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Genome-wide epigenetic modifications in cancer

Yoon Jung Park, Rainer Claus, Dieter Weichenhan, and Christoph Plass

German Cancer Research Center, Division of Epigenomics and Cancer Risk Factors, Heidelberg, Germany

Abstract

Epigenetic alterations in cancer include changes in DNA methylation and associated histone modifications that influence the chromatin states and impact gene expression patterns. Due to recent technological advantages, the scientific community is now obtaining a better picture of the genome-wide epigenetic changes that occurs in a cancer genome. These epigenetic alterations are associated with chromosomal instability and changes in transcriptional control which influence the overall gene expression differences seen in many human malignancies. In this review, we will briefly summarize our current knowledge of the epigenetic patterns and mechanisms of gene regulation in healthy tissues and relate this to what is known for cancer genomes. Our focus will be on DNA methylation. We will review the current standing of technologies that have been developed over recent years. This field is experiencing a revolution in the strategies used to measure epigenetic alterations, which includes the incorporation of next generation sequencing tools. We also will review strategies that utilize epigenetic information for translational purposes, with a special emphasis on the potential use of DNA methylation marks for early disease detection and prognosis. The review will close with an outlook on challenges that this field is facing.

1. Epigenetic modifications in healthy tissue

1.1. Epigenetic patterns in the human genome

The development of an organism relies on gene expression patterns that are regulated in a spatial and time-dependent manner. This is accomplished, in part, by altering the DNA and associated proteins without changing the DNA sequence; these alterations are collectively termed ‘epigenetic modifications’. Heritable information is carried by chemical modifications of both DNA and chromatin-associated proteins within the genomes of eukaryotes. Epigenetic modifications have profound influences on gene expression by modulating chromatin structure and DNA accessibility. Together, these epigenetic modifications on a genome-wide scale are referred to as the ‘epigenome’. Examination of the epigenome suggests that epigenetic phenomena contribute to global, cell-specific patterns of gene expression. The epigenome is highly dynamic depending on the tissue type and developmental stage within an organism. The biological and biomedical significance of epigenetic modifications becomes particularly evident in mouse models where alterations of genes responsible for the addition or removal of epigenetic modifications lead to embryonic lethality (for examples, (1–4)).

Epigenetic modifications mainly fall into two categories, DNA methylation and histone modifications. In mammals, DNA can be modified by addition of a methyl group to cytosine residues, typically in a CpG context. This is mediated by DNA methyltransferases, such as the *de novo* DNA methyltransferases DNMT3a and DNMT3b, and/or DNMT1, which

reestablishes DNA methylation at newly synthesized sequences after replication (reviewed in (5)). Conversely, it is not fully understood how DNA methylation is removed from the mammalian genome, although several proteins including DNA methyl binding domain-containing proteins, nucleotide excision repair factors, and DNA methyltransferases have been suggested to be part of the demethylation process (reviewed in (6)). Intriguingly, hydroxymethylcytosine was recently reported as an additional modification to cytosine residues in mouse brain and ES cells (7,8). Data indicated that it was generated from 5-methylcytosine by TET1 activity, which may be a key step for the demethylation process of 5-methylcytosine. Unlike plants or invertebrates, most of which have mosaic methylation patterns, mammalian genomes have “ubiquitous” DNA methylation patterns (reviewed in (9)). CpG dinucleotides are predominantly methylated, except for those in CpG islands (CGIs). CGIs are defined as GC-rich regions that retain a higher than expected frequency of CpG dinucleotides. They frequently co-localize with promoter regions of genes (reviewed in (10)). Unmethylated CGIs account for only 1–2% of the whole genome. A small yet significant portion of them are heavily methylated and are involved in directing tissue-specific patterns of gene expression, genomic imprinting and X chromosome inactivation.

In addition to cytosine residues of DNA, N-terminal tails of histone proteins are subject to modifications, including methylation, acetylation, phosphorylation, ribosylation and ubiquitination. Genome-wide analysis of histone modifications revealed that acetylation around transcriptional start sites is correlated with transcriptional activation and chromatin accessibility (11). The impact of lysine (K) methylation on gene expression varies depending on which Lys residue is modified and how many methyl groups it carries. For example, trimethylated K9 of histone H3 and K20 of histone H4 are enriched in constitutive heterochromatin while trimethylated K27 and K9 of histone H3 are enriched in the inactive X chromosome (12). These histone modifications are silencing marks of gene expression. In contrast, trimethylation at H3K4 and H3K36 is associated with active chromatin (13). High levels of monomethylation and low trimethylation at H3K4 are frequently observed in enhancer elements; such modifications have become a useful tool to identify potential enhancers together with cell type-specific expression patterns (14). The discovery of inter-dependent relationships between DNA methylation and specific histone modifications has just begun. DNA methylation can provide a template to maintain certain histone modifications after replication. Conversely, specific histone methylation can guide the establishment of DNA methylation patterns (reviewed in (15)).

1.2. DNA methylation and patterns of gene expression

The conventional view ascribes a repressive role for DNA methylation on gene expression. Promoters of transcriptionally active genes are usually unmethylated, but become silenced once targeted by DNA methylation. DNA methylation-associated gene silencing is mediated either by recruiting methyl binding domain proteins and repressive factors such as histone deacetylases or by blocking recruitment of transcriptional factors (reviewed in (16,17)). In contrast to DNA methylation in promoters, methylation within gene bodies is observed in transcriptionally active genes. It is not known, however, whether methylation reflects the default state in the genome or is targeted in a sequence- or locus-specific manner (reviewed in (18,19)). DNA methylation-dependent transcriptional activation has been shown at some imprinted loci by preventing interactions with enhancer blocking factors (20,21). Whether or not this mechanism directly applies to the function of genome-wide gene body methylation remains unclear.

Genome-wide DNA methylation analysis in various human tissues has revealed the presence of tissue-specific differentially methylated regions (19,22), which may play a role in cellular memory and tissue-specific genome function. Tissue-specific DNA methylation patterns are less frequently observed in the middle of CpG islands, instead differences have been

suggested to occur in sequences of intermediate CpG density up to 2kb away, referred to as CpG island shores (23). Distinct epigenetic patterns are also observed in genome-wide maps of ES cells versus differentiated cells in mice (24,25). A bivalent chromatin state consisting of H3K27me3 and H3K4me3 at the same genetic location was observed in embryonic stem (ES) cells (24). This state keeps genes in ES cells silenced but poised for either continued silencing or activation upon ES cell differentiation. Stable repression of genes associated with the pluripotent state also requires DNA methylation, which complements other regulatory mechanisms such as histone modifications or recruitment of transcription factors (26). Recent findings in induced pluripotent stem cells emphasize the importance of proper patterning of DNA methylation, in conjunction with the presence of specific transcription factors, to define pluripotency (27).

1.3. DNA methylation in genomic imprinting and X inactivation

In addition to transcriptional regulation during development, epigenetic regulation is required for proper patterns of genomic imprinting and X chromosome inactivation during germ cell development and early embryogenesis (reviewed in (5,28)). Imprinted genes are a subset of genes that show allele-specific expression defined by the allele's parent-of-origin and are controlled by epigenetic mechanisms. Allele-specific DNA methylation at imprinting control regions (ICRs) has long been known to be the main force behind imprinted gene expression (29). DNA methylation is established during gametogenesis in a sex-dependent manner, resulting in allelic differences after fertilization. DNA methylation in ICRs contributes to allele-specific expression patterns either by inhibiting transcription of regulatory non-coding RNAs or by blocking DNA binding factors such as CTCF (30). The parentally imprinted methylation is completely erased and is reestablished during primordial germ cell development. Epigenetic regulators of histone modifications such as G9a and the PRC2 complex have also been shown to be involved in maintenance of placental imprinting patterns (31,32). These imprinted modifications are faithfully maintained throughout development. Disruptions of the imprinting patterns are associated with human diseases, most notably cancer (reviewed in (33)).

Compared to the single active X chromosome in male somatic cells, inactivation of one of the X chromosomes in female mammalian somatic cells is necessary for dosage compensation of X chromosomal gene expression. X chromosome inactivation is regulated by imprinted and random patterns in extraembryonic and embryonic tissues, respectively. Similar to imprinted genes, epigenetic marks initiate and maintain inactivation of the X chromosome and undergo dynamic reprogramming during germ cell development and early embryogenesis. Embryonic random X inactivation utilizes DNA methylation to prevent *Tsix* expression, a non-coding RNA that blocks *Xist* expression, and allows for *Xist* transcription. There is, however, no consensus whether differential methylation in the control regions of *Xist*, *Tsix*, and *Xite* leads to imprinted X inactivation during early embryogenesis (reviewed in (34)). In addition to the primary role in random inactivation, DNA methylation also provides additional levels of repression for long-term inactivation across the whole X chromosome, together with the spreading of trimethylated H3K27(35).

1.4. DNA methylation and genome stability

In the human genome, DNA methylation resides predominantly in repetitive genomic regions, which include satellite DNA and retrotransposons such as LINEs, SINEs and LTRs. Satellite DNA, mainly located in centromeric and telomeric regions, consists of tandem repeats and forms heterochromatin characterized by DNA methylation and trimethylation of H3K9. About 40–50% of the mammalian genome consists of transposable elements. Their transcriptional repression by DNA methylation is likely to function as a host defense mechanism to maintain genomic integrity and stability (36). DNA methylation in

retrotransposons undergoes dynamic reprogramming during early embryogenesis and germ line development. Some repetitive elements such as intracisternal A particles (IAPs) are resistant to demethylation in both the primordial germ cells (PGCs) and the zygote, possibly a critical necessity for genome stability (37). Accordingly, establishment and maintenance of DNA methylation in transposable elements after reprogramming appears to be essential to prevent transpositions. Moreover, mutations in *Dnmt3L*, a DNA methyltransferase family member expressed during germ line development, cause loss of methylation and de-repression of transcripts in transposable elements, leading to meiotic defects and male infertility (38,39)). Argonaute family proteins, MILI and MIWI1/2, are also indispensable for maintenance of DNA methylation in retrotransposons during germ cell development (40). Together with genome instability, the loss of DNA methylation in retrotransposons is a hallmark of various cancers.

Altogether, epigenetic modifications are key regulators of tissue-specific gene expression, genomic imprinting, X inactivation and repression of retrotransposons during development. Understanding the epigenetic patterns in normal tissue and how they are disrupted in cancer will provide insights how to tackle pathogenesis.

2. Genome-wide epigenetic alterations in cancer

2.1 Hypermethylation of candidate genes

Epigenetic alterations in cancer have initially been investigated in candidate gene approaches. Following the hypothesis that epigenetic alterations have the ability to silence gene transcription, tumor suppressor genes were tested for epigenetic changes in their promoter regions. These tests were mainly building on the identification of aberrant DNA methylation as a marker for epigenetic alterations. The base 5-methylcytosine is a stable mark of the DNA sequence, and hence, can readily be traced even in archived materials. DNA methylation assays were developed that allowed rapid testing for changes in DNA methylation in comparisons of normal and tumor tissue DNA. PCR based assays following conversion of the DNA by sodium bisulfite treatment (41) proved sensitive, quantitative and scalable to high throughput applications (reviewed in (42)). Initial results quickly demonstrated that numerous GC-rich promoter regions in virtually every human malignancy are targets for epigenetic alterations and gene silencing. In these studies, known tumor suppressor genes, such as *MLH1* in colon cancer (43,44), *BRCA1* in breast cancer (45,46), *DAPK1* in chronic lymphocytic leukemia (47), or *p16^{INK4a}* in head and neck cancer (48,49) and lung cancer (50), were found to be epigenetically repressed.

2.2 DNA hypomethylation of cancer genomes

Early studies, in which the 5-methylcytosine content of cancer genomes was measured, indicated that global levels in a tumor genome are reduced as compared to those in normal tissues (51). It quickly became clear that loss of 5-methylcytosine occurs in sequences spread throughout the genome that are usually methylated in normal cells. spread throughout the genome. These sequences included centromeric repeats and alpha satellite sequences located in centromeric regions, but also interspersed repetitive elements such as LINE1 sequences. The consequence of hypomethylation at these repeat sequences is genomic instability caused by an opening of the chromatin and subsequent chromosomal breakage. This may explain the numerous chromosomal aberrations found as one of the hallmarks in cancer genomes. Numerically, hypomethylation events exceed the number of hypermethylation events by far if one considers the abundance of methylated CpG dinucleotides in GC-rich repetitive sequences (>80% of the total genomic CpG content) relative to the number of unmethylated CpG dinucleotides in CpG island sequences (1–2%).

2.3 Epigenetic changes in imprinted regions

Sequences that display both hypo- and hypermethylation include the imprinting control regions (ICRs). Both the gain and loss of methylation in these differentially methylated regions result in loss of genomic imprinting and dysregulation of genes controlled by protein complexes that detect the unmethylated ICR but not the methylated. Examples include loss of imprinting due to the hypermethylation of the ICR of the IGF2/H19 locus, resulting in overexpression of growth-activating *IGF2* (52,53).

2.4 Lessons learned from genome-wide approaches

Candidate gene approaches are not sufficient to evaluate the amount of epigenetic alterations in a cancer genome (Fig. 1). However, for a long time, assays to evaluate the DNA methylation status of all 28×10^6 CpG dinucleotides simultaneously in a human genome were not available (see also below 3.2 Methylome analysis). Thus, most first generation scanning assays focused on a representation of the genome rather than attempting to cover the entire genome. Assays that focused specifically on CpG island sequences included Differential Methylation Hybridization (DMH) (54) and Restriction Landmark Genomic Scanning (RLGS) (55). Both assays were designed to allow the calculation of overall CpG island methylation frequency. DMH was initially developed to identify methylated sequences in a cancer genome in a screen of CpG island clones (or later arrayed oligonucleotides) representing CpG island sequences. In this assay, DNAs were divided into two pools after *MseI* restriction digest and linker-ligation. The control pool was amplified without further treatment whereas the test pool was digested with the methylation-sensitive restriction enzyme *BstUI* prior to amplification. Subsequent to this procedure, both pools were labeled with different dyes and co-hybridized to arrayed CpG island sequences. RLGS, on the other hand, was built on the restriction digest of genomic DNA with a methylation sensitive restriction enzyme that preferentially cuts in CpG island sequences (e.g. *NotI* or *AscI*). Restriction ends were radioactively labeled. Subsequent to a second restriction digest with a more frequently cutting restriction enzyme, the DNAs were separated in a tube-like agarose gel followed by in-gel digestion with a third restriction enzyme and final separation in an acrylamide gel. The gels were dried and exposed to an X-ray film which displayed up to 2000 RLGS fragments that represented unmethylated *NotI* or *AscI* restriction sites.

While DMH and RLGS allow for the evaluation of overall levels of CpG island hypermethylation, other assays were designed specifically to identify hyper- or hypomethylated sequences from cancer genomes without providing data on the overall frequency of CpG island methylation in a tumor genome. For example, methylated CpG island amplification (MCA) was used as screening tools for the identification of novel methylated sequences in colon cancer (56): MCA identified cancer-specific methylation events and a panel of sequences that characterizes the CpG island-methylator phenotype (CIMP). The CIMP is present in the majority of sporadic colorectal cancers displaying microsatellite instability and being most frequently associated with *MLH1* hypermethylation. A surprise resulting from the data of the first genome scans performed by RLGS was the number of aberrantly methylated CpG islands. Leukemias, for example, demonstrated mean levels of CpG island methylation of 4.8% (chronic lymphocytic leukemia, CLL) and 1.9% (in acute myeloid leukemia, AML) (57,58). Similar numbers were found in solid tumors with mean levels of CpG island methylation of 5.3% in lung cancer (59), 1% in primary head and neck cancer (60), and 4.6% in ovarian cancer (61). These numbers exceed by far the estimated number of tumor suppressor genes. The frequency of CpG island methylation raises questions: which are the initial epigenetic silencing events? And what events are accumulating during tumorigenesis, perhaps due to a loss of DNA repair or accelerated growth? Lacking in this context is also the information on DNA methylation changes in regions outside of CpG islands. These regions might exhibit changes

that are of relevance in tumorigenesis. There is hope that novel genome-wide scans will provide this information in a comprehensive manner.

An additional surprising finding in these studies was that DNA methylation events are tumor-type specific (62). Tumors and tumor subtypes display specific patterns of aberrant CpG island methylation indicating specific, yet, unknown mechanisms that lead to the silencing of specific groups of genes within a tumor. Here, either direct targeting of genes by oncogene-encoded proteins, onco-fusion proteins, or a selection process are discussed as possible mechanisms (see (63) for a detailed discussion).

2.5 Genetic versus epigenetic alterations

While candidate gene approaches detected epigenetic effects in genes that had previously been identified as target genes for genetic (mutational) events, the epigenetic screens identified novel genes and gene families that were predominantly or even exclusively silenced by epigenetic mechanisms. The importance of these types of genes in normal development and their silencing in tumorigenesis is under investigation in many laboratories. One example is *DAPK1*, a gene frequently silenced in many tumor types by epigenetic alterations. However, there are no reports on genetic mutations in the coding region of *DAPK1* (64). An additional example is *CTNNA1*, a gene silenced by both epigenetic and genetic mechanisms in myelodysplastic syndrome (MDS) and AML cases with chromosome 5q deletions (65). The possibility of concordant genetic and epigenetic events in the inactivation of tumor suppressor genes is now being used to identify novel tumor suppressor genes in regions of chromosomal loss where searches for mutated genes have failed to pinpoint candidate cancer genes (66). The underlying assumption for this approach is that the two hits, postulated by Knudsen's "Two-Hit Hypothesis" (67), can be a combination of genetic and epigenetic events.

It is now becoming clear that DNA methylation changes are closely linked to alterations of other epigenetic modifications, especially histone tail modifications. An intriguing observation was the finding that many of the epigenetic target genes are targets for the polycomb repressor complex and are marked by the repressive histone tail modification H3K27me3, which is mediated by EZH2 a member of the polycomb group complex (68–70). Furthermore, cancer cells show a loss of monoacetylated and trimethylated forms of histone H4K16 and K20 residues of histone H4. These changes occur predominantly in hypomethylated, repetitive DNA sequences of the cancer genome (71).

3. Second generation methodologies for epigenome-wide scans

Microarray and novel sequencing techniques have facilitated the comprehensive analyses of whole transcriptomes and complex genomic sequences. These techniques also paved the way for the genome-wide scanning of DNA methylation states (methylome profiling). Methylome profiling covers the whole genome, yet historically concentrates on the methylation states in CpG islands because of their frequent overlap with/or their close vicinity to promoter sequences (see above). Here, we will concentrate on a few examples of recent technical achievements in whole genome profiling (2nd generation methylome profiling) rather than discuss approaches targeting single candidate genes. Basically, methylome profiling can be separated into two successive processes: sequence enrichment for potentially methylated CpG sites and sequence-based analysis (Table 1).

3.1. Enrichment for methylated CpG sites

The rationale for sequence enrichment is the reduction of genome complexity and sequence load in later analysis. Three technical alternatives are currently in use:

- i. Cutting with methylation-sensitive and methylation-resistant restriction isoschizomers followed by linker-mediated PCR amplification (developed in the late 1990th), still often employed in combination with modern analysis technology)
- ii. Affinity purification using antibodies or recombinant proteins with high affinity for methylated DNA
- iii. Sequence capture by hybridization to complementary oligonucleotides

A variety of enzymatic options are available to distinguish between methylated and non-methylated states at CpG sites. In two approaches, abbreviated MIAMI (72) and HELP (73), genomic DNA is cut with the methylation-sensitive restriction enzyme *HpaII* and, as internal control, the methylation-independent *MspI*; both enzymes recognize the sequence CCGG. This tetranucleotide occurs ~2.3 million times in the human genome, ~22% of them residing in CpG islands (74). After cutting genomic DNA to completion with either enzyme, linkers for final PCR amplification are ligated to the 5' G-overhangs of size fractionated (e.g., 200–2,000 bp) restriction fragments. In the later analysis, methylated CpG sites are recognized by their absence in the *HpaII*- and their presence in the *MspI*-treated sample. Recent technical improvement of the HELP approach considerably extended the representation of the addressed sequence regions (75).

Bisulfite treatment enables to discriminate between different methylation states by the conversion of unmethylated cytosine to uracil while methylated cytosine remains unconverted. In *MspI* reduced representation bisulfite sequencing (RRBS, (76)), genomic DNA is cut only with *MspI*, subsequently ligated to linkers containing only methylated but no unmethylated cytosines, and then bisulfite treated. PCR amplification then leads to a change from cytosine to thymine for every unmethylated cytosine while methylated cytosines are preserved as cytosines.

Affinity purification of randomly fragmented DNA (200–1,000 bp) employs antibodies or proteins with high affinity for methylated CpGs (77–79). The currently most widespread approach is meDIP (77) using antibodies against single-stranded methylated DNA. Alternatives apply recombinant human proteins MBD2 (MCIP, (78)) or complex MBD2/MBD3L1 (MIRA, (79)) which bind with high affinity to double-stranded methylated DNA. After binding, the methylated DNA fraction is eluted from the antibodies/proteins by a high-salt buffer or a gradient of buffers with increasing salt concentrations. The gradient discriminates between states of low, intermediate and high methylation. For microarray analysis, eluted DNA can be directly labeled in a linear amplification reaction. The labeling products reflect more reliably the relative abundance of enriched fragments than products of exponential PCR amplification. Another advantage of the affinity compared to the enzyme-based enrichment approaches is their independence of specific recognition sites, allowing, at least theoretically, examination of all potentially methylated sequence stretches. However, fragments with high methylation density are favored compared to those with low or moderate methylation density. Moreover, since affinity purification offers no direct proof for the presence of methylated CpGs, identified candidate genes need validation by a confirmatory method.

Sequence capture of bisulfite-treated DNA employs oligonucleotide capture probes which are complementary to specific target sequences. In two of three presented strategies, padlock probes were used. These probes are usually ~100 bases long and anneal via their end sequences to a target sequence, thereby forming a padlock- or horseshoe-like structure. In the first strategy, padlock probes were designed to target non-repetitive sequences covering ten bases with a 5' CpG which are flanked by at least 20 bases on each side free of CpGs (80). After annealing, the probes prime DNA synthesis of the targeted ten bases. Subsequent ligation leads to the formation of single-stranded DNA circles. Using primers derived from

the common backbone of the probes, all synthesis products can be amplified in a single PCR. In the second strategy, padlock probes were designed to target longer sequence stretches of up to 225 bases. Moreover, capturing arms were allowed to contain CpGs. Consequently, multiple probes were designed considering all possible sequence combinations after bisulfite treatment (81). The third strategy employed 60mer probes immobilized on a microarray (82). Similar to the second strategy, these probes were also allowed to contain CpGs (up to 15). However, only a binary probe design with respect to possible methylation states was applied: fully unmethylated or fully methylated, referring to reports that efficient hybridization tolerates polymorphic sites and even up to 6 distributed mismatches. Similar to enzyme-based enrichment, the presented sequence capture methods addressed specific target sequences rather than enabled to profile the whole methylome. All three capture methods are bioinformatically demanding and, therefore, require special expertise. However, coverage of substantial parts of the methylome in all three studies leads to the expectation that comprehensive capture addressing the unique sequences of the human methylome may be feasible in the near future.

3.2 Methylome analysis

Methylome analysis after sequence enrichment is either performed by hybridization on high-density oligonucleotide tiling microarrays or by next generation sequencing (NGS). Different commercial tiling microarray platforms are currently in use, offering the flexibility of custom-designed arrays or standard arrays covering, for example, promoter or CpG island sequences of the human genome. Tiling probes are usually 45–60 bps in length and cover the regions of interest like CpG islands in close spacing or even with overlaps. DNA samples are labeled with a fluorescent dye such as Cy3 or Cy5 and co-hybridized with a control sample, labeled with the complementary dye, to the microarray. Control samples, like DNA from healthy tissue, are usually enriched in the same way as the test sample or they may consist of the non-enriched test DNA. After hybridization, scanning of the array generates an image file displaying the different signal intensities of the two DNA samples on the oligonucleotide probes. Different types of feature extraction software evaluate the image file and provide both signal intensity ratios and a set of quality control values. Additional corrections by normalizing unequal distributions of bulk fluorescence intensity values may be necessary prior to final statistical data evaluation. Since microarray data provides no direct proof for the methylation state of CpG sites, validation by other methods involving bisulfite DNA treatment is mandatory. Recently, algorithms have been proposed that allow correlations of quantitative array data with DNA methylation levels (83,84).

NGS offers an attractive alternative to microarray analysis and has already been combined with the enrichment strategies described above. The power of NGS became particularly evident when applied on enriched bisulfite treated DNA samples. In three sequence capture studies (80–82), the Illumina Genome Analyzer was used which can provide hundreds of millions of short read sequences (~30–35 bases) in a single run. Prior to sequencing, a library of short (~100–300 bp) DNA fragments has to be prepared by ligation-mediated PCR. Use of different linkers for PCR priming allows sample multiplexing.

The huge output of a single run requires enormous data storage capacity and powerful software for proper quality check-up and final mapping of sequences on the human reference genome. Bisulfite treatment of DNA leads to the reduction of sequence complexity and to ambiguities at CpG sites, necessitating novel mapping algorithms. Repetitive sequences, such as the transposon-like repeats which are highly methylated in the human genome (74), are excluded *in silico* from further evaluation using masking algorithms (e.g. Repeatmasker) because they can not be mapped back to the reference genome. All capture studies reported encouraging experimental performance with respect to both specificity (discovery of false positives) and sensitivity (likelihood for detecting rare positives). In one

study, ~ 3 million reads were mapped to ~7,700 of ~10,000 targets (~77%) (80), while in another study ~5.5 million reads could be mapped to 10,364 of 10,582 targets (98% sensitivity) (81). In the latter study, the abundance of different captured fragments ranged from 1 to ~10,000-fold. This variation could be traced back to a combination of parameters including the size of the target sequence and the GC-content. Knowing these parameters and their influence should enable a more reliable sequence representation in NGS methylome profiling projects.

4. Epigenetic biomarkers

4.1 Epigenomic profiles as markers for cancer tissues

Biomarkers are biological parameters that can be objectively measured and evaluated as indicators of biological processes. Over the last years, biomarkers have gained an enormous impact on diagnosis and treatment of cancer and cancer-related diseases. They can be used as diagnostic tools and as prognostic factors that predict the outcome of individual patients in terms of a specific clinical endpoint. In addition, they may serve as predictive factors for the effect of a specific treatment and as surrogate endpoints that replace a clinical endpoint of interest. Recently, the term biomarker has become a synonym for “molecular biomarker”, which can be measured by molecular techniques in biological samples. Molecular biomarkers include changes in nucleic acid sequences such as mutations or polymorphisms and gene expression alterations, peptides, proteins, lipid metabolites, and other small molecules.

Human malignancies can be characterized by distinct epigenomic profiles as markers of the malignant cell clone (see 2.4, above). Increased DNA methylation at CpG islands is prevalent in basically every human cancer, and different types of cancer can be reliably distinguished by their unique DNA hypermethylation pattern (62). The list of the affected loci (genes) is rapidly growing and exerting its impact on clinical decisions (Table 2). Hypermethylation at distinct genomic loci has several properties that predispose for use as an attractive potential biomarker in disease. First of all, DNA hypermethylation of many distinct genes is characteristic for neoplastic cells. It is found to a significantly lower extent in healthy individuals. Early onset of DNA methylation changes is evident in the pathogenesis of many cancer types. This makes hypermethylation signatures an attractive tool in early detection and screening, particularly in patients who exhibit increased risk. Secondly, 5-methylcytosine is a chemically stable covalent mark that can be reliably detected in a variety of tissue sources. In contrast to RNA-based signatures, DNA methylation patterns are less prone to storage- or handling-dependent variations which could confound measurements and consecutive interpretations. Analyses can be performed on fresh tissues, archived frozen material or paraffin-embedded tissues. Samples can be long-term stored for intra- and inter-individual references. Detection is possible not only in tumor tissue, but also on tumor-derived DNA which may be present in body fluids (such as peripheral blood or serum). In addition, the detection of epimutations (epigenetic modifications that can be passed down from parents to offspring) which may be present in unaffected tissues becomes a promising approach for a biomarker based on DNA methylation (85). However, in assessment, evaluation and interpretation of DNA methylation analyses of clinical samples, it is important to consider potential cytological heterogeneity as possible confounder. Thirdly, the technology of DNA methylation measurement has greatly improved over the last years (see above). Sensitivity, reproducibility and applicability for clinical settings have significantly improved. The ability of quantitative measurements at single CpG dinucleotide resolution enables tight correlations with clinical endpoints, precise identification and separation of subgroups revealing previously unidentified differences. This gains particular importance considering

several reports that indicate superior prognostic or predictive significance of single CpG dinucleotides within CpG-rich areas.

A major challenge in utilizing DNA hypermethylation events for sensitive and specific diagnostic and prognostic markers is the selection of candidate genes (schematically depicted in Fig.1). For many entities, candidate gene selection using for example differential regulation or supposed function (“informed best-guess”) led to identification of successful DNA methylation markers. However, this approach relies on restricted observations and assumptions and might not consider independent potential markers. With the development of genome-wide tools and the tremendous effort of initiatives trying to decipher non-malignant and cancer methylomes, the exciting perspective of highly sensitive and specific DNA methylation signatures for various cancers and their respective subtypes becomes increasingly available. This might be of particular interest for distinction of tumors and non-tumor diseases with similar behavior, e.g. chronic inflammations. Moreover, precise discrimination between tumor subtypes by DNA methylation signatures might help to strengthen diagnostic efforts and to conduct improved disease- and stage-specific therapy decisions. In a large number of cases, diagnosis is based on biopsies from undifferentiated metastatic tissue that makes it difficult for conventional histological and immunocytochemical approaches to determine the tumor origin (e.g. “CUP syndrome”). Since particular therapeutic options may vary greatly between different tumor types, defined methylation signatures could significantly contribute to the management of such cases.

4.2 Epigenetic markers for (early) detection of cancer cells and screening

Evidence from various mouse models (e.g. *TCL1*-transgenic mouse model for CLL pathogenesis) show that DNA methylation events occur early during pathogenesis at particular genomic loci. They can already be detected in pre-malignant lesions (86). Thus, DNA hypermethylation of distinct loci/genes can frequently be assessed even before the histological onset of the disease. In humans, this could convincingly be shown for CpG island hypermethylation in *p16^{INK4a}*, *p14^{ARF}* and O6-methylguanine-DNA methyltransferase (*MGMT*) in colorectal adenomas (87), *p16^{INK4a}*, *RUNX3* and *HPPI* in Barrett’s esophagus (88), *MLH1* in atypical endometrial hyperplasia (89), *GSTP1* in prostate cancer (90), *p16^{INK4a}* and/or *MGMT* in squamous cell lung carcinoma (91) and many more. These data strongly underline that the analysis of the DNA methylation signature plays an important role in screening and early detection of different malignancies. Particularly, in predisposed patients with either infectious or inflammatory conditions, DNA methylation signatures may be useful as markers of increased cancer risk. Individuals with family history of cancer can notably benefit from successful early cancer detection by such innovative sensitive screenings. This was impressively affirmed by studies in colorectal cancer where DNA methylation patterns of familial cases exhibited striking similarity to those of sporadic cases (92). With respect to practical feasibility, the presence of DNA in body fluids offers easy, non-invasive accessibility to material for numerous cancer types. A large case-control study recently demonstrated that hypomethylation measured in peripheral blood lymphocytes was strongly associated with increased risk of bladder cancer (93). Indeed, hypomethylation of L1 LINE sequence elements in bladder cancer tissue had already been reported several years ago (94).

In reviewing the identification of new epigenetically altered candidate genes and the rapid development of technologies over the last few years, the challenging goal is now to set up panels for reliable early detection markers especially in high risk patients. At the same time, early detection using methylation signatures opens up novel treatment options that include epigenetic therapeutic strategies. In addition, sensitive epigenetic screening panels may not only allow effective identification of early cancer stages but may also be used as instruments for disease monitoring and detection of relapses. Continued sequential analyses of DNA

methylation signatures can be indicative for recurring disease at stages where clinical symptoms are still absent and conventional diagnostic tools do not offer sufficient sensitivity. First promising results come from a small prospective study demonstrating methylation of a gene panel in saliva for the early detection of relapses in head and neck squamous cell carcinomas (95).

4.3. DNA methylation profiles as marker for risk assessment, tumor progression and prognosis

Usually, detection of cancer is directly followed by assessments of stage and risk of the malignant disease. As DNA methylation signatures fulfill requirements for prognostic biomarkers (the baseline value of the biomarker, or changes in the biomarker over time, should be correlated with the clinical endpoint in untreated or in treated patients), they can be used to supplement conventional staging. Several studies have demonstrated that DNA hypermethylation of distinct genes can be correlated with clinical parameters or even substitute for them. This implies that DNA methylation signatures can significantly contribute to risk stratification in malignant diseases and may furthermore define new prognostic subgroups. Striking examples come from colorectal and lung cancer where methylation of *p16^{INK4a}* is accompanied by particularly poor prognosis (96). In breast cancer, *SFRP1* promoter hypermethylation is associated with unfavorable prognosis and poor overall survival in patients in early stages of the disease (97). Some studies extend the prognostic ability of epigenetic biomarkers from clinical endpoints like overall survival to distinct properties of the disease course. This has been demonstrated for the increased metastatic potential in cervical cancer assessed by hypermethylation in *MYOD1* CpG island (98). In chronic lymphocytic leukemia (CLL), methylation of single CpGs separates the disease into major prognostic subgroups in addition to established prognostic parameters like IgVH mutation status or ZAP70 protein expression (99). Precise and significant prognosis estimates are not only highly informative about the course of the disease but they allow more risk adapted treatment decisions.

4.4 Predicting therapy response by epigenomic profiles

Treatment decisions in oncology are based on risk-adapted procedures. However, the efficiency of a particular therapy and the sensitivity of an individual cancer are difficult to predict. The exciting possibility of treatment response prediction may be the most challenging and promising task for potential biomarkers. DNA methylation signatures have been demonstrated to serve as predictive markers, as their baseline value or their changes over time have been correlated with the effect of treatment. The most prominent example is *MGMT* hypermethylation in glioblastoma patients. Expression of *MGMT* leads to reduced toxicity of alkylating agents such as temozolomide due to rapid reversal of DNA adduct formation. DNA methylation of *MGMT* (and its methylation-associated silencing) is the best independent predictor for treatment response in glioblastoma (100). Further evidence for predictive DNA methylation signatures comes from hypermethylation of *hMLH1*, a DNA mismatch repair gene. *hMLH1* hypermethylation frequently occurs in various tumors and is associated with increased resistance to chemotherapeutics like cisplatin (101). In ovarian cancer, acquired *hMLH1* methylation in peripheral blood predicts for adverse response to chemotherapy (102). Interestingly, in human tumor xenograft models, demethylation of the *hMLH1* promoter resulted to sensitization to cisplatin (103).

4.5 Monitoring epigenetic therapies

Despite some advances in our understanding of the mechanisms underlying epigenetic therapies, we still do not understand completely how these drugs work. Epigenetic therapies have tremendously evolved over the last few years. The increasing number of studies that report the significance of epigenetic alterations in cancerogenesis and distinct tumor cell

properties build a strong rationale for the use of epigenetically modifying drugs in cancer treatment. In addition, frequent reports of epigenetic biomarkers and identification of hypermethylated genes sets that contribute to alteration of chemosensitivity support the rationale for reversal of DNA methylation patterns as an effective therapeutic approach. The DNA methyltransferase (DNMT) inhibitors 5-aza-2'-deoxycytidine/decitabine (Dacogen) and 5-azacytidine (Vidaza) have recently been approved by the U.S. Food and Drug Administration (FDA) for treatment of MDS. Therapeutic principles have been attributed to reversal of hypermethylation and reactivation of tumor suppressor genes (e.g. *p15^{INK4b}*). However, recent studies reported on particular DNA repair mechanisms and others to be involved in response to therapy. Several studies have used methylation signatures of single genes or gene combinations to monitor therapeutic effects of demethylating agents. Clinical trials in AML and MDS have shown decrease of DNA methylation at genome-wide levels (assessed by L1 LINE methylation) and at the *p15^{INK4b}* promoter upon therapy (104,105). Quantitative measurements of DNA methylation allow precise monitoring of even minor demethylation effects which are possibly indicative for effective therapy. Fetal hemoglobin (HbF) has been reported to be reactivated in patients upon treatment with demethylating agents (106). It appears reasonable to further investigate HbF as an *in vivo* marker for the application, efficacy and the assessment of treatment response. Markers for epigenetic therapy might be particularly important when considering an extended use of demethylating agents in solid tumors in the future, where early treatment response is often more difficult to assess than in patients with hematologic neoplasia.

Taken together, DNA methylation changes at single sites, single genes or panels of genes can serve as promising potent biomarkers that facilitate detection and clinical management of various cancer types.

5. Future directions in cancer epigenetics

5.1. Understanding the underlying mechanisms of epigenetic regulation

Despite significant advances in the field, there are many open questions and challenges that remain in the understanding of the methylome and utilizing this information in basic and translational research. We still do not understand the underlying mechanisms that lead to epigenetic alterations. Major efforts should be made to precisely characterize epigenomic patterns during development and to understand aberrant epigenetic processes, with the ultimate goal to perturb them in cancer development. Mouse models for cancer may be helpful tools that recapitulate epigenetic defects (86,107,108). These mice might allow us to dissect the cascade of events that leads to a global genome wide epigenetic defect and to develop strategies that might help to prevent these alterations and subsequently cancer development.

5.2. 3rd generation DNA methylome profiling

Although major advances have been made just recently in the development of novel scanning protocols, it can already be foreseen that further improvements will lead to a more detailed characterization of epigenetic patterns. Still ongoing improvements in probe density and design as well as relative simplicity in both performance and evaluation will assure that microarray technology will retain a share in methylome profiling studies during the next few years. In comparison, NGS is still reserved to only a few laboratories being able to cope with the relatively high equipment costs and technological and bioinformatical requirements. It can be envisaged, however, that overall costs for both equipment and consumables will considerably drop soon, making NGS available to a much broader scientific community. In depth methylome profiling of tumor samples, particularly in early stages, is often complicated or even prohibited by limited amounts of DNA, necessitating considerable

improvement in target-sequence enrichment and assay sensitivity. This has been already addressed by some of the cited studies (e.g., (75)), and a novel development of ultra-sensitive quantum-dot technology in profiling single candidate genes in large patient sample sets has just been published (109). Both technical and financial reasons make it presently more likely that only a limited set of candidate genes or sequences rather than the whole methylome will be analyzed in the clinical routine. It remains to be seen, whether such a candidate approach will be sufficient for proper molecular diagnostics, staging and prognosis of a malignant disease.

5.3 Integration of epigenetic markers in clinical settings

Epigenetic alterations, in particular DNA methylation have become promising new tools for cancer screening and diagnosis, risk assessment and prognosis estimation, as well as therapeutic management. However, more systematic evaluation in large, well characterized patient cohorts is strongly desirable. As an important part of translational investigations, putative epigenetic biomarkers should be frequently incorporated in clinical trials enabling prospective sampling and more comprehensive evaluations. Especially the development of predictive markers (foremost markers for prediction of therapy responses) is an important and urgent request for many cancer entities. Therapy response predictors (as described above for *MGMT* in glioblastoma) might lead to more patient-, risk- and disease stage-adapted therapeutic strategies that directly translate into clinical benefit for the patient.

It is obvious that epigenetics markers have started to move from bench to bedside. They are entering the clinical field and becoming essential factors in the clinical management of cancer patients.

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Fig. 1.
Schematic outline of steps in an epigenetic screen (see text for more information)

Table 1

Novel methods for methylome profiling

Method/Acronym	Enrichment	Analysis	Reference
HELP, MIAMI, RRBS	Restriction enzyme digestion with methylation-sensitive and -resistant isoschizomers	Microarray hybridization, NGS	(72,73,75, 76)
meDIP, MIRA, MCIp	Protein affinity purification	Microarray hybridization, NGS	(77–79,83)
Sequence capture	Hybridization of bisulfite-treated DNA to oligonucleotides	NGS	(80–82)

Table 2

Selection of prominent examples for established and potential epigenetic biomarkers

Methylated genes	Specimen	Lesion type	(Potential) Clinical use	Reference
GSTP1	Tumor tissue	Prostate cancer	Diagnosis and risk assessment	(110)
	Urine	Prostate cancer	Screening, detection	(111)
	Serum	Prostate cancer	Screening, detection	(112)
hMLH1	Tumor tissue	Colon cancer, gastric cancer, endometrial cancer	Classification and risk assessment	(113)
	Endometrial tissue	Atypical endometrial hyperplasia (endometrial cancer)	Screening and risk assessment	(89)
	Tumor tissue	Various tumors	Response to treatment	(103)
	Tumor tissue	Diffuse large B cell lymphoma (DLBCL)	Prognosis	(114)
	Serum	Ovarian cancer	Chemosensitivity	(102)
<i>p16^{INK4a}</i>	Esophageal tissue	Barrett's esophagus (esophageal carcinoma)	Screening and risk assessment	(88)
	Serum	Esophageal cancer	Screening, detection	(115)
	Cervical cytologic specimens	Cervical neoplasia	Screening and risk assessment	(116)
	Tumor tissue, lymph nodes	Non small cell lung cancer (NSCLC)	Prognosis (early recurrence)	(117)
	Sputum	Lung cancer	Screening, detection, risk assessment	(118)
	Serum	Hepatocellular carcinoma	Screening and detection	(119)
MGMT	Colon mucosa	Colorectal adenoma (colorectal cancer)	Screening and risk assessment	(87)
	Tumor tissue	Glioma/Glioblastoma	Treatment response in Glioma	(100,120)
SFRP1	Pancreatic excretion	Pancreatitis vs. pancreas carcinoma	Screening and risk assessment	(121)
	Tumor tissue	Breast cancer	Prognosis	(97)
e-cadherin	Tumor tissue	Bladder cancer	Prognosis	Reviewed in (122)
	Tumor tissue	Breast cancer	Prognosis, progression	(123)
	Tumor tissue	Skin cancer	Prognosis, staging	(124)
APC	Tumor tissue	Prostate cancer	Prognosis, marker for progression	(90,125)
	Colon (tumor) tissue	Colorectal cancer	Detection	
	Breast tissue	Breast cancer	Detection	
P15	Blood/myeloblasts	AML	Prognosis, treatment response	(105)