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Cancer epigenetics reaches mainstream oncology

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Epigenetics is one of the most promising and expanding fields in the current biomedical research landscape. Since the inception of epigenetics in the 1940s, the discoveries regarding its implications in normal and disease biology have not stopped, compiling a vast amount of knowledge in the past decade. The field has moved from just one recognized marker, DNA methylation, to a variety of others, including a wide spectrum of histone modifications. From the methodological standpoint, the successful initial single gene candidate approaches have been complemented by the current comprehensive epigenomic approaches that allow the interrogation of genomes to search for translational applications in an unbiased manner. Most important, the discovery of mutations in the epigenetic machinery and the approval of the first epigenetic drugs for the treatment of subtypes of leukemias and lymphomas has been an eye-opener for many biomedical scientists and clinicians. Herein, we will summarize the progress in the field of cancer epigenetics research that has reached mainstream oncology in the development of new biomarkers of the disease and new pharmacological strategies.

Introduction to epigenetics and its biological roles

When Conrad Waddington coined the word 'epigenetics' (literally 'over' or 'upon' genetics) in the early 1940s, the term was used 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]. But the word currently refers specifically to the study of mitotically and/or meiotically heritable changes in gene expression that occur without changes in the DNA sequence[2]. The disruption of such changes underlies a wide variety of pathologies, including cancer[3,4]. Epigenetic regulation includes DNA methylation (**Fig. 1**) and covalent histone modifications (**Fig. 2**), and we will discuss only these two epigenetic layers here.

DNA methylation usually takes place at the 5′ position of the cytosine ring within CpG dinucleotides, and its consequence is the silencing of genes and noncoding genomic regions. There are three main DNA methyltransferases (DNMTs): DNMT1, which maintains the existing methylation patterns following DNA replication, and DNMT3A and DNMT3B, *de novo* enzymes that target previously unmethylated CpGs[5]. CpG sites are concentrated either in CpG islands, short CpG-rich DNA regions located in approximately 60% of human gene promoters, or in regions of large repetitive sequences (for example, centromeres and retrotransposon elements)[5,6]. Although in the latter case most of the CpGs are methylated to prevent chromosome instability, the majority of CpG islands remain unmodified during development and in differentiated tissues[7]. Nevertheless, naturally occurring CpG island methylation takes place during developmental phenomena such as X chromosome inactivation or genomic imprinting[5]. Further investigation will be needed to elucidate additional roles of DNA methylation in non–CpG island promoters and in the origin and maintenance of pluripotency[8,9]. Recent findings also suggest that extensive DNA methylation changes caused by differentiation take place at CpG island 'shores', regions of comparatively low CpG density close to CpG islands[10,11]. Additionally, almost one-quarter of all DNA methylation found in embryonic stem (ES) cells occurs in a non-CpG context[12]. Finally, 5-methylcytosine (5-mC) can be converted into 5-hydroxymethylcytosine (5-hmC) by the 2-oxoglutarate– and Fe(II)-dependent oxygenases TET1, TET2 and TET3 (ref. 13). It will be necessary to gain insight into the role of this recently described modification, detected in ES cells and Purkinje neurons and involved in ES cell self renewal and embryonic inner cell mass specification[14].

Histones can undergo multiple post-translational modifications[15], which mainly occur along their N-terminal tails. The enzymes that add and remove such modifications are, respectively, histone acetyltransferases (HATs) and deacetylases (HDACs and sirtuins), methyltransferases (HMTs) and demethylases (HDMs), kinases and phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases, and so on[15,16]. Genome-wide studies have revealed that various combinations of modifications in a specific genomic region can lead, like a 'histone code', to a more 'open' or 'closed' state of chromatin structure and, therefore, to the activation or repression of gene expression[17]. For instance, trimethylation of lysines (K) 4, 36 or 79 on H3 (H3K4me3, H3K36me3 and H3K79me3, respectively),

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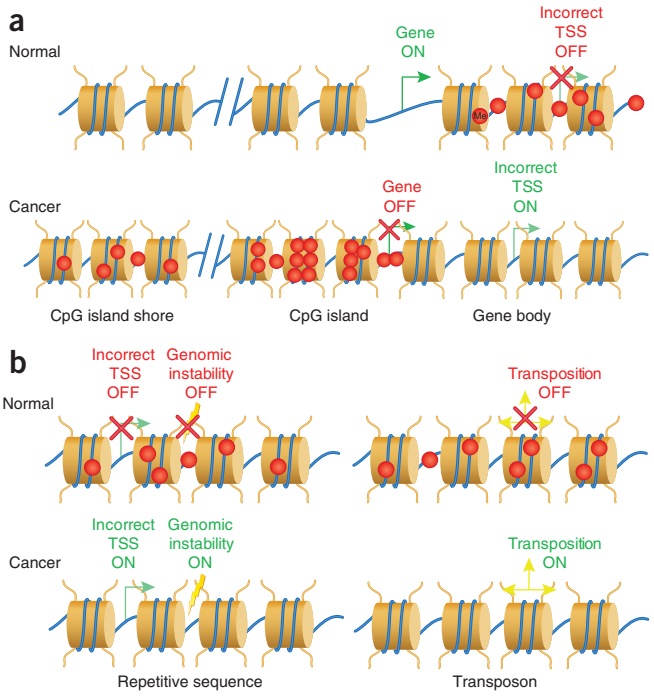

Figure 1 DNA methylation patterns in normal and cancer cells. DNA methylation takes place along the whole genome, and its disruption is a typical hallmark of cancer. (**a**) In normal cells (top), CpG islands and CpG island shores usually remain unmethylated, allowing gene transcription. Additionally, DNA methylation within the gene bodies avoids spurious transcription initiations. In cancer cells (bottom), by contrast, although both CpG islands and CpG island shores may be strongly methylated, gene bodies lack this modification. As a result, transcription of many genes gets blocked, and aberrant transcription may occur from incorrect transcription start sites (TSSs). (**b**) In normal cells (top), methylation of repetitive sequences prevents genomic instability and, again, spurious transcription initiations. Moreover, transposable elements cannot be activated in a methylated environment. In cancer cells (bottom), global hypomethylation triggers genomic instability and aberrant transcription initiations. Concomitant activation of transposons may lead to gene disruption.

monomethylation of H4K20 and H2BK5 (H4K20me and H2BK5me), and acetylation of H3K9 and H3K14 (H3K9ac and H3K14ac) result in gene activation, whereas di or trimethylation of H3K9 (H3K9me2 and H3K9me3) and trimethylation of H3K27 (H3K27me3) lead to gene repression[17–19]. In ES cells, key developmental genes remain poised for lineage-specific activation or repression as a result of their bivalent domains, a combination of two modifications in their promoter regions, H3K4me3 and H3K27me3, with typically opposite meanings[20].

Notably, all epigenetic processes work together to establish and maintain the global and local condensed or decondensed chromatin states that eventually determine gene expression. The continuous interplay of all these processes creates what Waddington called the 'epigenetic landscape' and today we call the 'epigenome'—the epigenetic status that determines the way a single eukaryotic genome may manifest itself in different cell types and developmental stages and that, if aberrant, gives rise to cancer and other diseases.

Epigenetic modifications in cancer

Cancer and many other human diseases show aberrant epigenetic regulation[21]. In particular, the cancer epigenome is characterized by global changes in DNA methylation and altered histone modification patterns. Because typical features such as global DNA hypomethylation and promoter-specific hypermethylation can be commonly observed in benign neoplasias and early-stage tumors, it is becoming apparent that epigenetic deregulation may precede the classical preliminary transforming events: mutations in tumor suppressors, protooncogenes, or both, and genomic instability[22]. Disruption of the epigenetic machineries, either by mutation, deletion or the altered expression of any of their components, is known to provoke aberrant gene expression patterns that give rise to all typical cancer characteristics[3]. In fact, these 'epimutations' sometimes provide the second hit for cancer initiation postulated by the two-hit model, as they can silence the remaining active allele of previously mutated tumor suppressors[23].

In terms of DNA methylation, cancer cells show genome-wide hypomethylation and site-specific CpG island promoter hypermethylation[3]. Additionally, a recent study comparing colorectal cancer tissue with its normal counterpart also suggests important changes at the CpG island shores[24] (**Fig. 1**). DNA hypomethylation occurs at many genomic sequences, such as repetitive elements, retrotransposons, introns and the like, resulting in genomic instability[3]. At repeat sequences, this is achieved by a higher rate

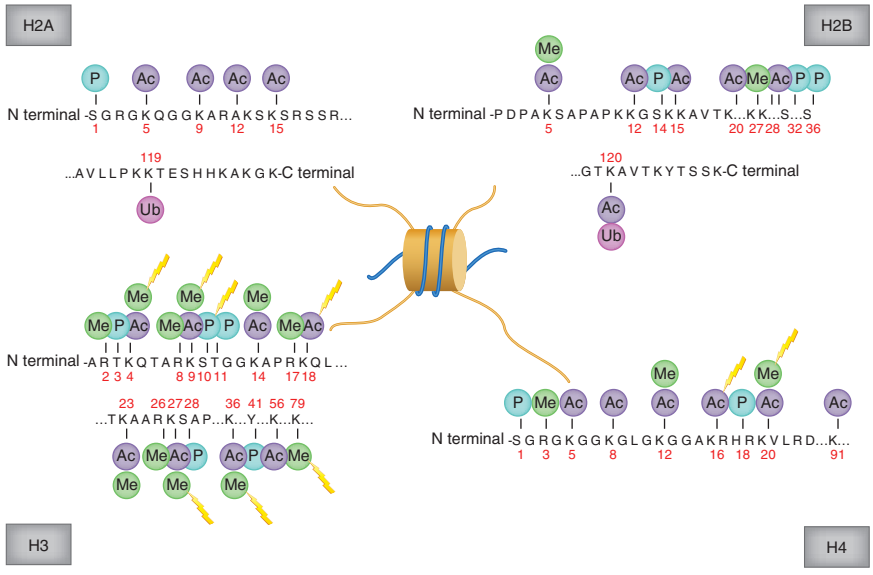

Figure 2 Histone modification patterns in normal and cancer cells. Mainly along their protruding N-terminal tails, but also within their C-terminal regions, histones can undergo diverse post-translational modifications. In the right combination and translated by the appropriate effectors, these modifications contribute to establishing the global and local condensed or decondensed chromatin states that eventually determine gene expression. This figure depicts the main modifications of the four core histones in normal cells (type and position in the amino acid sequence). Furthermore, and because disruption of their normal patterns is related to cancer, histone modifications typically associated with the disease have also been highlighted. Ac, acetylation; Me, methylation; P, phosphorylation; Ub, ubiquitination.

of chromosomal rearrangements and, at retrotransposons, by a higher probability of translocation to other genomic regions[25–27]. Furthermore, aberrant DNA hypomethylation can also account for the activation of some protooncogenes and lead to loss of imprinting, as in the case of the *IGF2* gene (encoding insulin-like growth factor-2) in Wilms's tumor[28–30]. However, the most recognized epigenetic disruption in human tumors is the CpG island promoter hypermethylation–associated silencing of tumor suppressor genes such as *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *MLH1* (mutL homolog-1), *BRCA1* (breast cancer–associated-1) and *VHL* (von Hippel-Lindau tumor suppressor)[3,4], an observation that has been expanded through the study of the inactivation of microRNAs with growth-inhibitory features by silencing[31–34]. The disturbance of the DNA methylation landscape in transformed cells has been recently supported by the finding of somatic mutations in *DNMT3A* in acute myeloid leukemia (AML)[35]. Finally, as *MLL-TET1* (*MLL* is the myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*) gene) fusions have been observed in some cases of AML and lymphocytic leukemias[36,37], and homozygous null mutations and chromosomal deletions involving the *TET2* locus have been described in various myeloid malignancies[38,39], the impairment of the conversion of 5-mC into 5-hmC might also be related to cancer.

Disruption of normal patterns of covalent histone modifications is another hallmark of cancer (**Fig. 2**)[40,41]. One of the most characteristic examples is the global reduction of the trimethylation of H4K20 (H4K20me3) and acetylation of H4K16 (H4K16Ac), along with DNA hypomethylation, at repeat sequences in many primary tumors[40]. Furthermore, there are many examples of alterations in enzymes that add, remove or recognize specific modifications in specific types of cancer (**Fig. 3**).

Regarding histone acetylation, chromosomal translocations involving HATs such as E1A-binding protein p300 (EP300), cAMP response element–binding binding protein (CREBBP), nuclear receptor coactivator-2 (NCOA2), MYST3 and MYST4 have been identified in hematological cancers[42,43]. In hematological and solid cancers, binding of adenoviral oncoproteins E1A and SV40 T to EP300 and CREBBP leads to cellular transformation through global hypoacetylation of H3K18 and concomitant activation of genes promoting cell growth and division[44,45]. Finally, whereas colorectal, gastric, breast and pancreatic tumors show missense mutations of *EP300*, monoallelic loss of *KAT5* (encoding lysine acetyltransferase-5) increases the potential for malignant transformation[46,47]. Overexpression of individual HDACs, such as HDAC1, HDAC2 and HDAC6, among others, has also been reported in tumors[48]. But the role of HDAC2 in tumor promotion is controversial, as the loss of its expression due to truncating mutations has also been documented in a subset of microsatellite-unstable colorectal cell lines and primary tumor samples[49]. Sirtuins are also overexpressed in a wide variety of tumors[50]. Intriguingly, inhibition of SIRT1 partially reactivated tumor suppressors, even when their promoters remained heavily methylated[51].

Anomalous expression or activity of HMTs and HDMs, due to chromosomal translocations, amplification, deletion, overexpression or silencing, has also been reported in cancer. In the case of the *MLL* gene, which encodes the most thoroughly studied H3K4 HMT, its partial tandem duplication (*MLL-PTD*) and more than 50 gene fusions account for 80% of infant leukemias and 5–10% of adult AML and lymphocyte leukemias[52]. Many MLL fusions seem to activate leukemia-promoting genes by abnormal recruitment of DOT1L, the non-SET-domain HMT for H3K79 (ref. 53). SMYD3, another H3K4 HMT, is frequently upregulated in colorectal and hepatocellular carcinoma cell lines, where it enhances cell growth and promotes transformation[54]. The H3K27-specific HMT EZH2 is overexpressed in solid tumors such as prostate, breast, colon, skin and lung cancer[55]. Notably, the increase in endothelial EZH2 expression promotes angiogenesis by silencing the gene encoding vasohibin-1, at least in ovarian cancer[56]. Although its oncogenic function has been the focus of main attention, recent discoveries also show inactivating mutations of EZH2 in follicular and diffuse large B cell lymphomas[53,57]. Other modifying enzymes also have a role in cancer. Nuclear receptor–binding SET domain protein-1 (NSD1), an HMT for H3K36 and, to a lesser extent, for H4K20, is involved in leukemogenic translocation and is silenced by promoter hypermethylation in gliomas and neuroblastomas, and its heterozygous mutation or loss

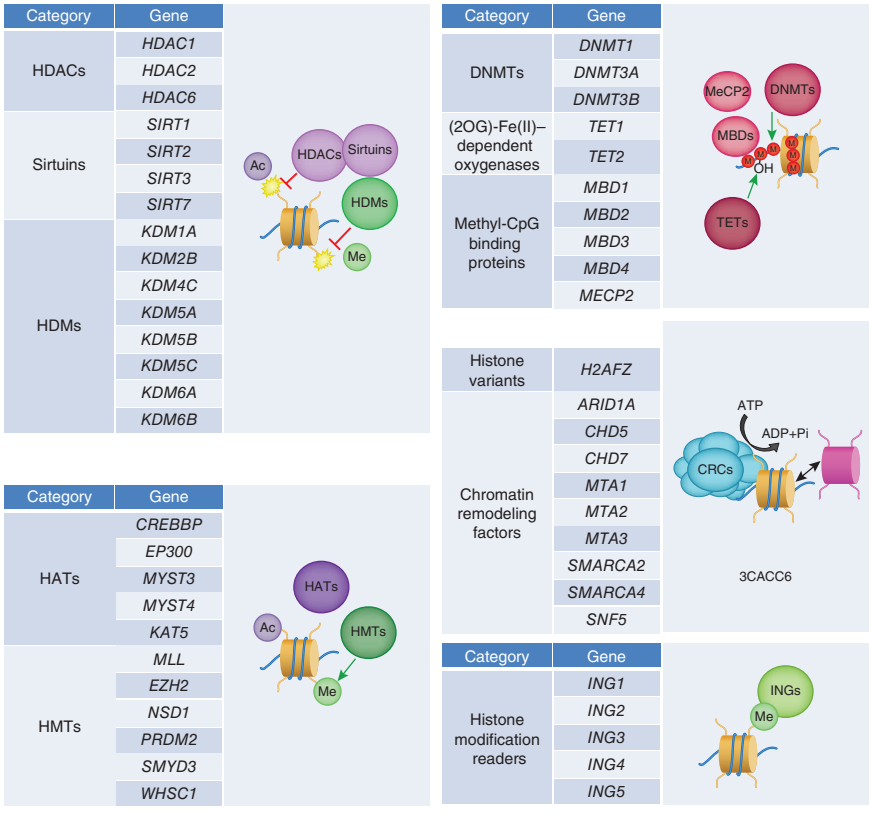

Figure 3 Selection of epigenetic genes disrupted in human tumors. Mutation, deletion and/or altered expression of genes encoding components of the various epigenetic machineries are typically observed in human tumors. The figure shows a selection of genes encoding enzymes that add, remove and recognize histone modifications, as well as members of the DNA methylation machinery, whose deregulation is connected to cancer. CRCs, chromatin remodeling complexes; Ac, acetylation; Me, methylation.

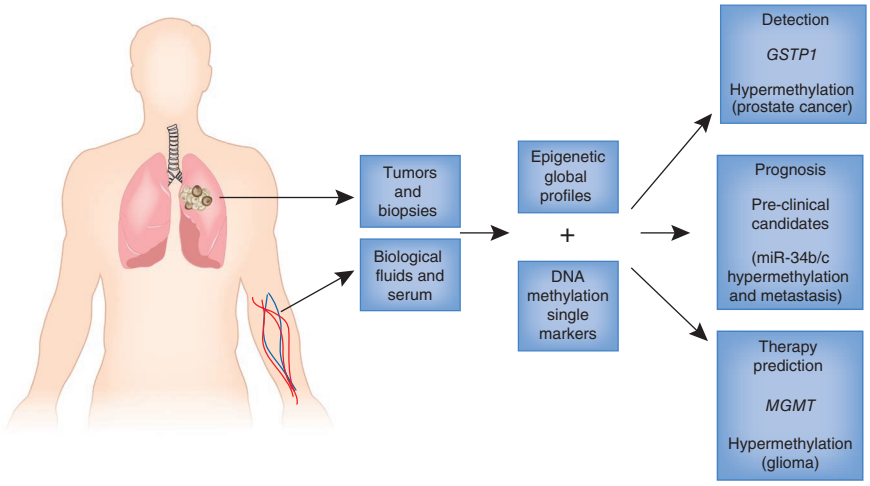

Figure 4 Epigenetic biomarkers in oncology. From all types of samples obtained from individuals with cancer, single and global epigenetic screenings have been developed to identify new molecular markers to manage the disease. To predict malignancy in prostate tumorigenesis and response to temozolomide in gliomas, the study of hypermethylation events in *GSTP1* and *MGMT*, respectively, is reaching the clinical stage.

of heterozygosity causes a childhood overgrowth syndrome with a higher risk of tumorigenesis called Sotos syndrome[58,59]. In contrast, recent data highlight the role of HDMs in cancer. Intriguingly, both overexpression and loss-of-function mutations of various members of the Jumonji/ARID domain–containing protein-1 (JARID1) family of H3K4me3/2 HDMs is believed to contribute to tumorigenesis in various cancer types.

Epigenetic biomarkers in individuals with cancer

The DNA methylation and histone modification patterns associated with the development and progression of cancer have a potential clinical use. Three major clinical oncology areas can potentially benefit from DNA methylation–based biomarkers: cancer detection, tumor prognosis and prediction of treatment responses, a field known as pharmacoepigenetics (**Fig. 4**).

Detection of tumoral cells. The last ten years have provided an extensive map of the aberrant DNA methylation events occurring in cancer cells, particularly for the hypermethylated CpG islands of tumor suppressor genes[3,60]. These data include examples from all classes of human neoplasia and have highlighted the existence of a unique profile of hypermethylated CpG islands that defines each tumor type[61,62]. The emphasis is now on focusing on those aberrant methylation events that are absent in normal cells and developing techniques that provide reliable, sensitive and fast results to study these potential biomarkers. The recent advances in technologies that couple bisulphite modification of DNA with PCR[63] have been key in the development of these quick and quantitative molecular methods. Recently, CpG island hypermethylation has been used as a tool to detect cancer cells in several types of biological fluids and tissue biopsies[63,64]. Thus, since it was first shown that cancer-specific hypermethylation events could be detected in the sera of individuals with cancer[65], a myriad of studies have used this easily accessible biological material in the translational and clinical setting. Good examples are the detection of cancer-specific hypermethylation events in feces from individuals with colorectal cancer[66], in urine for bladder cancer screening[67] or in sputum to predict lung cancer incidence[68]. Furthermore, new powerful techniques can now detect even minimal amounts of aberrant DNA methylation[69,70].

Numerous studies have shown that CpG island promoter hypermethylation of tumor suppressor genes occurs early in tumorigenesis. Thus, the presence of aberrant CpG island methylation alone does not necessarily indicate an invasive cancer, as premalignant or precursor lesions can also carry these epigenetic signatures. This finding has implications for early detection of cancer, especially in people with inherited genetic risk (for example, carriers of *BRCA1* and *BRCA2* mutations) or exposed to carcinogenic environment. One of the best examples of this is the detection of aberrant DNA methylation events in the early stages of lung tumorigenesis affecting smokers and miners[71].

Undoubtedly the best DNA methylation marker for cancer detection, and the one most likely to succeed as an epigenetic biomarker, is the hypermethylation of the glutathione S-transferase gene (*GSTP1*) in prostate cancer[72]. *GSTP1* is hypermethylated in 80–90% of men with prostate cancer and, at lower percentages, in other tumor types such as liver, breast and kidney[73]. Most important, although it is hypermethylated in a fraction of prostate intraepithelial neoplasias[74], it is not in benign hyperplastic prostate tissue[75]. Thus, the detection of *GSTP1* methylation could help distinguish between prostate cancer and benign lesions. As discussed earlier, hypermethylation of CpG islands can be detected in biologic fluids and biopsy specimens, which suggests that the detection of *GSTP1* hypermethylation in urine[76,77] and serum[78,79] has excellent prospects for clinical application.

Establishment of tumor prognosis. A longstanding goal of medical practitioners is the capacity to look at two morphologically identical tumors and distinguish which one will grow at a fast pace and which one will have a more indolent behavior. Molecular data from such studies is routinely used for detection of hematological malignancies through the presence of specific karyotypic abnormalities and certain oncogenic fusion proteins. Also, promising results using standardized gene expression arrays for the establishment of prognosis are also beginning to emerge in breast cancer[80]. Thus, it is logical that certain aberrant DNA methylation signatures might also be useful for this purpose. Research in the last decade has yielded an increasing number of hypermethylation events at single gene loci that indicate outcome in people with cancer. For example, hypermethylation of the genes encoding the HMT NSD1, the death-associated protein kinase DAPK, epithelial membrane protein-3 and CDKN2A has been linked to poor outcomes in neuroblastoma and lung, brain and colorectal cancer, respectively[3]. Larger multicenter and prospective studies are needed to further validate these and other single-gene DNA methylation markers.

In recent years there have been an increasing number of global DNA methylation approaches devoted to accomplishing the goal of identifying aberrant methylation signatures. Epigenomics unveils prognostic dendrograms, similar to those produced by gene expression microarray analyses, where a combination of aberrant DNA methylation markers from CpG arrays is used[81,82]. These epigenomic profiles are complementary to gene expression patterns and have the advantage that they can be developed with DNA extracted from

archived material. Most important, single markers with great clinical potential, such as predictors of recurrence in lung cancer[83], metastasis in colorectal cancer[84] or progression in virus-associated neoplasms[85], can be identified from these global DNA methylation screenings. Histone-based markers have also been incorporated later into this molecular race for epigenetic biomarkers, and a particular impairment of histone modifications has been associated with recurrence of prostate cancer[41].

Pharmacoepigenetics. The recent unmasking of genetic lesions in tumors has optimized cancer treatment regimens, specifically with the identification of the presence of *ERBB2* (v-erb-b2 erythroblastic leukemia viral oncogene homolog-2) amplifications, *BCR-ABL1* (breakpoint cluster region–c-abl oncogene-1) translocations and *EGFR* (epidermal growth factor receptor) mutations. It is predicted that, in coming years, the hypermethylation patterns of particular genes will also predict response to specific treatments.

The most promising epigenetic candidates to predict pharmacoepigenetic response are the DNA repair genes undergoing epigenetic inactivation in tumors, such as the O^6-methylguanine-DNA methyltransferase–encoding gene, *MGMT*, the DNA mismatch repair protein–encoding gene, *MLH1*, the Werner syndrome–associated gene, *WRN*, or *BRCA1*. In healthy tissues, these enzymes are responsible for repairing the DNA damage that occurs during a lifetime and prevent the formation of mutations and other types of genomic damage. In cancer cells, however, these enzymes can repair the DNA damage induced by many chemotherapy agents, thus generating chemoresistance. But these DNA repair genes can also undergo hypermethylation-associated silencing in a fraction of human tumors that progress with a mutator phenotype—an Achilles' heel, because this way they will not be able to repair the DNA damage caused by the chemotherapy agent. To date, the best example of hypermethylation of a DNA repair gene that has reached the clinical setting is that of the *MGMT* gene in gliomas, as a predictor of response to carmustine[86] and temozolomide[87,88]. MGMT reverses the addition of alkyl groups to the guanine bases of DNA and, in normal cells, protects DNA against the generation of transition mutations by carcinogens such as nitrosamides. The O^6 position of the guanine is also a point of attack for alkylating agents such as carmustine (BCNU), nemustine (ACNU), procarbazine, dacarbazine and temozolomide. Thus, those human primary tumors undergoing hypermethylation of *MGMT*[89] could be more sensitive to this category of drugs. Proof of principle of these observations is that CpG island hypermethylation of *MGMT* is an independent predictor of good clinical response to carmustine in gliomas[86]. These discoveries were subsequently expanded to show that hypermethylation of this gene is also an independent predictor of response to temozolomide, a newer drug, in glioblastomas[87]. Many studies have now confirmed these findings about temozolomide-based glioblastoma treatment in many different geographical areas[90]. It is also worth noting that methylation of *MGMT* might denote those rare glioma cases classified as long-term survivors[91] and predict cooperative responses with other therapeutic agents such as cilengitide[92] or even radiation[93]. In contrast, hypermethylation of *MGMT* in temozolomide-untreated patients is a marker of poor prognosis, and it is probably related to the accumulation of mutations in these tumors. In fact, a hypermutator phenotype consequent to mismatch repair deficiency occurs in temozolomide-treated glioblastomas[94]. Four final issues related to *MGMT* epigenetic inactivation and worthy of further exploration are the extension of the *MGMT* hypermethylation predictor value for temozolomide to other cancer types such as aggressive pituitary[95], colorectal and non–small-cell lung tumors; the review of the clinical trial data using temozolomide in tumors where there is very little CpG island methylation of *MGMT*, such as melanoma; the expansion of *MGMT* methylation to predict response to other types of DNA damage such as those mediated by cyclophosphamide[96]; and the possibility of using small molecules as inactivators of the MGMT protein in CpG island unmethylated tumors[97].

The potential to use the CpG island methylation status to predict response to chemotherapy has been described for other DNA repair genes such as *MLH1* in cisplatin treatment of ovarian cancer[98], *WRN* for irinotecan in colorectal tumors[99], insulin-like growth factor–binding protein-3 for cisplatin in lung tumors[100] and *BRCA1* for PARP (poly (ADP) ribose polymerase) inhibitors in breast cancer[101]. Other families of genes that undergo CpG island hypermethylation–associated silencing and have potential value as predictors of response to anticancer agents include the cell cycle checkpoint genes, such as *CHFR* (checkpoint with forkhead-associated and ring finger), related to docetaxel/paclitaxel[102,103], metabolite carrier genes, such as *SLC19A1* (solute carrier family 19 (folate transporter), member 1), related to methotrexate in lymphomas[104], and the *GSTP1* (glutathione S-transferase pi 1) gene, related to doxorubicin in breast cancer[105]. Finally, it is important to keep in mind that the lack of effectiveness of antisteroidal drugs such as tamoxifen, raloxifene and flutemide in some individuals with hormone-related malignancies may be a direct consequence of the epigenetic silencing of their respective cellular receptors, such as the estrogen and progesterone receptors[106]. A similar explanation can be applied to the lack of success with preventive retinoid treatment, which could be related to the epigenetic silencing of the gene encoding retinoic acid receptor β, *RARB2*, and the gene encoding cellular retinol binding protein I, *CRBP1*. With the emerging epigenomic technologies[107], researchers now have the techniques that will help address the DNA methylation profiles for chemosensitivity in an unbiased manner, as has been developed in ovarian cancer[108], and complete the promising pharmacoepigenetics landscape. Interestingly, the use of epigenetic drugs, such as DNA-demethylating agents, could resensitize some of the resistant cancer cells to classical chemotherapy agents[109], a finding that it is worthy of further exploration.

Epigenetic drugs for cancer treatment

As described above, and in contrast to genetic mutations, epimutations are reversible. This is why so much attention has been focused in recent years on the quest for epigenetic drugs, which could restore the normal epigenetic landscape in cancer cells by inhibiting enzymes of the epigenetic machineries (**Fig. 5**). To date, four have been approved by the US Food and Drug Administration (FDA) for cancer treatment: two DNMT inhibitors, vidaza and decitabine (5-aza- and 5-aza-2′-deoxycytidine, respectively), for patients with myelodysplastic syndrome who have few therapeutic options and develop acute leukemia; and two HDAC inhibitors, vorinostat and romidepsin (suberoylanilide hydroxamic acid and, formerly, FK-228, respectively), for the rare cutaneous T cell lymphoma (CTCL), among other hematological malignancies[110–113]. In addition to DNMT inhibitors and HDAC inhibitors, inhibitors of class I, II and IV HDACs, inhibitors of sirtuins (the class III HDACs), HATs, HMTs, HDMs and various kinases are also being intensively researched. These epigenetic drugs will become a crucial part of the therapeutic arsenal against cancer in the near future.

Regarding DNMT inhibitors, another cytidine analog stable in aqueous solution, 5-fluoro-2′-deoxycytidine, is undergoing clinical

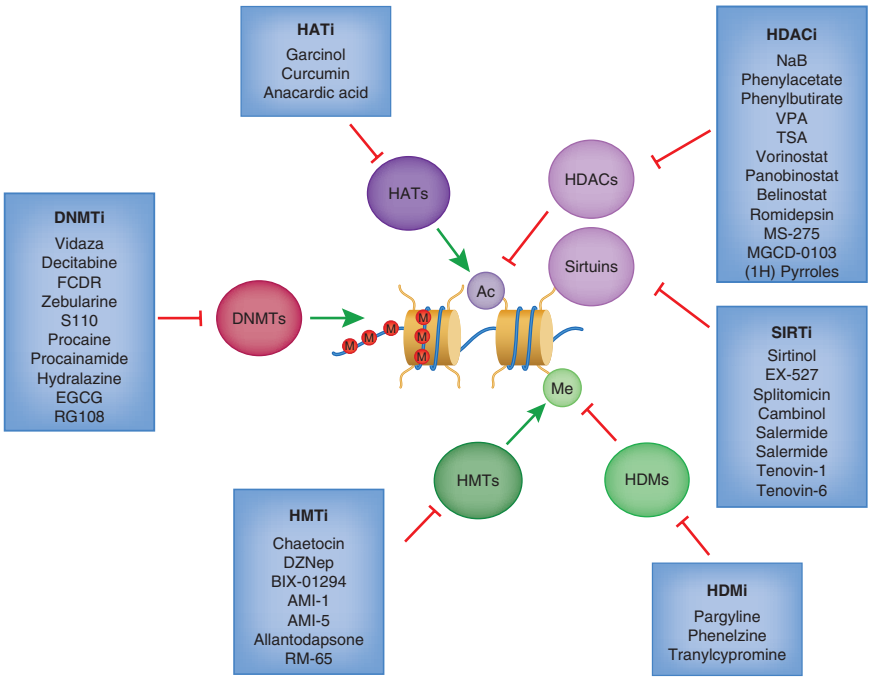

Figure 5 Epigenetic drugs for cancer therapy. Numerous compounds have been reported to be effective against cancer cells by inhibiting components of the epigenetic machineries. This figure shows the most important epigenetic drugs classified depending on their particular epigenetic targets.

trials in combination with other agents for the treatment of various tumors[114]. The effects on gene transcription of not only vidaza and decitabine, but also zebularine, another cytidine analog, are surprisingly different and involve genes relevant to leukemogenesis[115]. Some nonnucleoside analogs have also been described as inducing DNA demethylation in cancer cell lines[116–120].

The arsenal of HDAC inhibitors includes both natural and synthetic compounds that can be divided into four chemically distinct classes: short-chain fatty acids, hydroxamic acids, cyclic peptides and benzamide derivatives. All HDAC inhibitors are characterized by the presence of a metal-binding domain that can block substrate-Zn chelation at the HDAC active sites. This is why sirtuins, which, in contrast, require NAD^+ at their active sites, are unaffected by these inhibitors[121]. The main anticancer effects of HDAC inhibitors are cell cycle arrest in G1 or G2-M, induction of differentiation and apoptosis, but they can also inhibit angiogenesis and metastasis, as well as enhance the sensitivity to chemotherapy[122,123]. A summary of the most important HDAC inhibitors is shown in **Table 1**.

DNMT inhibitors and HDAC inhibitors are the most extensively studied anticancer epigenetic drugs. SIRT inhibitors are less studied, but, as most of them are SIRT1 inhibitors, they are likely to stop the formation of tumors and induce apoptosis by increasing p53 activity. Immediately before nicotinamide was reported to be a physiological inhibitor of sirtuins, sirtinol and splitomicin were described as SIRT inhibitors[124–126]. Sirtinol inhibits SIRT1 and SIRT2, is capable of

Table 1 HDAC inhibitors

Chemical class	Selected members	Comments	References
Short-chain fatty acids	Sodium *n*-butyrate (NaB) Phenylacetate Phenylbutyrate Valproate	Butyrates such as NaB inhibit proliferation of colon, prostate, endometrial and cervical carcinomas at high millimolar concentrations. Valproate is quite active against HDACs 1–5, 7 and 9 but less so against HDACs 6 and 10. It is more efficient as an inducer of differentiation in carcinoma cells, transformed hematopoietic progenitor cells and leukemic blasts from individuals with AML.	164–166
Hydroxamic acids	Trichostatin A Vorinostat (SAHA) Panobinostat Belinostat	Trichostatin A inhibits HDACs 1–7 and 9 at the single-digit nanomolar level and HDAC8 at the single-digit micromolar level. Despite its proven antitumoral activity, it has too many side effects to be used clinically. Vorinostat is FDA-approved for hematological malignancies. Panobinostat is highly active against HDACs 1–4, 7 and 9 but less so against HDAC6 and, especially, HDAC8. It is undergoing clinical trials for the treatment of CML, refractory CTCL and multiple myelomas. It may also be relevant to the treatment of hormone-dependent breast cancers, as it causes strong inhibition of their typically upregulated aromatase gene. Belinostat is quite active against HDACs 1–10. It is in clinical trials for the treatment of hematological malignancies and solid tumors.	112,167–170
Cyclic peptides	Romidepsin (formerly FK-228)	A natural, stable prodrug that, once converted to its active form (redFK) by cellular reducing activity, is capable of inhibiting HDACs 1, 2, 4 and 6. After showing strong preclinical antitumoral activity, it was approved by the FDA and has undergone clinical trials for the treatment of AML, CML and CTCL.	110,113,171
Benzamide derivatives	MS-275 (or entinostat) MGCD-0103	MS-275 inhibits HDACs 1–3 and 9 and has also been used in clinical trials in conjunction with other agents. MGCD-0103 can inhibit HDACs 1 and 2 and, to a lesser extent, HDACs 3 and 11. It is also in clinical trials for the treatment of hematological malignancies and solid tumors.	168,172–174

CML, chronic myeloid leukemia.

promoting growth arrest in cancer cells[127] and increases sensitivity to well-known anticancer drugs such as camptothecin and cisplatin[128]. Nevertheless, some sirtinol analogs have already shown higher capabilities. Cambinol and salermide are other SIRT inhibitors. Cambinol inhibits SIRT1 and SIRT2, inducing apoptosis in BCL6-expressing Burkitt's lymphoma cells[129]. In contrast, salermide, an inhibitor of SIRT1 and SIRT2 at the micromolar level, was recently found to induce p53-independent apoptosis only in cancer cells. Salermide has been shown to reactivate epigenetically repressed proapoptotic genes by SIRT1-mediated H4K16Ac deacetylation[130]. Finally, tenovins, such as tenovin-1 and the more water-soluble tenovin-6, are active against SIRT1 and SIRT2. They work on mammalian cells at single-digit micromolar concentrations and delay tumor growth *in vivo* as single agents[131].

Three naturally occurring small molecules have been described as HAT inhibitors: curcumin, garcinol and anacardic acid. Curcumin is an EP300- and CREBBP-specific inhibitor capable of repressing EP300-mediated p53 acetylation *in vivo*[132]. Its antitumor activities in a wide variety of cancers include the downregulation and upregulation of *CCND1* (cyclin D1) and *CASP8* (caspase-8), respectively, as well as the inhibition of constitutive nuclear factor-κB activation[133–135]. Garcinol and anacardic acid are both EP300 and KAT2B HAT inhibitors. Although garcinol has much better cell permeability than anacardic acid, they both may improve cancer therapy. Thus, whereas garcinol has been shown to induce apoptosis in HeLa cells, anacardic acid can sensitize cancer cells to ionizing radiation[136,137]. A few other small molecules have been described as HAT inhibitors, but, to date, only a series of isothiazolones affecting EP300 and KAT2B activity have been able to inhibit growth in colon and ovarian cancer cells[138].

As for HMT inhibitors, only three compounds have been described as lysine HMT inhibitors: chaetocin, DZNep and BIX-01294. First identified as a lysine-specific HMT inhibitor of *Drosophila melanogaster* Su(var)3-9, chaetocin also inhibits its human counterpart and shows anticancer properties against multiple myeloma (MM)[139,140]. DZNep induces apoptosis in breast cancer MCF7 and colorectal HCT116 cells, where it promotes the depletion of the polycomb-repressive complex-2 proteins (for instance, EZH2) and inhibits methylation of the H3K27 modification[141]. Additionally, the arginine-specific HMT inhibitor AMI-1 (arginine N-methyltransferase inhibitor-1) is believed to inhibit PRMT1, PRMT3, PRMT4 and PRMT6 (ref. 142). The fact that PRMT4 is overexpressed in hormone-dependent cancers may encourage research into these particular inhibitors[143]. Owing to structural similarities, analogs of the AMI-1 derivative AMI-5 can inhibit not only lysine and arginine-specific HMTs but also some HATs and sirtuins with the same potency, thus giving rise to the term 'epigenetic multiple ligands'[144].

With respect to HDM inhibitors, we know that inhibitors of monoamine oxidases (MAOis), such as pargyline, phenelzine and tranylcypromine, can also inhibit the HDM KDM1A, but currently there is no information about their putative anticancer properties[145]. Increasing the arsenal of HDM inhibitors against the many HDMs involved in cancer will be a major task in the coming years.

An increasing amount of evidence links kinases to epigenetic regulation, and important breakthroughs are taking place in the field of kinase inhibitors. Thus, the tyrosine kinase JAK2 has been reported to phosphorylate H3Y41, preventing the H3K9me2/3 'reader' CBX5 from binding to chromatin[146]. Moreover, aurora kinase B, which phosphorylates H3S10, thereby maintaining chromosomal stability during mitosis, has been found to be overexpressed in various human solid tumors[147,148]. It was also recently discovered that, together with aurora kinase A, it is essential for the progression of Myc-driven B cell lymphomas[149]. A considerable number of aurora kinase inhibitors are currently being examined in clinical trials. Danusertib (or PHA-739358) and MLN8237, currently in phase 2, are the most promising[147,150,151]. Finally, the use of HDAC inhibitors in conjunction with tyrosine kinase inhibitors such as AEE788 and imatinib mesylate was found to be effective against several types of cancer cells[152,153].

Future cancer therapies will surely exploit the synergistic effects between epigenetic drugs or between epigenetic drugs and other antitumoral agents. A recent work connects the emergence of cancer cell resistance to the chemotherapeutic agent erlotinib with an increased expression of the HDM KDM5 (ref. 154). Moreover, it also seems clear that epigenetic drugs such as HDAC inhibitors should synergize with DNA-damaging agents, as they may offer improved access to chromatin.

Relevant chemotherapeutic drugs have already been tested in combination with DNMT inhibitors, HDAC inhibitors and SIRT inhibitors. For instance, a recent phase 2 study combined the thrombopoietin mimetic romiplostim with 5-azacytidine in patients with myelodysplastic syndrome[155]. In other examples of successful alliance, romidepsin was capable of enhancing the effect of gemcitabine on hormone-refractory prostate cancer cells[156] and sirtinol was observed to increase sensibility to camptothecin and cisplatin in PC3 prostate cancer cells[128]. Numerous trials combining DNMT inhibitors and HDAC inhibitors have already taken place too[157,158]. Finally, it has been shown that panobinostat or anacardic acid can improve radiotherapy[137,159]. In the near future, combinations between epigenetic drugs and cyclin-dependent kinase inhibitors, HSP90AA1 antagonists or proteasome inhibitors will also be tested. Other interesting combinations may involve engineered transcriptional factors capable of selectively reactivating epigenetically silenced tumor suppressors[160].

Another challenge in combining different anticancer agents is determining the best sequence of their delivery. It was shown *in vitro* and in xenograft models that a combination of valproate with a topoisomerase II inhibitor was only effective inducing cancer cell death if valproate was administered first[161].

Notably, the therapeutic efficacy of any antitumoral drug is highly dependent on its cellular uptake. This could provide an explanation for the classical observation that clinical response rates to epigenetic drugs are generally low in solid tumors. In the particular case of 5-azacytidine, which depends on variably expressed nucleoside transporters for uptake, it has been recently observed that its delivery by elaidic acid esterification can markedly increase its anticancer activity[162]. Therefore, further investigation on cellular uptake mechanisms for different epigenetic drugs will be required to improve epigenetic cancer therapies.

Future prospects

We have already entered the epigenomics era. In recent years, the use of new high-throughput technologies has made it possible to study epigenetic processes at a much broader level than a single gene. Now, for instance, bisulphite treatment of methylated DNA (methylC-seq) or its immunoprecipitation with antibodies against methylated cytosine (MeDIP-seq) combined with next-generation sequencing allows the investigation of the DNA methylation status of human cells at nucleotide resolution[12,163]. Additionally, chromatin immunoprecipitation followed by modern high-density microarrays or, again, next-generation sequencing (ChIP-chip and ChIP-seq, respectively)

can determine accurately the location of different covalent histone modifications at a global level. With the latter techniques, finding out the genome-wide location of any member of the epigenetic machineries is also possible. This kind of epigenomic approach is already revolutionizing cancer research, and, of course, some examples have already been included in this review[10–12,18,20]. The integration of these data with the information coming from genomics and transcriptomics will exponentially expand understanding of tumorigenesis and yield better epigenetic biomarkers for detection, prognosis and therapy prediction.

Finally, in the use of epigenetic drugs for cancer treatment, it will be required to further the knowledge about key issues such as the optimal doses for single and combined therapies, the sequence of delivery in combined therapies and tumoral uptake of therapies. Moreover, and considering that the effects of most of the drugs are still so nonspecific that they may be a double-edged sword, causing undesirable side effects, it will be necessary to design new agents against specific enzymes of the epigenetic machinery, instead of global processes. In an era of an increasingly accurate and personalized medicine, new epigenetic drugs could be developed even against different isoforms or mutated variants of particular enzymes involved in very specific types of cancer.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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