Clin Chem Lab Med 2012;50(10):1733–1742 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/cclm-2011-0902

Review

Global DNA hypomethylation in cancer: review of validated methods and clinical significance

Estela G. Toraño[1,a], Sandra Petrus[1,a], Agustin F. Fernandez[1] and Mario F. Fraga[1,2,*]

[1] Cancer Epigenetics Laboratory, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), HUCA, Universidad de Oviedo, Oviedo, Spain
[2] Department of Immunology and Oncology, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain

Abstract

DNA methylation is one of the best-known epigenetic modifications in mammals. The alteration of DNA methylation patterns has been found to be related to many diseases, including cancer. It is well-known that during carcinogenesis, a site-specific DNA hypermethylation and a global DNA hypomethylation take place. This overall loss of DNA methylation has been proposed as a valid biomarker for cancer. Given its medical utility, in recent years it has become apparent that there is a need to develop methods for the analysis of DNA methylation using different approaches: global, locus-specific, or genome-wide. Here we review some of these techniques and discuss their potential clinical utility.

Keywords: biomarkers; cancer; DNA methylation; hypomethylation.

Introduction

The term "epigenetics" (literally "over" genetics) was coined by Conrad Waddington in 1942 to explain why genetic variations sometimes did not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype (1). However, the word currently refers specifically to the study of mitotically and/or meiotically stable heritable changes in gene expression that occur without changes in the DNA sequence (2); an important process for controlling patterns of gene expression during the cell cycle, development, differentiation and in response to environmental or biological variations (3). The disruption of these heritable changes underlies a wide variety of pathologies, including cancer (4, 5). Epigenetic regulation comprises DNA methylation, covalent histone post-transcriptional modifications (such as methylation, acetylation, ubiquitination and phosphorylation), chromatin organization (DNase hypersensitivity), and non-coding regulatory RNA (6). One of the best understood and predominant epigenetic modifications in mammals is DNA methylation, a covalent addition of a methyl (CH_3) group from the methyl donor S-adenosylmethionine (SAM) to the 5′ carbon of the pyrimidine ring of a citosine base that precedes guanines (CpG). DNA methylation can result in the silencing of specific genes and non-coding DNA sequences, such as introns, non-coding RNAs, repetitive elements, and transposable elements, which require effective mechanisms for their inactivation (7, 8). DNA methylation can also result in the recruitment of proteins that bind methylated CpG sequences [methyl-CpG-binding domain (MBD) proteins], histone deacetylases (HDACs) and HMTs (histone methyltransferases) prompting coordinated epigenetic modifications of the surrounding chromatin (9). Thus, DNA methylation is an important epigenetic mark involved in gene expression (10), X chromosome and transposon inactivation (11, 12), genomic imprinting (13), and chromosome condensation (14).

The DNA methyltransferases (DNMTs) carry out the methylation at dinucleotide CpG. So far, four members of the DNA methyltransferase family have been described in mammalians, based on sequence similarities, but only three of them have been shown to possess methyltransferase activity (15): DNMT1 (which maintains the existing methylation patterns following DNA replication), and DNMT3A and DNMT3B (two functional *de novo* cytosine methyltransferases that target unmethylated or hemimethylated CpGs without preference) (16). DNMT3L is structurally related to DNMT3A and DNMT3B, and it plays an important role in DNA methylation, although it appears to be inactive on its own. Furthermore, the sequence cues that target DNA methyltransferases to specific regions of the genome are still largely unknown. The distribution of CpGs in the genome is asymmetric: CpG sites are concentrated either in CpG islands, short CpG-rich DNA regions located in approximately 60% of human gene promoters (which are frequently unmethylated), or in regions of large repetitive sequences (i.e., centromeres and retrotransposon elements, which are methylated in 70%–80% of cases) (17, 18). Although in the latter case the majority of the CpGs

[a] Estela G. Toraño and Sandra Petrus contributed equally to this review.

*Corresponding author: Mario F. Fraga, Centro Nacional de Biotecnología (CNB-CSIC), Darwin, 3 Cantoblanco, 28049 Madrid, Spain
Phone: +34 985 109 475, E-mail: mffraga@cnb.csic.es

Received December 5, 2011; accepted June 8, 2012; previously published online July 15, 2012

are methylated, most CpG islands remain unmodified during development and in differentiated tissues (19).

DNA methylation takes place naturally during developmental phenomena, such as X chromosome inactivation or genomic imprinting (17). Methylation patterns that are transmitted by mitotic inheritance in both plants and animals (20) can be removed passively, i.e., by blocking methylation of newly synthesized DNA during DNA replication. However, both global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes (TSG) can occur in different pathologies, including cancer (21, 22). Hypomethylation and hypermethylation of DNA are relative terms and refer to "less" or "more" methylation with respect to some standard DNA (usually DNA from healthy tissues when applied to cancer epigenetics). Cancer-associated DNA hypomethylation (23, 24) was discovered before DNA hypermethylation. In fact, DNA hypomethylation in cancer often affects more of the genome than does hypermethylation which is why net losses of genomic 5-methylcytosine (5-MeC) are seen in many human cancers (24). DNA deregulation (understood as global DNA hypomethylation and promoter-specific hypermethylation) seems to precede the classical preliminary transforming events: mutations in tumor suppressors, proto-oncogenes, or both, and genomic instability (25). Disruption of the epigenetic machineries (either by mutation, deletion or the altered expression of any of their components) is known to play an important role in tumor initiation and development (4).

Global loss of DNA methylation in cancer generally occurs at centromeric repeats and repetitive sequences and it has been proposed that it is associated with chromosomal instability, reactivation of transposable elements and loss of imprinting (26). In fact, in cancer it is widely known that CpG islands in promoters and first exons of some tumor suppressor genes are hypermethylated (27) and, as a consequence, their role in cell cycle regulation, apoptosis, DNA repair, differentiation, cell adhesion, angiogenesis inhibition, and metastasis suppression is halted (26, 28). Unlike genetic mutations, epigenetic modifications can be reverted. Hence, the restoration of epigenetic patterns in premalignant cells could be a therapeutic target in the treatment or prevention of cancer (7).

Many genes have been described as hypermethylated in cancer and this trait has allowed the identification of specific biomarkers (29, 30). Transcriptional silencing via DNA hypermethylation is often associated with poor prognosis in some tumors (31–33).

In contrast to promoter hypermethylation, hypomethylation-dependent transcriptional activation in cancer is less frequent (34–37). However, this phenomenon has been found in the premalignant stages of many types of human cancers, which suggests that hypomethylation could be an early event in carcinogenesis (29). Currently, it is thought that the major contribution of this phenomenon to tumor development is through the enhancement of genomic instability (with hypomethylation of retrotransposons and pericentromeric repeats which may modify in trans the expression of crucial genes) (38, 39). Global DNA hypomethylation has been used recently as a marker of potential cancer risk. One study of head and neck cancer patients found them to have lower global cytosine methylation levels than controls (40). Another study showed that the level of global DNA methylation was significantly lower in patients with bladder tumors than in controls and was independently associated with cancer risk (41).

About 45% of the human genome is composed of repetitive elements, classified as interspersed repeats and tandem repeats. Hypomethylation of repeated DNA sequences can disrupt the functions of neighboring genes through transcriptional interference and the activation of transposable elements can potentially lead to mutagenesis (42). Long interspersed nucleotide element-1 (*LINE-1*) sequences (which constitute more than 18% of the human genome) and *Alu* repetitive elements (the most abundant short interspersed repeat that represents about 10% of the genome) are usually markedly methylated and contain most of the CpG methylation found in normal tissues (43, 44). More than one-third of DNA methylation is estimated to occur in repetitive elements and, for this reason, the detection of DNA methylation loss in these regions is an indirect evaluation of the genomic hypomethylation that characterizes human cancers (45–47). Weisenberger et al. demonstrated that analysis of *Alu* and *LINE-1* methylation could be used as a measure of global DNA methylation (48). For example, there is a close relationship between *LINE-1* hypomethylation and progression of prostate adenocarcinoma (49). Another study revealed that most *LINE-1* sequences were hypomethylated in cancer cell lines and tumor tissues (50).

Although DNA hypomethylation is described as "global", its arrangement in tumor genomes may adopt specific patterns (51). Researchers have found some regions, even transcription control regions, with the loss of DNA methylation related with cancer. There is an increasing number of genes that are normally methylated at promoters but which are hypomethylated and activated in tumors. For instance, the gene encoding the protease urokinase (*PLAU/uPA*) is overexpressed and hypomethylated in breast and prostate cancers (52, 53). In ovarian cancer, many cancer-associated genes reactivated by promoter DNA hypomethylation have been found: neuronal protein synuclein-gamma (*SNCG*); CCCTC-binding factor (zinc finger protein)-like (*CTCFL/BORIS*); Claudin-3 (*CLDN3*); Claudin-4 (*CLDN4*); DnaJ (Hsp40) homolog, subfamily C, member 15 (*DNAJC15[MCJ]*); T-cell differentiation protein (*MAL*); Homeobox A10 (*HOXA10*); and Class III β-tubulin (*TUBB3*) [see review by Kwon et al. (54)]. In pancreatic ductal adenocarcinoma, overexpression of S100 calcium binding protein A4 (*S100A4*) is associated with poor differentiation and DNA hypomethylation (55). Other examples include *R-RAS* in gastric cancer, melanoma antigen family A, 1 (*MAGE1*) in melanoma and also in gastric cancer. Another example of hypomethylation promoting tumor cell-growth is the paired box 2 (*PAX2*) gene in endometrial cancer (56).

Furthermore, aberrant DNA hypomethylation may induce chromosomal instability during multistage carcinogenesis and cause loss of imprinting, as in the well-known case of the encoding insulin-like growth factor-2 (*IGF2*) (42, 57).

Some DNA sequences show "opposite methylation levels" depending on the type of tumor (58). For example, *NBL2* (a DNA repeat) is hypermethylated in some cancers, but hypomethylated in others (59). This phenomenon has also been

found in the pericentromeric repeat D4Z4 (60). In addition, hypomethylation of juxtacentromeric *Sat2* repeat is observed in stomach and breast cancer, but not in colon cancer (61, 62).

More recently, hypomethylation has been found to directly cause the activation of a particular group of germline-specific genes that rely on methylation for repression in normal somatic tissues (63): the cancer-germline (*CG*) or cancer-testis (*CT*) genes. They are associated with transcription activation, which correlates with overall genome hypomethylation (46, 64, 65).

Thanks to the identification of single-copy genes (such as these *CG* or *CT* genes), DNA hypomethylation in tumors may now be further explored, since the experimental difficulties caused by the characteristic repeat elements where hypomethylation is also found can be avoided.

Methods to analyze global hypomethylation and their clinical significance

Healthy cells present specific DNA methylation patterns, and alterations of these patterns can lead to diseases, including cancer. This could be of potential clinical use at three levels: detection, prognosis and prediction of treatment responses (66). In addition, methylation status is currently used to classify and characterize cancers (67). In recent years there have been an increasing number of global DNA methylation approaches with the purpose of identifying aberrant methylation signatures. Almost all of them are based on the biological or chemical differentiation of methylated and unmethylated cytosines as a first step and then on the amplification of the differences between methylated and unmethylated DNA sequences. The combination of these methods with DNA sequencing technologies may be used to identify useful biomarkers for cancer research. The classification of patients using specific biomarkers is becoming the best way to subdivide the same tumor type (68).

As there are more studies on promoter hypermethylation than global hypomethylation, most of the current markers are based on aberrant DNA hypermethylation signatures. However, some studies have shown that global hypomethylation could also be useful. A challenge to global hypomethylation becoming a useful clinical marker will be to define, clarify and standardize the methodologies for its high-throughput determination in patients. Here we review some of the technologies used to analyze global DNA methylation and discuss the advantages and disadvantages for their clinical use. We classify these technologies as: 1. global approaches for the detection of gross DNA methylation, 2. locus-specific methods for the analysis of specific methylated CpG regions, and 3. genome-wide approaches developed to identify methylation hot-spots in the whole genome sequence (Figure 1).

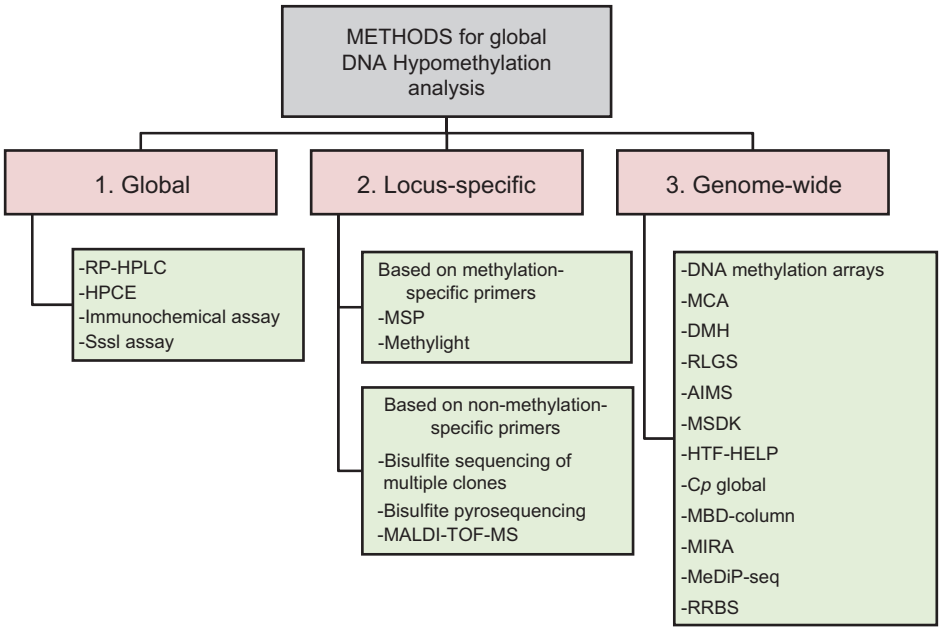

Figure 1 Flowchart summarizing the reviewed techniques for genomic DNA hypomethylation analysis.
The approaches were classified as global (1), locus-specific (2) and genome-wide (3). In the case of locus-specific approaches, techniques were grouped by the use of methylation-specific primers or not. Reverse-Phase high-performance liquid chromatography (RP-HPLC), high performance capillary electrophoresis (HPCE), methyl group acceptance assay (Sssl assay), methylation-specific PCR (MSP), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), methylation CpG island amplification (MCA), differential methylation hybridization (DMH), restriction-landmark genomic scanning (RLGS), amplification of inter-methylated sites (AIMS), methylation-specific digital karyotyping (MSDK), HPAII tiny fragment enrichment by ligation-mediated PCR (HTF-HELP), methylated DNA binding column (MBD column), methylated CpG island recovery assay (MIRA), methyl-DNA immunoprecipitation and sequencing (MeDIP-seq) and Reduced Representation Bisulfite Sequencing (RRBS).

Global approaches

In general, these methods involve the hydrolysis of the genomic DNA followed by the separation of nucleotides, bases, etc. by chromatography or similar analytical technology. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a chromatographic technique that utilizes non-polar stationary phases and mobile phases with different polarity grades. These phases allow for the separation of compounds according to their polarity which are then quantified by their UV absorbance at 254 and 280 nm. RP-HPLC is one of the earliest methylation analysis methods, described in 1980 by Kuo et al., who used it to measure DNA methylation levels of calf thymus and salmon sperm (69). The deoxyribonucleosides are separated based on their solubility in the mobile polar phase. With this technique, Gama-Sosa et al. demonstrated that DNA methylation levels decreased in human tumors (24). However, this approach sometimes shows low specificity and, furthermore, close attention must be paid to buffer composition and elution temperature, although the combination of HPLC with mass spectrometry (MS) allows for the identification of the bases in a more selective, sensitive and accurate way (70). An alternative to the HPLC is the high-performance capillary electrophoresis (HPCE) (71). This approach, following the hydrolysis of the DNA, separates and quantifies cytosine and methyl-cytosine by the use of an SDS micelle system, in which the order of nucleoside elution depends on their separation in the micelles. Whilst it is faster and cheaper than HPLC, the low injection volume, the high cost of the equipment and the quantity of good quality DNA required makes the medical use of this method difficult (Figure 2).

Global DNA methylation can also be measured by immunofluorescence using antibodies against 5-methylcytosine followed by incubation with fluorescein secondary antibodies. The first antibodies against 5-methylcytosine were developed by Achwal and Chandra (72) but over time, others raised improved antibodies against 5-methylcytosine (73) and currently, there are commercial kits available to measure global DNA methylation by immunofluorescence and quantify through an ELISA-like reaction. This technique can, however, show a fair amount of variability due to the different quality of antibodies used (74).

Another technique is the methyl group acceptance assay (Sssl assay) based on the activity of the bacterial CpG methyltransferase Sssl. This assay permits the evaluation of the genomic DNA methylation level by methylating specifically all unmethylated CpG dinucleotides using radiolabeled methyl groups (75). The use of radioactivity makes this approach unsuitable for clinical use.

Locus-specific approaches

As the DNA methylation status of specific repeated DNA loci reflects the global DNA methylation (45–47) an alternative way to measure the global DNA methylation levels of a sample is to analyze its locus-specific methylation status at repeated elements.

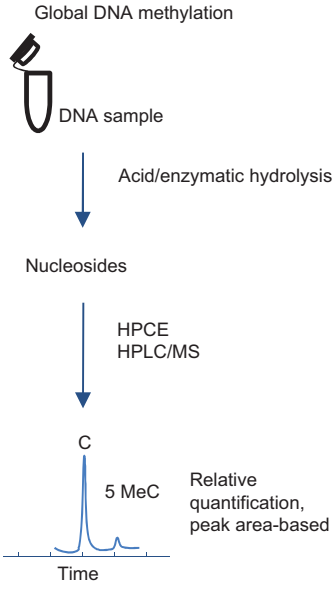

Figure 2 Simplified representation of the two alternative procedures used for quantification of global DNA methylation levels. DNA samples are hydrolyzed with acid or enzymes and then the nucleosides are analyzed by reverse-phase high-performance liquid chromatography combined with mass spectrometry (HPLC/MS) or high-performance capillary electrophoresis (HPCE). The peak area represents the amount of unmethylated cytosine (C) and 5-methylcytosine (5-MeC).

Most techniques to do this involve bisulfite modification as a first step. This modification converts unmethylated cytosine into uracil, while methylated cytosine remains unchanged. During PCR amplification a thymine nucleotide is incorporated into the PCR product for every uracil present in the bisulfite-treated template and a cytosine is introduced for every 5-methylcytosine in the template. In this way, the unmethylated and methylated PCR product can be distinguished by the different C or T content at CpG sites There are then two main alternatives which can be used to reveal changes in DNA methylation: designing primers which overlap CpG dinucleotides (methylation-specific primers) or designing non-overlapping primers (Non-Methylation-specific primers) with no CpG in the primer sequence and which are able to amplify both methylated and unmethylated alleles with equal efficiency. A critical point in both methods is the accurate bisulfite conversion necessary before analysis (76). Most of these techniques have been used to characterize the DNA methylation status of single copy genes but could also be used to analyze repeated DNA elements, such as *LINE-1* or *Alu* (77–79).

One of the best known methods based on methylation-specific primers is methylation-specific PCR (MSP) (80). This method relies on two sets of primers, one complementary to the methylated DNA (with unmodified cytosines after bisulfite treatment) and the other complementary to the unmethylated DNA (with cytosines modified to uracils after bisulfite treatment). PCR products are detected by gel electrophoresis

without the need for further restriction or sequencing analysis. MSP is of limited utility to determine global DNA methylation as it principally provides qualitative data. In contrast, MethyLight (81) combines the basis of MSP with real-time detection of the amplicons generated and thus provides quantitative data. This method has been used to measure DNA methylation levels of *LINE-1* in ovarian cancers (78) and DNA methylation levels of *LINE-1*, *Sat2* and *Alu* in girls with a family history of breast cancer (82).

The most widely used methods to determine global DNA methylation as a measure of the DNA methylation status of a repeated DNA element are those based on non-methylation specific primers. Of these, one of the first optimized and most extensively used assay is the bisulfite sequencing of multiple clones (83). This method is based on the sequencing of specific DNA regions in order to obtain data about the C or T content of the CpG sites of the fragment analyzed which in turn gives information about the methylation status of a CpG site. Bisulfite-converted DNA is amplified with specific primers by PCR and the resulting PCR products are cloned and the individual clones are then sequenced. The methylation percentage at each CpG position can be derived from the number of sequenced clones (10–20 colonies) that display methylated and/or unmethylated cytosines. Primer design is one of the most critical steps in this technique, since the complexity of the DNA is reduced after the bisulfite treatment and the increase in the number of thymines causes polymerase mistakes in many cases. This method, although extremely robust, is not cost effective and thus difficult to apply in routine clinical analyses.

An alternative to bisulfite sequencing of multiple clones is bisulfite pyrosequencing. It is a method of DNA sequencing based on the enzymatic synthesis of a single DNA strand and the luminometric detection of pyrophosphate release following nucleotide incorporation. This recently developed method has been adapted for DNA methylation studies (84). Apart from bisulfite conversion, a preliminary PCR amplification is needed, in which one of the primers carries a biotin label at its 5 -end, which purifies the PCR product. The sequencing primer complementary to the single-stranded template is then hybridized to the template and the pyrosequencing reaction is carried out by adding single nucleotides in a defined sequence. The complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in a pyrogram. Pyrosequencing provides robust quantitative data and is a fast and easy but reliable, and reproducible technique to use, which is why it has become one of the most popular tools for measuring the amount of DNA methylation at the locus-specific level. Moreover, this approach uses controls to check the bisulfite conversion efficiency, making it a good choice for clinical use. As such, it has been used to diagnose fetal and placental disorders by analyzing the DNA methylation of imprinted genes (85) as well as to determine the association between *LINE-1* DNA methylation levels and risk of renal cell cancer and with survival of melanoma patients (79, 86).

Locus-specific DNA methylation can also be analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (87–90). DNA is modified by bisulfite treatment, is PCR-amplified and the products transcribed in vitro into single-stranded RNA molecules. Afterwards, the ribonucleotides are cleaved using an endoribonuclease. Bisulfite conversion of unmethylated cytosine into uracil generates base-specific cleavage products that reflect underlying methylation patterns and that can be easily analyzed by MALDI-TOF-MS. In addition, the degree of methylation can be simultaneously determined at different CpG positions in a single reaction. A chip array can be used to obtain quantitative results (with an automated, high-performance analysis) from small amounts of starting material. Its principal disadvantage is the need for sophisticated equipment which makes it difficult to use in clinical analyses.

Genome-wide approaches

It is obvious that the best way to determine the global methylation status of samples is to analyze the methylation status of a large number of single CpG sites. These types of analyses are currently possible thanks to the next generation sequencing and DNA array-based technologies.

A type of DNA array particularly useful in DNA methylation analyses are the DNA methylation arrays. Methylation arrays use bisulfite modified DNA and determine the methylation status of single CpG sites using fluorescent assays. The development of different types of microarray-based methylation analysis, such as Illumina®, HumanMethylation27 DNA Analysis BeadChip, the Golden Gate Methylation Cancer Panel and VeraCode platforms have enabled the definition of methylation profiles in many tumors (91–94). Further microarray-based methylation studies comparing tumors will be required to evaluate the variation in hypomethylation patterns between individual tumor types in order for them to be exploited as prognostic markers. To date, these global DNA methylation screenings have helped to identify single markers with great clinical potential, such as predictors of recurrence in lung cancer (95), metastasis in colorectal cancer (96) or progression in virus-associated neoplasms (97).

Genome-wide DNA methylation can also be studied using non-bisulfite-based methods. Examples include methods based on the digestion of DNA with restriction enzymes, such as methylation CpG island amplification (MCA) (98), differential methylation hybridization (DMH) (99), restriction-landmark genomic scanning (RLGS) (100), amplification of inter-methylated sites (AIMS) (101), methylation-specific digital karyotyping (MSDK) (102), HPAII tiny fragment enrichment by ligation-mediated PCR (HELP) assay (103) and *CpG*lobal (104). All of which have been used in cancer studies, e.g., the application of *CpG*lobal to measure the changes in global DNA methylation in lung cancer resulted in substantial DNA methylation differences between healthy and tumor tissues (104).

Other approaches include those based on the affinity of MBD proteins, such as methylated DNA binding column (MBD column) chromatography, in which methylated and unmethylated DNA fragments can be discriminated and separated using methyl-CpG-binding protein 2 (MeCP2) and then analyzed with appropriate PCR primers (105). However, it

Table 1 Comparison of methylation arrays vs. ultra-deep sequencing at base resolution for DNA methylation analysis.

	Methylation arrays	Ultra-deep sequencing
CpG coverage	+	+++
Sensitivity	+++	++/+ (antibody-based)
Time consuming	++	++
Data analysis	+++	+
High-throughput	+++	+
Price	++	+/++ (price decreasing)

only results in a rough determination of DNA methylation status which must be compared with elution profiles of standard DNA (106). MBD column chromatography could be used in clinical studies for the detection of DNA methylation biomarkers in colorectal cancer (107). Another example derived from the affinity of MBD proteins is the "methylated CpG island recovery assay" (MIRA), which recognizes methylated CpG dinucleotides via the methyl-CpG-binding domain protein 2b (MBD2b) (108). This method is quite simple to use and yields few false-positives making it useful in genome-wide analysis in cancers (74).

Finally, the currently widely used methods based on next generation sequencing technology produce tremendous amounts of information on DNA methylation. Examples include the combination of this ultra-deep sequencing with methyl-DNA immunoprecipitation (MeDiP-seq) or its combination with digestion using methylation sensitive restriction enzymes (reduced representation bisulfite sequencing [RRBS]). Both are effective methods to analyze methylomes; RRBS has been used to analyze mammalian methylomes, and MeDiP-seq for human tumor and sperm methylomes (77, 109). However, MeDiP-seq suffers from the same dependence on antibody quality as the MeDiP approach, and RRBS offers less genome-wide coverage (74, 110).

In general, genome-wide approaches are very useful tools to measure global DNA methylation and to identify biomarkers with clinical outcome, but they are still too expensive and need to be improved before they can be routinely used in clinical studies (Table 1).

Concluding remarks

There are many approaches which can be applied to the analysis of global DNA methylation. Although each one has specific advantages and disadvantages, it is our view that sensitivity, reproducibility and the possibility of high-throughput analyses makes the bisulfite pyrosequencing one of the better choices for global DNA methylation analyses in a clinical context.

Acknowledgments

We would like to thank OIB (FYCIT) and M. Santirso for editorial assistance. EGT received support from FICYT (BP11138). AFF is supported by the IUOPA and Miguel Servet program. This work was supported by grants from the Spanish Ministry of Health (PI061267, PS09/02454), the Spanish National Research Council (CSIC 200820I172 to MFF), and the Community of Asturias (FICYT IB09-106). The IUOPA (Instituto Universitario de Oncología del Principado de Asturias) is supported by the Obra Social Cajastur, Spain.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

References

1. Waddington CH. The epigenotype. Endeavour 1942;1:18–20.
2. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes Dev 2009;23:781–3.
3. Wesley H, Brooks CL, Pers J-O, Youinou P, Renaudineau Y. Epigenetics and autoimmunity. J Autoimmun 2010;34:J207–19.
4. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358: 1148–59.
5. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
6. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell 2007;128:669–81.
7. Ellis L, Atadja PW, Johnstone RW. Epigenetics in cancer: targeting chromatin modifications. Mol Cancer Ther 2009;8:1409–20.
8. Lujambio A, Portela A, Liz J, Melo SA, Rossi S, Spizzo R, et al. CpG island hypermethylation-associated silencing of non-coding RNAs transcribed from ultraconserved regions in human cancer. Oncogene 2010;29:6390–401.
9. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286–98.
10. Stancheva I, Meehan RR. Transient depletion of xDnmt1 leads to premature gene activation in Xenopus embryos. Genes Dev 2000;14:313–27.
11. Beard C, Li E, Jaenisch R. Loss of methylation activates Xist in somatic but not in embryonic cells. Genes Dev 1995;9:2325–34.
12. Walsh CP, Chaillet JR, Bestor TH. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat Genet 1998;20:116–7.
13. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. Nature 1993;366:362–5.
14. Haaf T, Schmid M. Experimental condensation inhibition in constitutive and facultative heterochromatin of mammalian chromosomes. Cytogenet Cell Genet 2000;91:113–23.
15. Denis H, Ndlovu MN, Fuks F. Regulation of mammalian DNA methyltransferases: a route to new mechanisms. EMBO Rep 2011;12:647–56.
16. Robertson KD. DNA methylation, methyltransferases, and cancer. Oncogene 2001;20:3139–55.
17. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev 2002;16:6–21.
18. Wang Y, Leung FC. An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 2004;20:1170–7.
19. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 2008;9:465–76.

20. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Annu Rev Biochem 2005;74:481–514.
21. Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H, et al. A DNA methylation fingerprint of 1628 human samples. Genome Res 2012;22:407–19.
22. Issa JP. The epigenetics of colorectal cancer. Ann N Y Acad Sci 2000;910:140–53; discussion 53–5.
23. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983;301:89–92.
24. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 1983;11:6883–94.
25. Feinberg AP. Cancer epigenetics is no Mickey Mouse. Cancer Cell 2005;8:267–8.
26. Esteller M. The necessity of a human epigenome project. Carcinogenesis 2006;27:1121–5.
27. Herman JG. Hypermethylation of tumor suppressor genes in cancer. Semin Cancer Biol 1999;9:359–67.
28. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61: 3225–9.
29. Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 2006;310:251–74.
30. Esteller M. Relevance of DNA methylation in the management of cancer. Lancet Oncol 2003;4:351–8.
31. Bhalla KN. Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. J Clin Oncol 2005;23: 3971–93.
32. Das PM, Singal R. DNA methylation and cancer. J Clin Oncol 2004;22:4632–42.
33. Herranz M, Esteller M. DNA methylation and histone modifications in patients with cancer: potential prognostic and therapeutic targets. Methods Mol Biol 2007;361:25–62.
34. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002;21:5400–13.
35. Morey SR, Smiraglia DJ, James SR, Yu J, Moser MT, Foster BA, et al. DNA methylation pathway alterations in an autochthonous murine model of prostate cancer. Cancer Res 2006;66:11659–67.
36. Hoffmann MJ, Schulz WA. Causes and consequences of DNA hypomethylation in human cancer. Biochem Cell Biol 2005;83:296–321.
37. Rauch TA, Zhong X, Wu X, Wang M, Kernstine KH, Wang Z, et al. High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci USA 2008;105:252–7.
38. Ehrlich M. DNA hypomethylation in cancer cells. Epigenomics 2009;1:239–59.
39. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300:489–92.
40. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, et al. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 2007;16:108–14.
41. Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, et al. Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. Lancet Oncol 2008;9:359–66.
42. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. Biochim Biophys Acta 2007;1775:138–62.
43. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860–921.
44. Jordan IK, Rogozin IB, Glazko GV, Koonin EV. Origin of a substantial fraction of human regulatory sequences from transposable elements. Trends Genet 2003;19:68–72.
45. Kochanek S, Renz D, Doerfler W. DNA methylation in the Alu sequences of diploid and haploid primary human cells. EMBO J 1993;12:1141–51.
46. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, et al. Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 2004;95:58–64.
47. Ehrlich M, Woods CB, Yu MC, Dubeau L, Yang F, Campan M, et al. Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 2006;25:2636–45.
48. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005;33:6823–36.
49. Cho NY, Kim BH, Choi M, Yoo EJ, Moon KC, Cho YM, et al. Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol 2007;211:269–77.
50. Estecio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, He R, et al. LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. PLoS One 2007;2:e399.
51. De Smet C, Loriot A. DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. Epigenetics 2010;5: 206–13.
52. Pakneshan P, Tetu B, Rabbani SA. Demethylation of urokinase promoter as a prognostic marker in patients with breast carcinoma. Clin Cancer Res 2004;10:3035–41.
53. Pulukuri SM, Estes N, Patel J, Rao JS. Demethylation-linked activation of urokinase plasminogen activator is involved in progression of prostate cancer. Cancer Res 2007;67:930–9.
54. Kwon MJ, Shin YK. Epigenetic regulation of cancer-associated genes in ovarian cancer. Int J Mol Sci 2011;12:983–1008.
55. Rosty C, Ueki T, Argani P, Jansen M, Yeo CJ, Cameron JL, et al. Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. Am J Pathol 2002;160:45–50.
56. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;7:21–33.
57. Cui H, Onyango P, Brandenburg S, Wu Y, Hsieh CL, Feinberg AP. Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. Cancer Res 2002;62:6442–6.
58. Nishiyama R, Qi L, Lacey M, Ehrlich M. Both hypomethylation and hypermethylation in a 0.2-kb region of a DNA repeat in cancer. Mol Cancer Res 2005;3:617–26.
59. Nishiyama R, Qi L, Tsumagari K, Weissbecker K, Dubeau L, Champagne M, et al. A DNA repeat, NBL2, is hypermethylated in some cancers but hypomethylated in others. Cancer Biol Ther 2005;4:440–8.
60. Tsumagari K, Qi L, Jackson K, Shao C, Lacey M, Sowden J, et al. Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers. Nucleic Acids Res 2008;36:2196–207.
61. Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, Hirohashi S. DNA methyltransferase expression and DNA methylation of CPG

islands and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer 2001;91:205–12.
62. Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 1998;77:833–8.
63. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 1999;19:7327–35.
64. Cadieux B, Ching TT, VandenBerg SR, Costello JF. Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 2006;66:8469–76.
65. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci USA 1996;93:7149–53.
66. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med 2011;17:330–9.
67. Tost J. DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. Mol Biotechnol 2010;44:71–81.
68. Costa FF. Epigenomics in cancer management. Cancer Manag Res 2010;2:255–65.
69. Kuo KC, McCune RA, Gehrke CW, Midgett R, Ehrlich M. Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. Nucleic Acids Res 1980;8:4763–76.
70. Annan RS, Kresbach GM, Giese RW, Vouros P. Trace detection of modified DNA bases via moving-belt liquid chromatography-mass spectrometry using electrophoric derivatization and negative chemical ionization. J Chromatogr 1989;465:285–96.
71. Fraga MF, Rodriguez R, Canal MJ. Rapid quantification of DNA methylation by high performance capillary electrophoresis. Electrophoresis 2000;21:2990–4.
72. Achwal CW, Chandra HS. A sensitive immunochemical method for detecting 5mC in DNA fragments. FEBS Lett 1982;150:469–72.
73. Braunschweig MH. Quantification of global DNA methylation with infrared fluorescence in liver and muscle tissues of differentially fed boars. Luminescence 2009;24:213–6.
74. Mitchell N, Deangelis JT, Tollefsbol TO. Methylated-CpG Island Recovery Assay. Methods Mol Biol 2011;791:125–33.
75. Balaghi M, Wagner C. DNA methylation in folate deficiency: use of CpG methylase. Biochem Biophys Res Commun 1993;193:1184–90.
76. Harrison J, Stirzaker C, Clark SJ. Cytosines adjacent to methylated CpG sites can be partially resistant to conversion in genomic bisulfite sequencing leading to methylation artifacts. Anal Biochem 1998;264:129–32.
77. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, et al. Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 2011;21:515–24.
78. Zeimet AG, Fiegl H, Goebel G, Kopp F, Allasia C, Reimer D, et al. DNA ploidy, nuclear size, proliferation index and DNA-hypomethylation in ovarian cancer. Gynecol Oncol 2011;121:24–31.
79. Liao LM, Brennan P, van Bemmel DM, Zaridze D, Matveev V, Janout V, et al. LINE-1 methylation levels in leukocyte DNA and risk of renal cell cancer. PLoS One 2011;6:e27361.
80. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–6.
81. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.
82. Wu HC, John EM, Ferris JS, Keegan TH, Chung WK, Andrulis I, et al. Global DNA methylation levels in girls with and without a family history of breast cancer. Epigenetics 2011;6:29–33.
83. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 1992;89:1827–31.
84. Tost J, Dunker J, Gut IG. Analysis and quantification of multiple methylation variable positions in CpG islands by pyrosequencing. Biotechniques 2003;35:152–6.
85. Bourque DK, Peñaherrera, MS, Yuen RK, Van Allen MI, McFadden DE, Robinson WP. The utility of quantitative methylation assays at imprinted genes for the diagnosis of fetal and placental disorders. Clin Genet 2011;79:169–75.
86. Sigalotti L, Fratta E, Bidoli E, Covre A, Parisi G, Colizzi F, et al. Methylation levels of the "long interspersed nucleotide element-1" repetitive sequences predict survival of melanoma patients. J Transl Med 2011;9:78.
87. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 1988;60:2299–301.
88. Tanaka K. Protein and polymer analyses up to mlz 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1988;2:151–3.
89. Tost J, Schatz P, Schuster M, Berlin K, Gut IG. Analysis and accurate quantification of CpG methylation by MALDI mass spectrometry. Nucleic Acids Res 2003;31:e50.
90. Sauer S, Lechner D, Berlin K, Lehrach H, Escary JL, Fox N, et al. A novel procedure for efficient genotyping of single nucleotide polymorphisms. Nucleic Acids Res 2000;28:E13.
91. Kanduri M, Cahill N, Goransson H, Enstrom C, Ryan F, Isaksson A, et al. Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia. Blood 2010;115:296–305.
92. Martin-Subero JI, Ammerpohl O, Bibikova M, Wickham-Garcia E, Agirre X, Alvarez S, et al. A comprehensive microarray-based DNA methylation study of 367 hematological neoplasms. PLoS One 2009;4:e6986.
93. Hernandez-Vargas H, Lambert MP, Le Calvez-Kelm F, Gouysse G, McKay-Chopin S, Tavtigian SV, et al. Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. PLoS One 2010;5:e9749.
94. Martin-Subero JI, Kreuz M, Bibikova M, Bentink S, Ammerpohl O, Wickham-Garcia E, et al. New insights into the biology and origin of mature aggressive B-cell lymphomas by combined epigenomic, genomic, and transcriptional profiling. Blood 2009;113:2488–97.
95. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, et al. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med 2008;358:1118–28.
96. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, et al. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 2008;105:13556–61.
97. Fernandez AF, Rosales C, Lopez-Nieva P, Grana O, Ballestar E, Ropero S, et al. The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. Genome Res 2009;19:438–51.

98. Toyota M, Ho C, Ahuja N, Jair KW, Li Q, Ohe-Toyota M, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999;59:2307–12.
99. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. Hum Mol Genet 1999;8:459–70.
100. Rush LJ, Plass C. Restriction landmark genomic scanning for DNA methylation in cancer: past, present, and future applications. Anal Biochem 2002;307:191–201.
101. Frigola J, Ribas M, Risques RA, Peinado MA. Methylome profiling of cancer cells by amplification of inter-methylated sites (AIMS). Nucleic Acids Res 2002;30:e28.
102. Hu M, Yao J, Polyak K. Methylation-specific digital karyotyping. Nat Protoc 2006;1:1621–36.
103. Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, et al. Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res 2006;16:1046–55.
104. Anisowicz A, Huang H, Braunschweiger KI, Liu Z, Giese H, Wang H, et al. A high-throughput and sensitive method to measure global DNA methylation: application in lung cancer. BMC Cancer 2008;8:222.
105. Cross SH, Charlton JA, Nan X, Bird AP. Purification of CpG islands using a methylated DNA binding column. Nat Genet 1994;6:236–44.
106. Shiraishi M, Sekiguchi A, Oates AJ, Terry MJ, Miyamoto Y, Tanaka K, et al. Variable estimation of genomic DNA methylation: a comparison of methyl-CpG binding domain column chromatography and bisulfite genomic sequencing. Anal Biochem 2002;308:182–5.
107. Zou H, Harrington J, Rego RL, Ahlquist DA. A novel method to capture methylated human DNA from stool: implications for colorectal cancer screening. Clin Chem 2007;53:1646–51.
108. Rauch T, Pfeifer GP. Methylated-CpG island recovery assay: a new technique for the rapid detection of methylated-CpG islands in cancer. Lab Invest 2005;85:1172–80.
109. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A. High-throughput bisulfite sequencing in mammalian genomes. Methods 2009;48:226–32.
110. Harris RA, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL, et al. Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. Nat Biotechnol 2010;28:1097–105.

Estela G. Toraño is a first year PhD student in Cancer Epigenetics under the supervision of Dr. Mario F. Fraga. She has a fellowship from FICYT (Fundación para el fomento en Asturias de la investigación científica aplicada y la tecnología). Before she began her phD, she graduated in Biology and enrolled a master in cancer research at the University of Oviedo. Her work is focused on changes in post-translational modifications of histones during cellular differentiation and tumorigenesis. Also, she works in the study of methylomes of cancer and is familiar with the methods to analyze DNA methylation.

Originally from Menorca (Spain), graduated in Biotechnology and currently Biomedical Sciences master student (Cancer Genomics and Developmental Biology Programme) at the University of Utrecht (The Netherlands). Relevant research experience in genetic epidemiology of bladder cancer, epigenetics in monozygotic twins and developmental biology of blood endothelial cells in zebrafish. Awarded with a Summer-Training fellowship by the *Spanish National Cancer Research Centre* (CNIO) in 2010 and with a Collaboration grant from the *Spanish Ministry of Education and Science* (MEC) in 2010–2011.

Agustin F. Fernandez is, at present, a Miguel Servet researcher at the University Institute of Oncology of Asturias (IUOPA) where he works in the Cancer Epigenetics laboratory located at the Asturias Central University Hospital (HUCA), Oviedo, Spain. He received his PhD in Genetics from the University of Oviedo, and worked as postdoctoral fellow in the Laboratory of Cancer Epigenetics at the Spanish National Cancer Research Centre (CNIO), Madrid, Spain, and, later, at the Bellvitge Institute for Biomedical Research (IDIBELL), Barcelona, Spain. His main research areas are in the field of epigenetics and comprise the epigenetic mechanisms involved in cell differentiation, and their alterations in aging and different pathologies including cancer.

Mario F. Fraga is a Tenured Scientist at the National Centre of Biotechnology (CNB-CSIC) and the Head of the Cancer Epigenetics Laboratory at the Institute of Oncology of Asturias (IUOPA), Spain. He received his PhD in plant epigenetics from the University of Oviedo, and was a "Ramon y Cajal" postdoctoral fellow at the Spanish National Cancer Research Centre (CNIO), where he focused his research on cancer epigenetics and the study of human interindividual epigenetic variation. The research interests of his team are epigenetic processes involved in cell differentiation and their alterations in cancer and aging.