.R., Lanza J.R., Leamon J.H., Lefkowitz S.M., Lei M., Li J., Lohman K.L., Lu H., Makhijani V.B., McDade K.E., McKenna M.P., Myers E.W., Nickerson E., Nobile J.R., Plant R., Puc B.P., Ronan M.T., Roth G.T., Sarkis G.J., Simons J.F., Simpson J.W., Srinivasan M., Tartaro K.R., Tomasz A., Vogt K.A., Volkmer G.A., Wang S.H., Wang Y., Weiner M.P., Yu P., Begley R.F., Rothberg J.M. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. **437**, 376–380.

115. Braslavsky I., Hebert B., Kartalov E., Quake S.R. 2003. Sequence information can be obtained from single DNA molecules. *Proc. Natl. Acad. Sci. USA*. **100**, 3960.

116. Lagerqvist J., Zwolak M., Di Ventra M. 2006. Fast DNA sequencing via transverse electronic transport. *Nano Lett*. **6**, 779–782.

117. Vire E., Brenner C., Deplus R., Blanchon L., Fraga M., Didelot C., Morey L., van Eynde A., Bernard D., Vanderwinden J.M., Bollen M., Esteller M., Di Croce L., de Launoit Y., Fuks F. 2006. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*. **439**, 871–874.

118. Schlesinger Y., Straussman R., Keshet I., Farkash S., Hecht M., Zimmerman J., Eden E., Yakhini Z., Ben-Shushan E., Reubinoff B.E., Bergman Y., Simon I., Cedar H. 2007. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nature Genet*. **39**, 232–236.

119. Ohm J.E., McGarvey K.M., Yu X., Cheng L., Schuebel K.E., Cope L., Mohammad H.P., Chen W., Daniel V.C., Yu W., Berman D.M., Jenuwein T., Pruitt K., Sharkis S.J., Watkins D.N., Herman J.G., Baylin S.B. 2007. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nature Genet*. **39**, 237–242.

120. Ohm J.E., Baylin S.B. 2007. Stem cell chromatin patterns: An instructive mechanism for DNA hypermethylation? *Cell Cycle*. **6**, 1040–1043.